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Transient State Imaging of live cells by Single Plane Illumination Microscopy.

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Abstract

Long-lived, photoinduced transient dark states of fluorophores have a great potential for environmental sensing. Transient State (TRAST) Imaging based on time-modulated excitation has recently been shown to provide imaging of the kinetics and populations of these states in biological samples and has been realized for confocal, wide-field and total internal reflection microscopy settings. In this study, we demonstrate the applicability of Single Plane Illumination Microscopy to TRAST imaging, offering optical sectioning and reduced overall excitation light exposure of the specimen. This concept was first verified by showing that transition rates of free dye in solution can be determined accurately. Thereafter, experiments on MCF-7 cells were performed, showing that fluorophore triplet state decay and oxidation rates can be resolved pixel-wise. Furthermore, a new theoretical framework for analyzing TRAST data acquired with arbitrary duty cycle pulse trains has been derived. By this analysis it is possible to reduce the overall measurement time and thereby enhance the frame rates in TRAST imaging.

Introduction

In fluorescence spectroscopy and imaging, long-lived, photo-induced, dark states, such as triplet, redox and isomerized states of the fluorophores, are often considered undesirable. They reduce the fluorescence signal (1, 2) and may act as precursor states for photobleaching (3, 4). However, in recent years these transient states have also been found to be quite useful as a basis for high-resolution microscopy (5–7) and they hold great potential for environmental sensing purposes (8). The fluorescence lifetime of a fluorophore is in the range of $\sim 10^{-9}$ s, whereas the transient dark states typically decay within $\sim 10^{-6}$ - 10^{-3} s. Consequently, dyes in such states have orders of magnitude longer time to interact with their environment, compared to dyes in the excited singlet state. Still, the use of long-lived transient states as readouts in biomolecular research has to date been relatively limited. Different methodologies to quantify these states and their kinetics do exist. Transient state absorption techniques allow detailed investigations of a broad range of photoinduced dark states (9–12). However, these techniques usually require advanced equipment, micromolar sample concentrations, suffer from overlap of spectra from different dark states, and are typically restricted to cuvette measurements. Triplet states can be observed via phosphorescence intensity and lifetime measurements (13), albeit the achievable signal intensity is usually considerably lower than in fluorescence-based studies. In addition, triplet states are often subject to quenching, especially by oxygen. Consequently, deoxygenation of the samples is usually required (14), or engineered macromolecules with limited quenchability need to be used (15).

As an alternative, Fluorescence Correlation Spectroscopy (FCS) has been found to be a versatile tool to investigate dark state dynamics, combining the high detection sensitivity of the fluorescence readout with the environmental sensitivity of long-lived states (16–19). However, FCS experiments are limited to nanomolar concentrations, and to relatively bright and photostable dyes. Moreover, since triplet state investigations require high temporal resolution (microsecond time scale) and quantum yield of detection, FCS measurements are mainly restricted to single point detectors.

To circumvent these limitations, we recently developed Transient State (TRAST) imaging, which also measures the dynamics of photoinduced dark states by using fluorescence as a read-out parameter (20). In TRAST, the fluorescent sample is subject to a modulated excitation. Depending on the excitation pulse train characteristics (e.g. pulse duration, separation and height) long-lived photo-induced states (e.g. triplet, photo-isomerized and photo-ionized states) of the fluorophores in the sample are populated to different extents. Upon transient state population build-up in the sample, the fluorescence intensity drops. By systematically varying the excitation pulse train characteristics, and registering how the time-averaged fluorescence intensity changes, the population kinetics of the transient states can be retrieved. In contrast to FCS, the TRAST technique does not rely on fluorescence detection with high sensitivity and time resolution, or on a certain dye concentration in the sample. TRAST is thus widely applicable and enables

imaging of triplet and other dark state kinetics also in a massively parallel fashion, using a CCD with low temporal resolution for detection.

So far, TRAST imaging has been performed in regular and laser scanning confocal (20, 21), wide-field (22) and total internal reflection (23) mode. To obtain reliable results in TRAST measurements, it is necessary that a significant population difference in the monitored transient states is generated upon variation of the excitation pulse train characteristics. For standard rhodamine dyes in air-saturated aqueous environments, significant triplet state population requires excitation irradiances in the range of 100 kW/cm^2 (21, 22). However, the doses following from such high irradiation levels can easily exceed hundreds of J/cm^2 and are likely to be toxic to live cells (24). As a first remedy, we show in this work that these irradiances can be reduced by several orders of magnitude by using dyes with high triplet state yields.

Second, for both wide-field and confocal setups, the excitation beams are not confined: all heights of the sample are illuminated, although fluorescence collection only takes place in certain positions. The exposure can be reduced significantly by illuminating only the plane, from which fluorescence is actually detected, using Single Plane Illumination Microscopy (SPIM) (25). In this approach, an asymmetric excitation profile with sheet-like character is created by inserting a cylindrical lens, which focal plane concurs with the rear focal plane of the illumination objective, mounted perpendicularly to the detection objective (26, 27). Within the last decade, the interest in SPIM and other sheet-like illumination techniques has increased considerably. The main applications so far are for three-dimensional and high-resolution fluorescence imaging (26, 28–31), but also the combination of SPIM and FCS has been demonstrated (32–34). In this work, we introduce the SPIM technique with time-modulated excitation for TRAST imaging, thereby providing an additional means to reduce the excitation light exposure of the sample during transient state measurements.

We demonstrate the applicability of SPIM to TRAST imaging, first by solution measurements, then by imaging of live cells, thereby further extending the range of fluorescence-based methods for quantitative biomolecular imaging.

Materials and Methods

TRAST model

In order to extract the fluorophore's transition rates between electronic states, experimental acquisitions are compared to a theoretical prediction. We base the approach on a model comprising a singlet ground state $|S_0\rangle$, an excited singlet state $|S_1\rangle$, a triplet state $|T\rangle$ and a redox state $|R\rangle$ as shown in Fig. 1 (top, middle graph). In general, several different redox states are conceivable, but in many cases, a single redox state can properly describe the observations (23, 35). The probability of each state to be populated at a

time t reads $S_0(t)$, $S_1(t)$, $T(t)$ and $R(t)$, respectively.

The excitation and de-excitation rates between the ground and the first excited singlet state are denoted by k_{01} and k_{10} , k_{isc} and k_t represent the rates of intersystem crossing into $|T\rangle$ and triplet decay back into $|S_0\rangle$, respectively. The redox state $|R\rangle$ is populated and depopulated with the rates k_{ox} and k_{red} . This notation suggests that $|R\rangle$ always represents an oxidized intermediate. However, it may also be a reduced product (see e.g. (36)), then with different names of the rate variables.

The measurements in this work were performed with rectangular pulse trains, and mostly with low excitation duty cycles ($\eta \leq 1\%$). This ensured that almost all fluorophores were relaxed back to their ground state at the onset of each and every pulse in the pulse train. Simulations (see supporting material) and experimental data (see Results and Discussions) further validated this approach. Considering a fluorophore, described by the electronic four-state model, $S_{01}TR$, depicted in Fig. 1, its average fluorescence intensity within the N excitation pulses of a rectangular pulse train of low duty cycle is given by (23):

$$\langle F_s \rangle_{Nt_p}(t_p) = \frac{F_0}{t_p} \left(\frac{1}{\lambda_1} (1 - e^{-\lambda_1 t_p}) + \frac{k'_{isc}}{\lambda_2^2} (1 - e^{-\lambda_2 t_p}) - \frac{k_t k'_{ox}}{\lambda_2 \lambda_3^2} (1 - e^{-\lambda_3 t_p}) + \frac{k_t k_{red}}{\lambda_2 \lambda_3} t_p \right) \quad (1)$$

A detailed derivation of $\langle F_s \rangle_{Nt_p}$ is included in Eq. S1-S17 in the supporting material. In Eq. 1, t_p denotes the pulse width of the excitation pulses, and the eigenvalues of the transition matrix between states have been approximated by: $\lambda_1 = -(k_{01} + k_{10})$, $\lambda_2 = -(k'_{isc} + k_t)$ and $\lambda_3 = -(k'_{ox} + k_{red})$, with the equivalent intersystem and oxidation rates given by: $k'_{isc} = k_{isc} \frac{k_{01}(\bar{r})}{(k_{01}(\bar{r}) + k_{10})}$ and $k'_{ox} = k_{ox} \frac{k'_{isc}}{(k'_{isc} + k_t)}$. The fluorescence

emitted by a single fluorophore in the sample in the absence of any dark state population is given as:

$F_0 = \Phi_F \frac{k_{10} k_{01}}{k_{10} + k_{01}}$, where Φ_F represents the fluorescence quantum yield. For dyes with moderate to low

triplet yields, the two singlet states can be merged in the STR model shown in Fig. S1. This model results in a similar expression, without the term in Eq. 1 related to λ_1 .

Single Plane Illumination

The illumination profile for SPIM is created as suggested by Greger et. al (37). Assuming a Gaussian beam, the irradiance distribution reads:

$$I(x, y, z) = \frac{2P_0}{\pi \omega_y \omega_z(x)} e^{-\frac{2y^2}{\omega_y^2}} e^{-\frac{2z^2}{\omega_z^2(x)}} \quad (2)$$

Here, the coordinate system is defined as shown in Fig. 1 (top left): The excitation beam propagates in the x -direction, and after the objective, the y -dimension is collimated and the z -dimension is focused. The

peak irradiance in the focus of the excitation ($x = x_0$) is defined as $I_0 = 2P_0/(\pi\omega_y\omega_z(x_0))$, where ω_y and $\omega_z(x)$ are the e^{-2} radii. Considering a beam of wavelength λ that is focused down to a waist $\omega_{0,z}$ at the focus point x_0 , the beam waists evolve along the propagation axis as follows:

$$\omega_z(x) = \omega_{0,z} \left(1 + \left(\frac{x - x_0}{x_{R,z}} \right)^2 \right)^{\frac{1}{2}} \quad (3)$$

The parameter $x_{R,z} = \pi\omega_{0,z}^2/\lambda$ in Eq. 3 is referred to as Rayleigh range and is a good measure for a region with almost homogeneous excitation conditions (27, 38). Using the razor blade edge tests described in the context of Fig. S4, we characterized the beam profile experimentally and found $x_{R,z} = (21.8 \pm 2.7) \mu\text{m}$ and $\omega_{0,y} = 40 \mu\text{m}$ in the collimated direction.

The excitation rate, k_{0I} , is calculated using the illumination profile and the cross section σ of the fluorophores for the transition $|S_0\rangle \rightarrow |S_1\rangle$ at the laser wavelength λ . In our TRAST experiments, k_{0I} is given by:

$$k_{0I}(\bar{r}, t) = \sigma I(\bar{r}) f(t)$$

$$f(t) = \begin{cases} 1 & \text{for } jT \leq t < jT + t_p, j = 0, 1, 2, \dots, N \\ 0 & \text{otherwise} \end{cases} \quad (4)$$

The modulation function $f(t)$ represents the time dependence of the rectangular pulsed laser modulation, with period T and pulse width t_p . The fluorescence detected by a camera can then be estimated by calculating the three-dimensional fluorescence in the sample using Eq. 1-4 and convoluting the fluorescence with the collection efficiency function of each pixel, as detailed in Eq. S18. To speed up the execution time of the iterative algorithm used for fitting the experimental data, the model is scaled down to a two-dimensional system and the detected fluorescence is calculated directly in a hypothetical plane using a two-dimensional effective excitation rate k_{0I}^{eff} for each pixel. The derivation of this approach and its validation by simulations are described in the context of Eqs. S19-S20 and Fig S2.

SPIM-TRAST Setup

The used setup is an extension from previously described implementations (20, 23). As depicted in Fig. 1, the 491 nm light from a continuous wave (CW) diode laser (Cobolt Calypso 491 nm, Cobolt AB, Solna, Sweden) is pulsed by an acousto-optic modulator (AOM) (MQ 180-AO, 25-VIS, AA Opto-Electronic Company, Orsay Cedex, France), positioned in the focus of a telescope (L1, L2). Before passing L2, the first order of the pattern, created by the AOM, is selected by an iris (not shown). Optical density filters are used to decrease the laser power as needed. The cylindrical lens (LJ1558RM, f=300 mm, Thorlabs, Newton, USA) is focusing one dimension of the initial beam into the rear focal plane of the illumination

objective (EO M Plan Apo, 20x, NA 0.42, Edmund Optics, Karlsruhe, Germany), which has a long working distance of 20 mm, leaving sufficient space for sample mounting.

A large focusing angle is required to tightly focus the illumination light sheet and puts high demands on the sample holder, which should not hinder the incident illumination. In this study, two different chambers were used. For solution measurements, an aluminum compartment as shown in Fig. 1 (top, right) was built with two orthogonal cover glasses (both Nr. 1, Menzel-Gläser, Brunswick, Germany) glued (Twinsil, picodent, Wipperfürth, Germany) and with an aluminum wire in the edge. For in vivo investigations, cells are mounted in agarose and the chamber does not have to be waterproof. Here, a home-build, U-shaped aluminum piece is used as a holder for two glued, orthogonal cover glasses (60x24 mm², Nr. 1, Menzel-Gläser).

The chamber is mounted on a piezo stage (NanoMax TS 3-Axis Flexure Stage, Thorlabs, Newton, USA). The detection objective (solution measurements: EC Plan-Neofluar, 40x, NA 0.75, Ph2, Carl Zeiss AG, Oberkochen, Germany; cell measurements: C-Apochromat, 40x, NA 1.2W, Carl Zeiss AG) is followed by a dichroic mirror (F500-Di01, Semrock Inc., Rochester, USA), which together with a band-pass filter (HQ 550/80, Chroma Technology Corp., Bellows Falls, USA) in front of the camera (not shown) discriminates scattered light. An achromatic lens ($f=200$ mm) focuses the detected light onto the EM-CCD (electro-magnified charge coupled device, Luca, Andor Technology, Belfast, Northern Ireland). For these components, the actual magnification is 49x. The data acquisition is controlled via a graphical user interface, programmed in MATLAB 7.11.0 (The Mathworks Inc., Natick, USA), as described previously (22, 23).

Confocal measurements were performed on the same setup, but in these cases the laser was coupled into the detection objective (40x Zeiss C-Apochromat) using the dichroic mirror (DM). The detection pathway is identical to SPIM-TRAST, and a droplet of free dye in solution on a cover glass is used as a specimen.

Solution Measurements

Measurements on Rhodamine 110, Eosin Y (Sigma-Aldrich Co. LLC, St. Louis, USA), Fluorescein (Life Technologies Corp., Carlsbad, USA), and Atto 495 (ATTO-TEC GmbH, Siegen, Germany) were performed in phosphate buffered saline (PBS, pH 7.4). Table 1 presents irradiances and concentrations used in the measurements of the dyes, together with the photophysical parameters used in the data analysis, based on own measurements and literature values (39–42).

To establish a focal overlap between the illumination and detection pathways in the SPIM configuration, the cylindrical lens was taken off to create a symmetrically focused illumination beam. Its shape was imaged through the detection objective using the fluorescence response of a dye solution. The size of the illumination beam's image could then be minimized to co-align the foci of the two objectives.

For every dye, 56 different illumination pulse trains with different t_p were applied onto the sample. Generally, η was kept constant at 1 % and for the consecutive pulse trains, t_p was increased logarithmically from 100 ns up to 10 ms. The total illumination time, that is the total time within a pulse train during which the specimen was subject to excitation, was kept constant for all sequences and equal to the longest t_p applied (10 ms). Each pulse within a sequence was considered to be perfectly rectangular, as previously validated (20).

The AOM does not completely block the light in between the pulses, but approximately 0.2% of the initial irradiance is transmitted, which artificially increases the counts in an acquired image. This is corrected for by subtracting a background image from each measurement. Inherent bleaching is monitored and corrected for by taking a control image with t_p set to 100 ns after every fourth pulse train. These control images are fitted pixel-wise by a single exponential decay, yielding a set of correction factors for every image.

The focus of the illumination in the propagation direction was obtained by exploiting the fluorescence depletion in an image of free dye in solution. For CW excitation, the signal in the focus of illumination is depleted, because this region is subject to higher irradiances, causing an increase in dark state build-up. The propagation direction of the excitation beam was checked by fitting the intensity distributions along several vertical lines by Gaussian functions, yielding a center position along each line.

All fitting procedures were performed using a Levenberg-Marquardt algorithm, implemented on a custom MATLAB 7.11.0 software. In order to obtain sufficient statistics and to reduce calculation time, the images are resized by binning into pixel sizes of $1 \times 1 \mu\text{m}^2$.

The detection volume in confocal TRAST is estimated by fitting a two-dimensional Gaussian function to the intensity distribution in a non-depleted fluorescence image. For the used alignment, the e^{-2} excitation beam waists in focus were found to be 770 nm and 860 nm.

The recorded SPIM-TRAST curves for Rhodamine 110, Atto 495 and Fluorescein were fitted by Eq. S15, corresponding to an STR model. For Eosin Y, the analysis was based on the four-state S_0I TR model (Eq. 1) to account for its large intersystem crossing rate, which renders the simplifications of the STR model inappropriate. In all cases, the excitation cross section and the $|S_1\rangle$ decay rates were fixed to the values provided in table 1 and k_{0I} was calculated using Eq. S20 (see supporting material).

Cell Preparation Protocol for Live Cell SPIM-TRAST Imaging

Michigan cancer foundation cell line 7 (MCF-7) cells were stained with the fluorescein derivative 5-(and-6)-Carboxyfluorescein diacetate succinimidyl ester (CFDA-SE, Life Technologies Corp., Carlsbad, USA) or the Eosin derivative 5-(and-6)-Carboxyeosin diacetate succinimidyl ester (CEDA-SE, Life Technologies Corp.). MCF-7 cells were routinely cultured in DMEM (Dulbecco's Modified Eagle

Medium) or MEM (Minimum Essential Medium, both Life Technologies Corp.) with 10% FBS (Fetal Bovine Serum, Life Technologies Corp), 0.4% of a 100x non-essential amino acids solution (Sigma Aldrich, St. Louis, MO, USA) and 1 mM sodium pyruvate (M7145, Sigma Aldrich).

First, the adherent cells grown in a 35 mm Petri dishes were washed twice with 37°C PBS, then incubated (10 min, at 37 °C, 5% CO₂) in the staining solution (either 20 µM CEDA-SE or 3 µM CFDA-SE in colorless RPMI without FBS). Afterwards, the cells were washed twice in Roswell Park Memorial Institute medium (RPMI, incl. 10% FBS) and incubated for 30 minutes.

After staining, the adherent cells were washed with PBS and detached from the surface during five minutes of incubation in 0.5 ml of TrypLE (Life Technologies Corp.). The entire volume was transferred to a tube and spinned (5 min at 1500 rpm, Centrifuge 5804 R, Eppendorf AG, Hamburg, Germany). The supernatant was taken off, the cells were dissolved in RPMI (incl. 10% FBS) and the concentration of cells was estimated in a cell counting device.

A 1.2 w% agarose (Boehringer, Mannheim, Germany) in ddH₂O mixture was heated in a microwave (43). For cell mounting, equal volumes of 1.2 w% agarose, 2×RPMI (prepared from powder form, Life Technologies Corp) and the prepared stock of stained MCF-7 cells were mixed. This isotonic liquid was transferred into the sample chamber before it became a gel. Labeled MCF-7 cells were thus randomly distributed in an agarose gel having a similar refractive index as water (26). To minimize scattering from cells present in the illumination beam before the focus (30), but to still find sufficient numbers of MCF-7 cells, a concentration of $\sim 2 \cdot 10^6$ cells per milliliter sample volume was found to be a good trade-off.

Live Cell Imaging

The majority of steps for in vivo SPIM-TRAST are similar to solution measurements. In an initial step, the chamber was loaded with free dye dissolved in agarose and the alignment of illumination and detection foci was done as described previously for the solution measurements. To reduce bleaching and photodamage in cell measurements, the maximum pulse widths were set to 1 ms for CEDA-SE staining and to 0.2 ms for CFDA-SE. The number of applied pulse trains was set to 19 for both dyes and the chosen excitation irradiances are listed in table 1.

The center of the illumination profile was localized as in the solution measurements, exploiting the low background fluorescence from phenol red, contained in the medium. For this procedure, the stage was moved to a region containing no cells and 100× longer illumination times as in cell measurements were used to detect the background fluorescence.

Since the Zeiss C-Apochromat objective used for cell imaging has a better collection efficiency than the one used in solution measurements, the images are resized to smaller bins prior to fitting ($0.6 \times 0.6 \mu\text{m}^2$). The excitation rates were found to be significantly reduced in cell measurements compared to solution

measurements. This can be due to the adjustment of the vertical position of the cells, which might result in a non-perfect overlap between the excitation and detection foci. Moreover, scattering due to cells present in the illumination pathway can decrease the irradiance and may distort the beam profile. To correct for both effects, the effective excitation rate was reduced by a factor between 0 and 1 in the evaluation of the TRAST curves. This reduction factor was determined separately for each cell by an initial fit over the mean fluorescence TRAST curve averaged over the whole cell of consideration. For this procedure, we considered only the first part of the TRAST curve corresponding to the triplet state decay (up to a pulse width of five times the characteristic time of the triplet decay), which was fitted by Eq. S17. In this fit, the excitation rate, calculated according to Eq. S20, was averaged over the cell and the intersystem crossing rate remained fixed to $120 \mu\text{s}^{-1}$ for CEDA-SE and $10 \mu\text{s}^{-1}$ for CFDA-SE. The measurements on free dye in solution do not demand this step, since scattering is negligible in these measurements.

In the histograms used to present the results, the bin sizes are set with respect to the Freedman-Diaconis rule (44), which gives a consistent rule for the binning sizes. The obtained distributions are normalized to the total number of events and present frequency distributions.

Results and Discussion

Free dye in solution

Transition rates for fluorophores in aqueous solution were determined from data recorded by the SPIM-TRAST instrument, following the procedures described in the methods and materials section above. The determined rates were compared to previously published data to validate the overall approach as a tool for measuring these parameters. To verify the estimation of the excitation, SPIM-TRAST measurements were performed at several excitation powers (Fig. 2 and S5).

Fig. 2 A shows representative TRAST curves for measurements on Rhodamine 110 at different peak excitation irradiances, I_0 . Each of the curves corresponds to the mean over an area of $40 \times 18 \mu\text{m}^2$ around the center of illumination. Fig. 2 C and D show the intersystem crossing rates, k_{isc} , and the triplet decay rates, k_t , obtained by separately fitting the experimental TRAST curves recorded in each pixel at different excitations I_0 using Eq. S16 (equivalent to Eq. 1, except that it is based on the simpler STR model). The data points correspond to the mean rates and the error bars to their standard deviations over the mentioned area. Both k_{isc} and k_t are constant within the standard deviations, indicating that the considered fitting model and the assumed excitation rate distribution are suitable. It is important to achieve consistent results within the fitting area, to enable local differences in k_t within cells to be resolvable in the *in-vivo* measurements. Under isotropic conditions, the obtained distributions of k_{isc} and k_t values were found to have standard deviations around 10% of their means, which should make it possible to resolve

local differences of k_{isc} and k_t of a corresponding size in the cellular measurements. Representative histograms for both rates at a selected irradiance are depicted in Fig. 2 B. Fig. 2 C and D show that the k_{isc} and k_t values obtained by fitting do not depend on the applied irradiance. Their standard deviation decreases however for higher irradiances since both the fluorescence brightness and the contrast of the TRAST curves increase the certainty of the fitting results.

For the transitions into and out of the redox state, an irradiance dependence is discernible in Fig. 2 E and F. Such an effect may be caused by higher excited states, which have higher probabilities to be populated at increasing photon densities, and which also show a stronger yield of redox state formation (18). Those transitions are not accounted for within the applied model and the fitting procedure may therefore yield increasing k_{ox} with higher I_0 . However, apart from Rhodamine 110, no increase in oxidation rate could be seen for the other dyes that were tested in this study (see Fig. S5 D,I,N). These dyes were excited at considerably lower irradiances and therefore the population of higher excited states was less probable.

The exact determination of redox rates for freely diffusing fluorophores would require consideration of the diffusion term, as discussed in the supplementary part. Absolutely accurate values of k_{ox} and k_{red} are therefore not accessible within the applied fitting model. Nevertheless, as previously shown by simulations in (23), the model allows relative changes in redox parameters to be followed.

We conducted similar experiments on several organic fluorophores as shown in Fig. S5. The obtained transition rates are provided in tables 2 and S2. All outcomes for SPIM-TRAST imaging were reproduced by confocal TRAST measurements and were in good agreement with literature values, when available. The TRAST curves were acquired starting from pulse widths of 500 ns for Fluorescein and Atto 495 and 700 ns for Rhodamine 110 and Eosin Y, to ensure equilibration between the two singlet states. For Atto 495, a third dark state appears to be populated for pulses longer than 1 ms, which manifests itself in an additional decay in the TRAST curves. To minimize the effect of the additional transition when applying the STR model, we analyzed these data sets only up to 1 ms.

Taken together, the results from the solution measurements on several dyes and at many different I_0 , indicate that the SPIM-TRAST setup, including the data analysis and the assumptions on the illumination, provide a suitable approach to access the transition rates between the transient dark states of fluorophores.

Acquisition with Arbitrary Duty Cycle Pulse Trains

Next, we evaluated under what conditions in the TRAST measurements the assumption is valid, that all molecules are in their ground state at the onset of an excitation pulse. First, simulations were performed following an STR model (Eqs. S23- S24), and based on parameter values for Rhodamine 110 ($k_{I0} = 243 \mu s^{-1}$, $k_{isc} = 1 \mu s^{-1}$, $k_t = 0.4 \mu s^{-1}$, $k_{ox} = 1.4 ms^{-1}$, $k_{red} = 0.15 ms^{-1}$, $\sigma = 2.9 \cdot 10^{-20} m^2$ at $\lambda=491$ nm excitation

wavelength), $I_0 = 25 \text{ kW/cm}^2$, and illumination profile and CEF as found in the setup ($\omega_{0,y} = 40 \text{ }\mu\text{m}$, $\omega_{0,z} = 1.6 \text{ }\mu\text{m}$, $\text{NA} = 1.2$, $n = 1.33$, pixel size $10 \text{ }\mu\text{m}$, magnification 49x). The simulations indicated that the total dark state population at the beginning of each pulse indeed is small at a duty cycle of 1% (see Fig. S3). This indication was then tested experimentally by SPIM-TRAST measurements on Rhodamine 110 in aqueous solution, acquired with different duty cycles η (Fig. 3). The experimental TRAST curves were then analyzed based on the electronic state model in Fig. S1, now using the solution to the system of differential equations obtained when no constraints on η are applied, as derived in the supporting material (Eqs. S23-S24). The intersystem crossing and triplet decay rates obtained based on this model were found to be in good agreement with the results shown in Fig. 2. As the computational effort to process the data is considerably higher, a smaller area of $19.5 \times 7.5 \text{ }\mu\text{m}^2$ and larger pixel sizes $1.5 \times 1.5 \text{ }\mu\text{m}^2$ were analyzed. The mean rates and standard deviations over this area were determined to $k_{\text{isc}} = (1.03 \pm 0.07) \text{ }\mu\text{s}^{-1}$ and $k_t = (0.35 \pm 0.01) \text{ }\mu\text{s}^{-1}$.

From the rate parameters in Fig. 3, no trend is visible between the fitted k_{isc} and k_t and the used duty cycle. These results validate the newly derived model for fitting TRAST data, acquired at arbitrary duty cycles. Second, this new model makes it possible to decrease measurement times significantly without loss of precision. The gain in measurement times comes however at the cost of longer analysis times. Moreover, it can be inferred from these results that the previously used approach is suitable for low duty cycles.

The rates related to the redox state reveal however a dependence on the duty cycle. For this, different causes are conceivable. In particular, the contrast in the TRAST curves decreases with increasing duty cycle, causing less certainty in the outcome. Moreover, as the duty cycle increases, the pause between two pulses, which allows for fluorophore recovery, decreases. As discussed in the previous section, we can, with the model used in this work, only expect to be able to follow relative changes in the redox rates. However, these relative changes can be well reflected in the obtained fitting results, independent of the applied duty cycle.

Live Cell SPIM-TRAST Imaging

The SPIM-TRAST images on MCF-7 cells were analyzed in a different manner from the solution data. First, the excitation rate was determined by a global fit, as described in the Materials and Methods section. Then, the TRAST measurements on cells were fitted in a pixel-wise manner using the $S_{01}\text{TR}$ model (Eq. 1) for CEDA-SE and the STR model (Eq. S15) for CFDA-SE. As in previous works (22), the amount of free parameters was decreased in order to obtain a robust fit at the decreased signal-to-noise ratio (SNR) experienced in live cell imaging. For the Eosin derivate CEDA-SE, we assumed $k_{\text{isc}} = 120 \text{ }\mu\text{s}^{-1}$ and $k_{\text{red}} = 0.01 \text{ ms}^{-1}$, and kept k_t and k_{ox} as free parameters. The chosen k_{isc} value showed the best overall

agreement between experimental data and the fits. As the whole decay of R cannot be followed in the TRAST curves within the maximally applicable pulse widths, k_{red} was fixed to a low boundary in order to increase the stability of the fitting procedure. The lower k_{isc} of CEDA-SE when bound to proteins, as compared to Eosin Y in solution, can be explained by O_2 shielding effects and viscosity changes of the protein environment. Fluorescence lifetime imaging microscopy (FLIM) also yields faster fluorescence decay rates ($1/\tau_f = k_{10} + k_{\text{isc}}$) for Eosin Y, as compared to protein bound CEDA-SE, as summarized in table 1. This further supports the view that k_{isc} is indeed higher for Eosin Y in solution. Although the assumed rates may introduce a bias in the fitted k_t and k_{ox} values, the capability of imaging relative changes between different measurement conditions or local differences within a cell is maintained. The same arguments hold for CFDA-SE, for which we assumed $k_{\text{isc}}=10 \mu\text{s}^{-1}$ and $k_{\text{red}}=0.05 \text{ ms}^{-1}$.

A representative MCF-7 cell stained with CEDA-SE is depicted in Fig. 4. An almost homogeneous labeling could be achieved, as seen in the fluorescence intensity image in Fig. 4 A. The TRAST curves acquired in each of the pixels representing the cell were fitted separately, as described in the paragraph above. Fig. 4 D shows the average fluorescence TRAST curve and the average of the fit applied to each pixel over the pixels of the MCF-7 cell in Fig. 4 A. The residuals were found to be slightly larger than in the solution measurements, which could be due to small cell movements, or the fixed rates possibly deviating from the real values. However, the TRAST curve approaches the measurement data properly, and allows for the spatially resolved determination of k_t as shown in Fig. 4 B. The average and standard deviation of k_t over the pixels of the cell in Fig. 4 B read $k_t = (91.0 \pm 10.2) \text{ ms}^{-1}$. In Fig. 4B, the k_t image shows gradients, with faster rates being measured in the cell center, a feature we found for practically all the measured cells. No obvious spatial relation between the fluorescence image and the obtained k_t -image can be noticed, indicating that the k_t -image does not seem to be biased by the local fluorescence intensity levels. Instead, the k_t rates can be expected to be proportional to the concentration of molecular oxygen, which is a predominant triplet state quencher (17). The found k_t rate distribution in the cells thus rather indicates a depletion of oxygen in the periphery of the cell.

In contrast, the k_{ox} -images (Fig. 4 C) revealed patterns much more influenced by noise. This is partly a result of that the maximum pulse width was shorter than the full redox decay in the TRAST curves. Moreover, due to the large triplet yield, CEDA-SE mainly populates its triplet state upon excitation, and thus has its strengths primarily for the investigation of triplet states. The mean rate and the standard deviation over the cell in the k_{ox} image in Fig. 4 D was determined to $k_{\text{ox}} = (0.4 \pm 0.2) \text{ ms}^{-1}$.

To distinguish between reasonably fitted and noise governed outcomes, the starting value of k_{ox} in the fitting procedure of the experimental data was set to values below the detectable limit (1 s^{-1}). After fitting, a distinct peak in the distribution of k_{ox} could be well separated from the lower threshold, and all

determined oxidation rates, which were equal or lower than this starting value, were disregarded. In Fig. S6 A, a histogram of the determined k_{ox} values and their 95% confidence intervals is shown, as determined in 88 measurements on 21 different cells. From this histogram, it is clear that for the disregarded pixel values falling below the threshold, the confidence intervals are significantly larger. This further justifies the omission of these values from subsequent analyses.

In Fig. 4E, the obtained rates from the 399 pixels comprising the image of the cell are displayed in a k_{ox} - k_t 2D-histogram. The obtained k_t distribution in Fig 4E is broader than in the case of solution measurements (Fig. 2) where a comparable amount of pixels is considered. As a clear spatial pattern is obtained in the k_t images, not related to the local fluorescence intensity levels, the main reason for the broadening of the k_t distribution is likely the heterogeneous intracellular environment and oxygen depletion. Moreover, the k_{ox} - k_t 2D-histogram reveals an inclination, indicating a correlation between the fitted k_t and k_{ox} values.

To further investigate these findings, we compiled a corresponding 2D-histogram from 88 measurements on 21 different cells, comprising a total of 40590 pixels (Fig. S6 B). This histogram includes data from measurements on different days since the day-to-day variation was small compared to the intrinsic spread. The k_t - and the k_{ox} -distributions show distinct peaks. Their means and standard deviations were found to be: $k_t = (92.5 \pm 19.7) \text{ ms}^{-1}$ and $k_{ox} = (0.5 \pm 0.3) \text{ ms}^{-1}$. Again, the distribution of the triplet decay rates is broader than that from the solution measurements.

Concerning the k_{ox} -distribution, both the intracellular distributions in reducing and oxidating agents, and the limited observable t_p range of the redox decay in the TRAST curves can be possible reasons for the large spread of k_{ox} -values. Nevertheless, the distribution reveals a distinct peak and thus a relative, mean oxidation rate. SPIM-TRAST measurements thus provide a relative measure for the detection of the redox state of investigated cells, potentially to be used e.g. to screen potentially toxic substances and their influence on cellular redox levels.

The inclination of the 2D-histograms for CEDA-SE in Fig. 4E is seen also in the histogram presented in Fig. S6 B, representing the compiled results for many cells. The inclination could at least partly be due the fact that both values are not estimated independently. Another possible reason for the correlation between k_{ox} and k_t could be the oxygen concentration, since oxygen can enhance both triplet state decay rates and oxidation rates of fluorophores (18). The tilted ellipse may thus be a manifestation of different local oxygen concentrations in the cells.

Similarly, we also performed measurements on MCF-7 cells, using the Fluorescein derivative CFDA-SE (Fig. 5 A-D). The previous discussion for CEDA-SE is also valid for CFDA-SE. However, the applied irradiance, the determined rates and hence the dark state build-up are different. The following mean and standard deviations were obtained from the pixel values of the cell presented in Fig. 5: $k_t = (179.5 \pm 14.9) \text{ ms}^{-1}$ and $k_{ox} = (5.0 \pm 0.9) \text{ ms}^{-1}$. Compared to CEDA-SE, the Fluorescein derivate is

better suited for imaging k_{ox} . Although its high bleaching rate does not allow using illumination schemes with pulse widths over 200 μs , a larger part of the redox process is still visible in the curves, since the rates are significantly faster. Therefore in general only very few pixels had to be omitted for reasons of non-convergence to a reasonable solution (as described above for CEDA-SE).

Similar to the results on CEDA-SE, the 2D-histograms indicate a slight correlation between the k_t and k_{ox} rates (Fig. 5E). The same holds, when the results from 27 measurements on 13 different MCF-7 cells are taken together, as shown in Fig. S6 C. Both the triplet decay and the oxidation rates appear to be distributed around central, unique peaks, with the following means and standard deviations: $k_t = (180.9 \pm 47.3) \text{ms}^{-1}$ and $k_{\text{ox}} = (5.3 \pm 3.0) \text{ms}^{-1}$. The anticipated reason for the broadness of the distributions is also in this case the heterogeneous intracellular environment. Moreover, Fluorescein is known to have a strongly pH-dependent brightness (45, 46) and the corresponding protonation-deprotonation reactions may therefore also partly contribute to the broadening.

Conclusions

We have demonstrated a novel combination of Transient State (TRAST) imaging and Single Plane Illumination Microscopy (SPIM). By this concept, the overall excitation light exposure of the specimen can be considerably reduced. Transition rates of free dye in solution were found to be determined accurately, and imaging of oxygen gradients, as well as relative oxidation rates in live cells was demonstrated. A new theoretical framework was derived, and validated by simulations and experiments, which allows the analysis of TRAST data, acquired with arbitrary duty cycle pulse trains. This approach makes it possible to increase the obtainable frame rates of TRAST imaging by more than a factor of 10 compared to previous experiments. Taken together, these developments reduce the constraints further and extend the applicability of TRAST imaging for live cells studies.

Supporting material

Derivations, simulations, supporting figures, tables and equations are available at [www.biophys.org/biophysj/supplemental/S0006-3495\(XX\)XXXXX-X](http://www.biophys.org/biophysj/supplemental/S0006-3495(XX)XXXXX-X).

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Supporting Citations

References (47–53) appear in the Supporting Material.

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Fluorophore	c [μM]	I_0 [kW/cm ²]	$\sigma \cdot 10^{-20}$ [m ²]	k_f [μs^{-1}]
Rh110	0.04	~ 25	2.9 (39, 40)	244 (39)
Fluorescein	0.30	~ 10	3.0 (39)	250 (39)
Atto 495	0.44	~ 10	2.9 (41)	1042 $\pm$ 19
Eosin Y	2.00	~ 2	1.7 (42)	1029 $\pm$ 11
CFDA-SE	-	~ 5	3.0 (39)	342 $\pm$ 14
CEDA-SE	-	~ 1	1.7 (42)	583 $\pm$ 25

Table 1: Concentrations c , peak irradiances I_0 and assumed parameter values used in the data analysis of the free dye measurements in PBS and for the live cell imaging. The provided irradiances correspond to an approximate value in the center of the light sheet. The $|S_i\rangle$ decay rates $k_f = 1/\tau_f = k_{10} + k_{isc}$ given with a standard deviation are determined by FLIM as described in the supporting material and are, if available, in good agreement with previously reported values (41, 42). For cytosolic measurements, the fluorophore concentration cannot be determined precisely.

		$k_{isc} [\mu s^{-1}]$	$k_t [\mu s^{-1}]$
Rh110	SPIM-TRAST	1.05±0.11	0.37±0.04
	Conf TRAST	1.18±0.06	0.33±0.02
	Reference	1.3±0.1 (39)	0.49±0.03 (39)
		1.0±0.1 (39)	0.45±0.04 (39)
		1.2±0.2 (23)	0.37±0.02 (23)
Fluorescein	SPIM-TRAST	9.29±0.55	0.58±0.03
	Conf TRAST	10.30±0.60	0.42±0.03
	Reference	13.5±1.0 (39)	0.66±0.04 (39)
		11.1±1.0 (39)	0.45±0.04 (39)
Atto 495	SPIM-TRAST	33.64±4.06	1.12±0.13
	Conf TRAST	36.43±2.39	0.95±0.05
Eosin Y	SPIM-TRAST	746±34	0.48±0.01
	Conf TRAST	757±79	0.42±0.02
	Reference	420±50 (42)	
		840±80 (48)	

Table 2: Triplet transition rates for Rhodamine 110 (Rh110), Fluorescein, Atto 495 and Eosin Y in PBS (pH 7.4). The measurements were performed in SPIM-TRAST (720 pixels) and confocal (Conf) TRAST mode (12 measurements). All error bars correspond to the standard deviations. If found, reference values are provided. The cited values for Fluorescein were measured in Tris buffer (pH 8.2), whereas the TRAST measured have been performed in PBS at pH 7.4. However, for both solvents, the majority of Fluorescein molecules is expected to be deprotonated.

Figure titles and legends

Figure 1: Setup and measurement principle for SPIM-TRAST imaging.

A pulse train is created by an AOM from the emission of a CW laser. The use of a cylindrical lens results in a sheet-like excitation profile in the sample. The fluorophores in the sample can be described by a simplified, electronic four-state model, comprising two singlet, one triplet and one redox state. The detection takes place perpendicularly to the excitation sheet and the image is focused and detected on an EMCCD chip. For each pulse train, with a certain width of the pulses, t_p , one image, representing the total acquired fluorescence, is detected. When plotting this signal as a function of t_p , as used in the excitation schemes, a TRAST curve (bottom left) is obtained in each and every pixel. The acquired points are subsequently fitted using Eq. 1 or S15, depending on the considered electronic state model, and Eqs. S19-S20.

Figure 2: TRAST measurement data from free Rhodamine 110 in solution, recorded at different excitation irradiances.

I_0 indicates peak irradiances used in the measurements. Excitation modulation duty cycle of all measurements: 1%. **(A)**: Representative TRAST curves (+) showing the normalized average fluorescence intensity over a rectangle of 40x18 pixels, each 1x1 μm^2 large, and the variation of this intensity with the excitation pulse width. For every I_0 , each of the pixels is fitted according to Eqs. S15 and S19-S20 and the obtained average fitting curves (-) approximate well the measurement data as the residuals appear to be randomly distributed and do not reveal any systematic deviations. **(C, D, E, F)**: From every fitted TRAST measurement, a set of four rates is extracted from the TRAST curve of every pixel and the averages and standard deviations of the rates within the mentioned region is shown. The measurements were taken for several powers, with the peak irradiance I_0 in the center of excitation ranging from $I_0 = 11 \text{ kW/cm}^2$ to $I_0 = 33 \text{ kW/cm}^2$ (for clarity not all of these measurements were shown as TRAST curves in (A)). All standard deviations for k_{isc} and k_t over the 720 pixels are around 10% of their means, or lower. **(B)** A representative histogram for a measurement at $I_0 = 24 \text{ kW/cm}^2$ is provided, showing distinct peaks in the distributions of k_{isc} and k_t .

Figure 3: Data from TRAST measurements on free Rhodamine 110 in solution, acquired with different duty cycles, η , of the excitation pulse trains.

Excitation irradiance used in the measurements: $I_0 = 33 \text{ kW/cm}^2$. All values depicted in the TRAST curves of **(A)** correspond to the mean fluorescence averaged over a 65 pixels large area, each of them $1.5 \times 1.5 \text{ }\mu\text{m}^2$ big. The TRAST curves were fitted pixel-wise according to the newly derived model (Eqs. S22-S24). The residuals indicate that the model accurately represents the measurement data. **(B, C, D, E)**: The mean and standard deviations of the determined rate parameters within the mentioned region are shown for each of the TRAST curves in **(A)**, recorded at different η of the excitation pulse trains. The fits yield consistent results for intersystem crossing **(B)** and triplet decay rates **(C)**, independent of the length of the interval between the pulses within each excitation scheme. Their means are in good agreement with the simplified model (Fig. 2). The obtained redox rates **(D and E)** however, reveal a slight dependence on η .

Figure 4: Representative measurement on a CEDA-SE stained MCF-7 cell.

The excitation was performed by the previously characterized light sheet at a peak irradiance $I_0 = 0.8 \text{ kW/cm}^2$ and all fits are based on a $S_{01}\text{TR}$ model (Eq. 1). **(A)** shows the fluorescence image of the cell, **(B)** the k_t -image and **(C)** the k_{ox} -image. All scale bars correspond to $8 \text{ }\mu\text{m}$. **(D)**: The acquired average TRAST curve (blue) over the whole cell is adequately fitted by the proposed model (red). **(E)**: The 2D-histogram of the k_t and k_{ox} values from **(B)** and **(C)**.

Figure 5: Representative measurement on a CFDA-SE stained MCF-7 cell.

Representative fluorescence image **(A)**, k_t -image **(B)** and k_{ox} -image **(C)** recorded at $I_0 = 8.9 \text{ kW}/\mu\text{m}^2$ and fitted pixel-wise with a STR model (Eq. S16) (scale bar $8 \text{ }\mu\text{m}$). **(D)**: The acquired mean TRAST (blue) and fitted (red) curve averaged over the whole are in good agreement. **(E)**: The 2D-histogram of the k_t and k_{ox} values presented in **(B)** and **(C)**.

Figures

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Supporting Material

Electronic State Model

The $S_{01}TR$ model of Fig. 1 includes some simplifications, as summarized below. In the model, only oxidations originating from the triplet state are considered, although such transitions can also occur from excited singlet states (1). Since the redox- rates k_{ox} and k_{red} are much slower than all the other rates of the $S_{01}TR$ model, $|S_0\rangle$, $|S_1\rangle$ and $|T\rangle$ are after onset of excitation usually equilibrated well before population and equilibration of $|R\rangle$ has taken place. For e.g. organic dyes in air-saturated aqueous solution, redox state build-up typically equilibrates in the range of milliseconds after onset of excitation, much later than the triplet formation, which is usually equilibrated within microseconds (2, 3). In the $S_{01}TR$ model, the photoinduced formation rates of $|R\rangle$, from $|S_1\rangle$, and from $|T\rangle$, thereby differ only by a scaling factor, which justifies considering oxidations only to originate from the triplet state (4).

Higher excited singlet and triplet states are disregarded in the model, because the corresponding excitation cross section is for conventional organic dyes smaller than the cross section from the ground to the first excited singlet state (5), and such higher excited states usually decay orders of magnitude faster, within picoseconds (6).

Moreover, for any sample with a spatial extension, the excitation rate, k_{01} , depends on the spatial position as defined by the illumination profile and has in case of pulsed excitation also a temporal dependence. For the sake of simplicity, the spatial dependence of k_{01} is not denoted explicitly in the following. The same holds for k_{isc} , k_t , k_{ox} and k_{red} , which also may have spatial dependencies in non-homogenous samples. Regarding the temporal dependence of k_{01} , rectangular excitation pulse trains are used in this study, with time-independent excitation rates within the pulses.

The probabilities of populating the four different states of a fluorophore, described by the $S_{01}TR$ model of Fig 1, and with the fluorophore subject to an excitation pulse with constant k_{01} , can be derived from the general form of a set of first order differential equations.

$$\frac{d}{dt}\vec{S}(t) = \mathbf{M}\vec{S}(t), \quad (S1)$$

where the state vector $\vec{S}$ and the transition matrix $\mathbf{M}$ take the form:

$$\vec{S}_{S_{01}TR} = \begin{pmatrix} S_0(t) \\ S_1(t) \\ T(t) \\ R(t) \end{pmatrix}, \quad \mathbf{M}_{S_{01}TR} = \begin{pmatrix} -k_{01} & k_{10} & k_t & k_{red} \\ k_{01} & -k_{10} - k_{isc} & 0 & 0 \\ 0 & k_{isc} & -k_t - k_{ox} & 0 \\ 0 & 0 & k_{ox} & -k_{red} \end{pmatrix} \quad (S2)$$

For the majority of organic dyes, k_{01} and the de-excitation rate, k_{10} , are orders of magnitude faster than the triplet-related transition rates. The introduced model can then be simplified by unifying both singlet states, as they are equilibrated before any significant triplet state build-

Figure S1: The simplified Jablonski diagram for a four-state model (Fig. 1) can be narrowed down to a three-level system if at onset of excitation both singlet states are equilibrated before the transient dark states are populated to a noteworthy extent.

up occurs (Fig. S1). The system is then reduced to a three-state STR model. Such an assumption requires the introduction of an effective intersystem crossing rate k'_{isc} , which corresponds to the product of the actual intersystem crossing and the $|S_1\rangle$ population at equilibrium (3) :

$$k'_{isc} = k_{isc} \frac{k_{01}}{k_{01} + k_{10}} \quad (S3)$$

For a STR model Eq. S2 simplifies to:

$$\vec{S}_{STR} = \begin{pmatrix} S(t) \\ T(t) \\ R(t) \end{pmatrix}, \quad \mathbf{M}_{STR} = \begin{pmatrix} -k'_{isc} & k_t & k_{red} \\ k'_{isc} & -k_t - k_{ox} & 0 \\ 0 & k_{ox} & -k_{red} \end{pmatrix} \quad (S4)$$

For both the $S_{01}TR$ and the STR model, the sum of all elements of $\vec{S}$ are assumed to be conserved and equal to 1 for all times. This assumption presumes constant concentrations and hence no photo-bleaching. Although photo-bleaching is present to some extent in our experiments, it can be properly corrected for, and the assumption is therefore still valid.

It should be noted that the assumptions namely $k_{01}, k_{10} \gg k_{isc}, k_t$, made for introducing the STR model, is applicable for a wide range of organic dyes, but does not hold for the rates found for Eosin Y. Thus, this fluorophore's dynamics have to be approached using the $S_{01}TR$ model.

Sets of differential equations like Eq. S1 have been solved before (3, 4, 7). A unique solution of the set of differential equations can only be found for a given initial condition. Often, only $|S_0\rangle$ is assumed to be populated at the time $t_0=0$. Here, a general solution is derived by introducing a propagator $\mathbf{U}(t, t_0)$, which, based on an initial state $\vec{S}(t_0)$, maps the corresponding vector at time t (8).

$$\begin{aligned}\bar{S}(t) &= \mathbf{U}(t, t_0) \bar{S}(t_0) \\ U(t, t_0) &= \exp\left(\int_{t_0}^t \mathbf{M}(t') dt'\right)\end{aligned}\quad (\text{S5})$$

The propagator, $\mathbf{U}(t, t_0)$, is thus a matrix exponential and the integral can be evaluated trivially as long as $\mathbf{M}$ is time-independent. Since the exponential of a diagonal matrix consists simply of the exponentials of the diagonal elements, a transfer to the eigenspace of $\mathbf{M}$ is useful. Both, $\mathbf{M}_{S_0TR}$ and $\mathbf{M}_{STR}$ are diagonalizable. We define $\mathbf{P}$ as the eigenvector matrix of $\mathbf{M}$, containing in the i -th column the eigenvector $\bar{v}_i$ and $\mathbf{\Lambda}$ as the corresponding eigenvalue matrix having as diagonal elements the eigenvalues λ_i of $\mathbf{M}$: $\mathbf{\Lambda}_{ij} = \lambda_i \delta_{ij}$.

Under these conditions the propagator can be found using the projection matrix $\mathbf{P}$ to the eigenspace and its inverse $\mathbf{P}^{-1}$. The inverse projection exists in every case, as $\mathbf{P}$ consists of linearly independent basis vectors. Hence, its determinant cannot be equal to zero.

$$\mathbf{U}(t, t_0) = \mathbf{P} \exp(\mathbf{\Lambda}(t - t_0)) \mathbf{P}^{-1} \quad (\text{S6})$$

From now on, the solution will be applied to the STR model, but the solution to S_0TR follows an analogous derivation. The projection of $\mathbf{M}_{STR}$ into the eigenspace reads:

$$\mathbf{P} = \begin{pmatrix} k_{\text{red}}(k_t + k_{\text{ox}}) & k_t \lambda_2 + k_{\text{red}}(k_t + k_{\text{ox}}) & k_t \lambda_3 + k_{\text{red}}(k_t + k_{\text{ox}}) \\ k'_{\text{isc}} k_{\text{red}} & (k'_{\text{isc}} + \lambda_2)(k_{\text{red}} + \lambda_2) & (k'_{\text{isc}} + \lambda_3)(k_{\text{red}} + \lambda_3) \\ k'_{\text{isc}} k_{\text{ox}} & k_{\text{ox}}(k'_{\text{isc}} + \lambda_2) & k_{\text{ox}}(k'_{\text{isc}} + \lambda_3) \end{pmatrix} \quad (\text{S7})$$

$\mathbf{P}$ is based on the following eigenvalues λ_i :

$$\begin{aligned}\lambda_1 &= 0 \\ \lambda_{2,3} &= -\frac{k'_{\text{isc}} + k_t + k_{\text{ox}} + k_{\text{red}}}{2} \mp \frac{1}{2} \sqrt{(k'_{\text{isc}} + k_t + k_{\text{ox}} + k_{\text{red}})^2 - 4(k'_{\text{isc}} k_{\text{red}} + k_t k_{\text{red}} + k_{\text{ox}} k_{\text{red}} + k'_{\text{isc}} k_{\text{ox}})}\end{aligned}\quad (\text{S8})$$

The first eigenvalue corresponds to the equilibrium situation, while λ_2 and λ_3 are the inverse characteristic times of triplet and redox equilibration. As long as the eigenvalues are clearly separated (i.e. $|\lambda_2| \gg |\lambda_3|$), the eigenvalues can be approximated as (4):

$$\lambda_2 = -(k'_{\text{isc}} + k_t) \text{ and } \lambda_3 = -(k'_{\text{ox}} + k_{\text{red}}) \quad (\text{S9})$$

$$\text{with } k'_{\text{ox}} = k_{\text{ox}} \frac{k'_{\text{isc}}}{k'_{\text{isc}} + k_t}.$$

When observing fluorescence emission, the evolution of the singlet state population, $S(t)$, is of particular interest. An expression for $S(t)$ upon onset of continuous wave excitation is accessed by applying the propagator to an initial vector $\bar{S}(t_0)$. Using the conservation of probability $S(t) = 1 - T(t) - R(t)$, $\forall t$ yields:

$$S(t) = \frac{k_{\text{red}}(k_t + k_{\text{ox}})}{\lambda_2 \lambda_3} + \frac{k'_{\text{isc}} + k_t + \lambda_3}{\lambda_2(\lambda_2 - \lambda_3)} \left(k'_{\text{isc}} + \lambda_2 T(t_0) + \frac{k_{\text{red}}(k_{\text{red}} + \lambda_3) - k'_{\text{isc}} k_{\text{ox}}}{k_{\text{ox}}} R(t_0) \right) e^{\lambda_2(t-t_0)} \\ - \frac{k'_{\text{isc}} + k_t + \lambda_2}{\lambda_3(\lambda_2 - \lambda_3)} \left(k'_{\text{isc}} + \lambda_3 T(t_0) + \frac{k_{\text{red}}(k_{\text{red}} + \lambda_2) - k'_{\text{isc}} k_{\text{ox}}}{k_{\text{ox}}} R(t_0) \right) e^{\lambda_3(t-t_0)} \quad (\text{S10})$$

When taking $\bar{S}(t_0) = [1, 0, 0]^T$ as an initial condition, and considering that $k'_{\text{isc}}, k_t \gg k_{\text{ox}}, k_{\text{red}}$, a Taylor series expansion yields a simplified evolution of the singlet state population:

$$S(t) = \frac{k_{\text{red}} k_t}{(k'_{\text{isc}} + k_t)(k'_{\text{ox}} + k_{\text{red}})} + \frac{k'_{\text{isc}}}{k'_{\text{isc}} + k_t} e^{\lambda_2(t-t_0)} + \frac{k_t k'_{\text{ox}}}{(k'_{\text{isc}} + k_t)(k'_{\text{ox}} + k_{\text{red}})} e^{\lambda_3(t-t_0)} \quad (\text{S11})$$

As this model assumes an instantaneous equilibration between both singlet states, the fraction of S , which corresponds to $|S_1\rangle$, is obtained by considering the equilibrium conditions of a hypothetical two-level system:

$$S_1(t) = \frac{k_{01}}{k_{01} + k_{10}} S(t) \quad (\text{S12})$$

Average fluorescence signal upon pulsed excitation schemes of low duty cycle

The emitted fluorescence $F_S(t)$ from a fluorophore in the sample is directly proportional to the population of the excited singlet state:

$$F_S(t) = \Phi_F k_{10} S_1(t) = \Phi_F k_{10} \frac{k_{01}}{k_{01} + k_{10}} S(t) \quad (\text{S13})$$

TRAST imaging is based on integrating the fluorescence over an entire pulse train, consisting of N excitation pulses with constant height, width t_p and period time T . The corresponding duty cycle is defined as $\eta = \frac{t_p}{T}$. Consequently, the mean signal, that is the total fluorescence in the sample, averaged over the total exposure time Nt_p of one excitation pulse train, reads:

$$\langle F_S \rangle_{Nt_p}(t_p) = \Phi_F k_{10} \frac{k_{01}}{k_{01} + k_{10}} \sum_{j=0}^{N-1} \left(\int_{jT}^{jT+t_p} \frac{1}{Nt_p} S(t) dt \right) \quad (\text{S14})$$

Here k_{01} can be considered as time-independent, even though the excitation is pulsed. Only the on-state yields a fluorescence response within the STR model. For our rectangular pulse trains with constant height of the excitation pulses, all fluorescence contributing to $\langle F_S \rangle_{Nt_p}(t_p)$ is generated by a constant k_{01} . In case an S_{01} TR model is assumed, there is some fluorescence for a short time after a pulse has ended. This corresponds to the still excited molecules, which emit fluorescence, but are relaxed within nanoseconds after the excitation is turned off. The amount of photons released during this time is small compared to the signal acquired during one pulse, which is always chosen to be at least 100 nanoseconds long, and can thus be neglected. Consequently, all summands in Eq. S14 become equivalent when the time is

formally reset to zero after each pulse period, as the initial condition at the onset of each pulse is always identical. This holds if the pause between two pulses is long enough to allow for a complete relaxation of the fluorophore into its ground state. Under these conditions, the mean fluorescence response to a pulse train is obtained from Eq. S11 and S14:

$$\langle F_S \rangle_{Nt_p}(t_p) = \Phi_F k_{10} \frac{k_{01}}{k_{01} + k_{10}} \left(\frac{k_{\text{red}} k_t}{(k'_{\text{isc}} + k_t)(k'_{\text{ox}} + k_{\text{red}})} + \frac{k'_{\text{isc}}}{\lambda_2 t_p (k'_{\text{isc}} + k_t)} (e^{\lambda_2 t_p} - 1) + \frac{k'_{\text{isc}} k_{\text{ox}}}{\lambda_3 t_p (k'_{\text{isc}} + k_t)(k'_{\text{ox}} + k_{\text{red}})} (e^{\lambda_3 t_p} - 1) \right) \quad (\text{S15})$$

To compare Eq. S15 to Eq. 1 in the main article, it can be rewritten using the approximated eigenvalues introduced in Eq. S9.

$$\langle F_S \rangle_{Nt_p}(t_p) = \Phi_F \frac{k_{10} k_{01}}{k_{01} + k_{10}} \frac{1}{t_p} \left(\frac{k'_{\text{isc}}}{\lambda_2^2} (1 - e^{\lambda_2 t_p}) - \frac{k'_{\text{isc}} k_{\text{ox}}}{\lambda_2 \lambda_3^2} (1 - e^{\lambda_3 t_p}) + \frac{k_{\text{red}} k_t}{\lambda_2 \lambda_3} t_p \right) \quad (\text{S16})$$

In this notation, the two models can be seen to be equal for $t_p \gg |1/\lambda_1|$, (as in the main text, the eigenvalue $\lambda_1 = -(k_{01} + k_{10})$, represents from now on the equilibration of the singlet states) indicating that the STR model is valid as long as all t_p -values are larger than the singlet state equilibration time.

The $S_{01}T$ model can be derived from Eq. 1 by considering the case of a $S_{01}TR$ model, where the dark state is much faster depopulated than populated: i.e.: $k_{\text{red}} \gg k'_{\text{ox}}$

$$\langle F_S \rangle_{Nt_p}(t_p) = \frac{F_0}{t_p} \left(\frac{1}{\lambda_1} (1 - e^{\lambda_1 t_p}) + \frac{k'_{\text{isc}}}{\lambda_2^2} (1 - e^{\lambda_2 t_p}) - \frac{k_t}{\lambda_2} t_p \right) \quad (\text{S17})$$

Derivation of the effective excitation rate.

In the TRAST measurements, each pixel on the CCD array, assigned a position (α, β) , is detecting the fluorescence originating from a volume centered around a position (x_α, y_β) in the sample (from the focal plane of the detection objective $z = 0$). The detected fluorescence $F_D(\alpha, \beta, t)$ in a single pixel of the CCD with coordinates (α, β) can be expressed by a two-dimensional convolution:

$$F_D(\alpha, \beta, t) = \phi_D \iiint c(\bar{r}, t) F_S(x, y, z, t) \text{CEF}(x - x_\alpha, y - y_\beta, z) dx dy dz \quad (\text{S18})$$

Here, ϕ_D is the detection efficiency of the detection path and the CCD and $c(\bar{r}, t)$ denotes the concentration of fluorophores. F_S is given by Eq. S13, and CEF is the collection efficiency function (3). Fluctuations of the dye concentration are neglected (4, 7), since TRAST comprises short pulses, which yield only low photodegradation. Furthermore, bleaching is corrected for separately.

For a known excitation rate distribution, the acquired fluorescence signal expected upon a pulse train excitation scheme comprising equally long pulses with large duty cycles can be predicted from Eq. 1 or S11 together with Eq. S18. However, when fitting Eq. S18 to experimental data, the execution of the convolution integral is time consuming as it involves several Fourier transforms and a three-dimensional calculation of the expected fluorescence in the sample. Therefore, the model is scaled down to a two-dimensional system. The fluorescence $\langle F_S \rangle_{Nt_p}(\alpha, \beta, t_p)$ in a hypothetical plane is calculated directly using a two dimensional effective excitation rate $k_{01}^{\text{eff}}(\alpha, \beta, t_p)$ calculated for each pixel. $k_{01}^{\text{eff}}(\alpha, \beta, t_p)$ comprises two effects, the irradiance distribution in the sample as well as the collection efficiency. The coordinate system is chosen as before: the light sheet propagates in $+x$ -direction, is collimated in y , and centered at $z=0$. The normalized fluorescence signal detected by a pixel (α, β) on the CCD would then read:

$$\bar{F}_D(\alpha, \beta, t_p) = \phi_D c \frac{\langle F_S \rangle_{Nt_p}(\alpha, \beta, t_p)}{F_{\text{norm}}(\alpha, \beta, t_p)} \quad (\text{S19})$$

Here, $F_{\text{norm}}(\alpha, \beta, t_p)$ is a normalization factor, corresponding to the value of $\langle F_S \rangle_{Nt_p}(\alpha, \beta, t_p)$ in the absence of transitions to $|T\rangle$ or $|R\rangle$ (see Eq. S21 below). The fluorescence in the sample has a spatial distribution, mainly due to the applied illumination profile. In order to account for the collection efficiency in the real system, we define the effective excitation rate as (3):

$$k_{01}^{\text{eff}}(\alpha, \beta, t_p) = \frac{\iiint k_{01}(x, y, z) \langle F_S \rangle_{Nt_p}(x, y, z, t_p) \text{CEF}(x - x_\alpha, y - y_\beta, z) \mathbf{d}x \mathbf{d}y \mathbf{d}z}{\iiint \langle F_S \rangle_{Nt_p}(x, y, z, t_p) \text{CEF}(x - x_\alpha, y - y_\beta, z) \mathbf{d}x \mathbf{d}y \mathbf{d}z} \quad (\text{S20})$$

In this approach, k_{01}^{eff} is weighted with the fluorescence in the sample. As given by Eq. 1, it depends on the rates of transition, especially the excitation rate itself. The quality of this model requires a proper choice of these parameters when describing the three-dimensional, theoretical mean fluorescence $\langle F_S \rangle_{Nt_p}$ in the sample. Ideally, it is a Gaussian shaped response, but fluorescence depletion may distort this profile. At the irradiances used in TRAST, we can neglect saturation effects caused by excessive $|S_1\rangle$ build-up (9, 10). As a further simplification, fluorescence depletion due to dark state build-up may also be neglected when calculating k_{01}^{eff} . This assumption is equivalent to considering two singlet states, which equilibrate in the nanosecond range. Since the applied pulses are always much longer than this equilibration time, all excitation schemes would be equally affected, and the effective excitation rate becomes pulse width invariant (see Fig. S3 B). However, since TRAST is based on populating dark states, we expected an improvement of the model, by taking non-fluorescent states into account when calculating k_{01}^{eff} , using the full, pulse width dependent Eq. 1 for the mean fluorescence distribution in Eq. S20. Within this approach, k_{01}^{eff} decreases with increasing dark state build-up. This decay of k_{01}^{eff} with increasing pulse width has two major effects: Firstly, it corrects for fluorescence depletion, which is wanted on purpose.

Secondly, it makes pulse trains with different pulse widths incomparable. It has been assumed that longer pulses yield lower average fluorescence rates within the pulses, which is accounted for by finding an adjusted $k_{01}^{\text{eff}}(\alpha, \beta, t_p)$. On the other hand, a decreased excitation rate also causes a drop of the fluorescence, due to a lower build-up of $|S_1\rangle$. To correct for this additional effect, Eq. S19 contains a normalization factor, which is dependent on the excitation scheme, namely its pulse width. In essence, it represents the undepleted fluorescence signal for an assumed excitation rate. The relation, describing this particular value, is Eq. S14 in the limit $t_p \rightarrow 0$. Consequently, the expression used for fitting CCD-based SPIM-TRAST measurements is Eq. S19, with $F_{\text{norm}}(\alpha, \beta, t_p)$ given by:

$$F_{\text{norm}}(\alpha, \beta, t_p) = \Phi_F k_{10} \frac{k_{01}^{\text{eff}}(\alpha, \beta, t_p)}{k_{01}^{\text{eff}}(\alpha, \beta, t_p) + k_{10}} \quad (\text{S21})$$

Simulations on the Effective Excitation Rate

The performance of the approach of Eqs. S19-S21 was further tested by simulations. The simulations were performed for Eosin Y in solution, using the parameters mentioned in Fig S3, and for an area of $40 \times 18 \mu\text{m}^2$, comprising $1 \times 1 \mu\text{m}^2$ large pixels. The larger side of this rectangle is oriented in parallel to the illumination beam propagation. Its length is chosen with respect to the Rayleigh range of the excitation profile. The shape of the illumination beam is simulated using parameters found in the setup (see Fig. S3). All excitation cross sections and the fluorescence rates are selected with respect to table 1.

Since the calculation of k_{01}^{eff} yields slightly better results when the fluorescence saturation due to dark states is not disregarded, we decided to base the data analysis in this study on that approach. The derived fitting model is a powerful tool as it simplifies the real system and reduces computational processing. However, it assumes starting values of transition rates in Eq. S20. Since the overall objective is to extract exactly these rates, the initial guesses ought to be close to the real values and the procedure has to be iterated until convergence occurs.

With increasing pulse width of the pulsed excitation scheme in TRAST and thus growing dark state build-up, the fluorescence response to a Gaussian shaped excitation flattens towards a rectangular profile as indicated in Eq. 1 and as shown in Fig. S3A. When calculating the effective excitation rate (Fig. S3 B) for the pixel, which images the center of the light sheet, there is no pulse width dependence, if fluorescence saturation due to dark state build-up is neglected (blue line). However, with the dark state build-up taken into account, k_{01}^{eff} reveals the expected pulse width dependent decrease. Although the depicted decrease amounts only to a few percent, its effect still needs to be evaluated.

Fig. S3 C compares both approaches. As a reference, we calculated the convolution integral (Eq. S23) for several pulse widths (+ in Fig. S3 C). Second, we used Eq. S19-21 with and without taking dark state build-up into account. Although the deviations are small, the model which disregards fluorescence saturation due to dark state build-up (blue line) does not fit the simulated data as well, as when the depletion is taken into account (red line).

Figure S2: Simulations performed for Eosin Y, assuming typical transition rates ($k_{10} = 329 \mu s^{-1}$, $k_{isc} = 700 \mu s^{-1}$, $k_t = 0.4 \mu s^{-1}$, $k_{ox} = 1 ms^{-1}$, $k_{red} = 0.5 ms^{-1}$), an excitation cross section $\sigma = 1.7 \cdot 10^{-20} m^2$ for $\lambda_0 = 491 nm$ excitation wavelength and a peak irradiance of $I_0 = 10 \mu W/\mu m^2$. The illumination profile and the CEF are simulated with parameters as found in the setup ($\omega_{0,y} = 40 \mu m$, $\omega_{0,z} = 1.6 \mu m$, NA = 1.2 refractive index $n = 1.33$, pixel size $10 \mu m$, magnification 49x). **(A)** The fluorescence response to a Gaussian excitation of width $1.6 \mu m$ reshapes towards a rectangular profile. This results from fluorescence saturation, which is caused by increasing dark state build-up as the applied pulse width increases. **(B)** The corresponding, effective excitation rate (—, and * for the particular cases from above) decreases respectively, unless fluorescence saturation is neglected. In this case, k_{01}^{eff} remains constant for all excitation schemes. **(C)** Several TRAST curve are calculated in the different, discussed fashions. We consider the calculation based on the convolution integral (Eq. S20) as the true decay (crosses). Obviously, it is matched best, when k_{01}^{eff} is calculated without disregarding fluorescence saturation (red). Nevertheless, even if fluorescence saturation due to dark states is neglected when calculating k_{01}^{eff} , the obtained curve is still close (blue). If the introduced normalization factor F_{norm} is neglected, this deteriorates the outcome by an overestimation of the decay (green).

Moreover, it is shown that the normalization factor F_{norm} is crucial, as it corrects for the drop in the calculated fluorescence signal, which accompanies the pulse width dependent decrease of k_{01}^{eff} . Following the same calculation as above, with depletion taken into account when calculating k_{01}^{eff} , but omitting the pulse-width dependence of the normalization factor, yields larger deviations compared to the reference values. For this neglect (green line), the TRAST curve decays too fast compared to the prediction based on Eq. S19.

Apparently, both approaches for k_{01}^{eff} match the prediction from the convolution integral quite well. However, this is not the prior focus of TRAST imaging. We are rather seeking to extract the transition rates as accurately as possible. To validate both models with respect to this demand, we simulated CCD images of TRAST measurements for the organic dyes

Rhodamine 110, Fluorescein, Atto 495 and Eosin Y. These calculations are based on Eq. S15 and S18. In contrast to all other investigated dyes, the simplified STR model does not hold for Eosin Y. Accordingly, the S_{01} TR (Eq. 1 instead of Eq. S15) is applied in this case.

The simulated results are fitted in two different ways. First, the effective excitation rate is considered to be the same for all pulse trains ($k_{01}^{\text{eff}}(\alpha, \beta)$). Its dependence on fluorescence depletion is neglected. Subsequently, this particular effect is taken into consideration and the effective excitation rate ($k_{01}^{\text{eff}}(\alpha, \beta, t_p)$) is pulse width dependent. The starting values for k_{isc} , k_t , k_{ox} and k_{red} were set 10% off the assumed value, in order to ensure that a convergence occurs within the fitting procedure. The results of these simulations are provided in table S1. Indeed, the approach, which takes the effect of fluorescence depletion into consideration when calculating k_{01}^{eff} , yields better results.

In most cases the differences between both models are negligible. In order to show deviations, the values are presented with higher accuracy than it is achievable in actual measurements. Analogous simulations for different irradiances reproduced the assumed values equally well. Even though both fitting procedures yielded sufficiently good results, data processing in this study is performed using the slightly more precise model including fluorescence depletion due to dark state build up.

		$k_{\text{isc}} [\mu\text{s}^{-1}]$	$k_t [\mu\text{s}^{-1}]$	$k_{\text{ox}} [\text{ms}^{-1}]$	$k_{\text{red}} [\text{ms}^{-1}]$
Rhodamine 110 $I_0 = 35 \frac{\text{kW}}{\text{cm}^2}$	assumed	1.00	0.45	1.00	0.20
	$k_{01}^{\text{eff}}(\alpha, \beta)$	0.99 ± 0.01	0.46 ± 0.01	0.98 ± 0.01	0.21 ± 0.01
	$k_{01}^{\text{eff}}(\alpha, \beta, t_p)$	1.00 ± 0.01	0.45 ± 0.01	1.00 ± 0.01	0.20 ± 0.01
Fluorescein $I_0 = 20 \frac{\text{kW}}{\text{cm}^2}$	assumed	13.50	0.66	5.00	0.50
	$k_{01}^{\text{eff}}(\alpha, \beta)$	13.30 ± 0.02	0.70 ± 0.01	4.62 ± 0.02	0.59 ± 0.01
	$k_{01}^{\text{eff}}(\alpha, \beta, t_p)$	13.55 ± 0.01	0.67 ± 0.01	5.00 ± 0.01	0.51 ± 0.01
Atto 495 $I_0 = 20 \frac{\text{kW}}{\text{cm}^2}$	assumed	30.00	1.00	4.00	2.00
	$k_{01}^{\text{eff}}(\alpha, \beta)$	29.94 ± 0.01	1.03 ± 0.01	3.88 ± 0.01	2.08 ± 0.01
	$k_{01}^{\text{eff}}(\alpha, \beta, t_p)$	30.01 ± 0.01	1.01 ± 0.01	4.02 ± 0.01	2.01 ± 0.01
Eosin Y $I_0 = 1 \frac{\text{kW}}{\text{cm}^2}$	assumed	840	0.40	0.60	0.30
	$k_{01}^{\text{eff}}(\alpha, \beta)$	836 ± 2	0.42 ± 0.01	0.57 ± 0.01	0.32 ± 0.01
	$k_{01}^{\text{eff}}(\alpha, \beta, t_p)$	842 ± 2	0.40 ± 0.01	0.60 ± 0.01	0.30 ± 0.01

Table S1: Assumed and subsequently obtained transition rates of Rhodamine 110, Fluorescein, Atto 495 and Eosin Y. The TRAST curves were simulated with respect to the given values. All obtained results were fitted with two different approaches. Either assuming the same effective excitation rates for all pulse widths ($k_{01}^{\text{eff}}(\alpha, \beta)$), or taking the effect of fluorescence depletion into account ($k_{01}^{\text{eff}}(\alpha, \beta, t_p)$).

Average fluorescence signal upon application of pulsed excitation schemes of arbitrary duty cycles

As derived in the section above, the theoretical treatment of TRAST data simplifies significantly with low excitation duty cycles (4). However, it may be desired to reduce the acquisition time and thus the pause between pulses. In the following, an adequate model for arbitrary duty cycles is derived, based on the propagation operators introduced in Eq. S5. To calculate the mean fluorescence for one pulse train, the average singlet state population within the pulses needs to be calculated. Any spatial dependence arising from the excitation profile is not considered for the moment. Following Eq. S14, we denote the time-average of $S(t)$ within the excitation pulses as:

$$\langle S \rangle_{Nt_p}(t_p) = \sum_{j=0}^{N-1} \left(\int_{jT}^{jT+t_p} \frac{1}{Nt_p} S(t) dt \right) \quad (\text{S22})$$

Without loss of generality, the time can be reset to zero after each period. $S(t)$ is given by Eq. S10. The initial triplet and redox populations $T_j(0)$, $R_j(0)$ at the onset of the j -th pulse strongly depend on the length and number of prior pulses, as well as on the pauses in-between them. These initial conditions constitute the only difference between each and every addend in Eq. S22. As the excitation rate is piecewise constant, the integration yields:

$$\begin{aligned} \langle S \rangle_{Nt_p}(t_p) = & \frac{k_{\text{red}}(k_t + k_{\text{ox}})}{\lambda_2 \lambda_3} \\ & + \frac{k'_{\text{isc}} + k_t + \lambda_3}{\lambda_2(\lambda_2 - \lambda_3)} \left(\frac{k'_{\text{isc}}}{\lambda_2} + \langle T_{\text{init}} \rangle + \frac{k_{\text{red}}(k_{\text{red}} + \lambda_3) - k'_{\text{isc}} k_{\text{ox}}}{\lambda_2 k_{\text{ox}}} \langle R_{\text{init}} \rangle \right) \frac{e^{\lambda_2 t_p} - 1}{t_p} \\ & - \frac{k'_{\text{isc}} + k_t + \lambda_2}{\lambda_3(\lambda_2 - \lambda_3)} \left(\frac{k'_{\text{isc}}}{\lambda_3} + \langle T_{\text{init}} \rangle + \frac{k_{\text{red}}(k_{\text{red}} + \lambda_2) - k'_{\text{isc}} k_{\text{ox}}}{\lambda_3 k_{\text{ox}}} \langle R_{\text{init}} \rangle \right) \frac{e^{\lambda_3 t_p} - 1}{t_p} \end{aligned} \quad (\text{S23})$$

Interestingly, $\langle S \rangle_{Nt_p}(t_p)$ depends on the average initial occupation of both dark states $\langle T_{\text{init}} \rangle$, $\langle R_{\text{init}} \rangle$ over all pulses, as defined in Eq. S24. At this point, it becomes beneficial to have time evolution operators.

No assumptions have been made about k'_{isc} in the derivation so far, except for the physical demand to be positive semidefinite as all other rates. The case $k'_{\text{isc}} = 0$ has not been excluded. Consequently, if the excitation is turned off, the eigenvalues and eigenvectors of $\mathbf{M}^{\text{off}} = \mathbf{M}(k'_{\text{isc}} = 0)$ and the corresponding propagator $\mathbf{U}^{\text{off}}$ are obtained directly from $\mathbf{U}$ by setting the effective intersystem crossing rate to 0. As the analytical expression of $\mathbf{U}$ is quite long and not of particular interest in the derivation, it is included in the appendix below. For the sake of clarity, the time evolution operators, which describe the dynamics when the excitation is turned on, are denoted as $\mathbf{U}^{\text{on}}$ in the following. Applying both propagators for excitation turned on and off subsequently to the initial state at the onset of the j -th pulse, yields the corresponding initial population for the following pulse. This allows for turning the calculation of the means of initial populations into a geometric matrix series:

$$\begin{pmatrix} \langle S_{\text{init}} \rangle \\ \langle T_{\text{init}} \rangle \\ \langle R_{\text{init}} \rangle \end{pmatrix} = \frac{1}{N} \sum_{j=0}^{N-1} \begin{pmatrix} S_j(0) \\ T_j(0) \\ R_j(0) \end{pmatrix} = \frac{1}{N} \sum_{j=0}^{N-1} \left(\mathbf{U}^{\text{off}}(T, t_p) \mathbf{U}^{\text{on}}(t_p, 0) \right)^j \begin{pmatrix} 1 \\ 0 \\ 0 \end{pmatrix} \quad (\text{S24})$$

It has been assumed that at the very first pulse, the fluorophore is in its ground state. Unfortunately, both propagators do not commute, neither is the standard solution of geometric series

$$\sum_{j=1}^N \left(\mathbf{U}^{\text{off}}(T, t_p) \mathbf{U}^{\text{on}}(t_p, 0) \right)^{j-1} = \left[\mathbf{E} - \mathbf{U}^{\text{off}}(T, t_p) \mathbf{U}^{\text{on}}(t_p, 0) \right]^{-1} \left[\mathbf{E} - \left[\mathbf{U}^{\text{off}}(T, t_p) \mathbf{U}^{\text{on}}(t_p, 0) \right]^N \right] \quad (\text{S25})$$

applicable, because as the system tends to equilibrium, the product of both time evolution operators approaches the unity operator $\mathbf{E}$. As an alternative, the numerical determination of the eigenvalues of $\left[\mathbf{U}^{\text{off}}(T, t_p) \mathbf{U}^{\text{on}}(t_p, 0) \right]$ and a subsequent projection into the eigenspace can save computation time when calculating $\langle T_{\text{init}} \rangle$ and $\langle R_{\text{init}} \rangle$.

If the duty cycle is kept sufficiently small, $\langle T_{\text{init}} \rangle$ and $\langle R_{\text{init}} \rangle$ will tend to zero, because the pause will be long enough to allow full relaxation back into the ground state.

A solution to this kind of problem has been already presented by Sandén et al. (7). However, that approach does not rely on time evolution operators. The benefit of this derivation is the representation in term of matrices, which makes the computational implementation for fitting purposes substantially faster and easier to implement.

Simulations on the initial conditions at the onset of a pulse

As described above, the assumption of low duty cycles makes it possible to simplify the data analysis. We compared this simplifying approach with the thorough solution, based on Eqs. S23-S24, including calculations of the fluorescence signal from Rhodamine 110 in solution detected in a single pixel, using Eq. S18 and suitable parameters, as stated in Fig. S3. Firstly, as indicated in Eq. S22-S24, it is of interest to consider the mean of all initial singlet, triplet and redox populations for all pulses of the applied excitation scheme ($\langle S_{\text{init}} \rangle$, $\langle T_{\text{init}} \rangle$ and $\langle R_{\text{init}} \rangle$). As shown in Fig. S3 A-C, these populations can only be neglected for excitation pulse trains with low duty cycles η , having long pauses between the pulses. The simulations reveal that $\langle R_{\text{init}} \rangle$ is always higher than $\langle T_{\text{init}} \rangle$. This is in agreement with the much slower redox dynamics, compared to the orders of magnitude faster triplet decay.

Figure S3: (A, B, C): The average population at the onset of all pulses depends in a STR model on the applied pulse width and the duty cycle η . The shorter the time between two pulses, the less the assumption of relaxed fluorophores at the onset of a pulse is valid. **(D):** With increasing duty cycle, the contrast in the TRAST curves decreases. The simplified approach by Spielmann et al. (4) is similar to the thoroughly derived approach in the limit of $\eta \rightarrow 0$. For $\eta = 0.01$, the triplet related decay is still described properly by the simplified model, but deviations occur. These data have been simulated for Rhodamine 110 ($k_{10} = 243 \mu\text{s}^{-1}$, $k_{\text{isc}} = 1 \mu\text{s}^{-1}$, $k_t = 0.4 \mu\text{s}^{-1}$, $k_{\text{ox}} = 1.4 \text{ms}^{-1}$, $k_{\text{red}} = 0.15 \text{ms}^{-1}$, $\sigma = 2.9 \cdot 10^{-20} \text{m}^2$ at $\lambda = 491 \text{nm}$ excitation wavelength, peak irradiance $I_0 = 0.25 \mu\text{W}/\mu\text{m}^2$), exposed to pulse trains of different pulse widths, but identical total exposure time (Nt_p) of 1ms. The illumination profile and the CEF are simulated with parameters as found in the setup ($\omega_{0,y} = 40 \mu\text{m}$, $\omega_{0,z} = 1.6 \mu\text{m}$, NA = 1.2, refractive index $n = 1.33$, pixel size $10 \mu\text{m}$, magnification 49x).

If only the ground state is populated at the onset of each excitation pulse, ensured by infinitely long pauses between pulses ($\eta \rightarrow 0$), Eq. S23, with a prefactor of $F_0\eta$, is equivalent to Eq. S15 as shown in Fig. S3 D. This justifies the simplifying assumption for small duty cycles, providing sufficient time for relaxation between two subsequent pulses. The dashed green line, which is based on Eq. S16, slightly overestimates the dark state population compared to the full calculation based on Eq. S23 for a duty cycle of 1% (blue line). Nevertheless, the triplet state decay is described very accurately. Redox rates, determined in fitting procedures may be biased to a larger extent. However, since k_{ox} and k_{red} rates are also influenced by diffusion of fluorophores, the simulated mismatch does not seem to be as crucial, as these rates cannot be determined absolutely by the proposed method.

In general, any effects of diffusion are assumed to be negligible in the derivations given here. The intersystem crossing and the triplet decay rate are not affected by this assumption. Considering one-dimensional diffusion through the light sheet, a fluorophore of diffusion constant D needs a time of $\tau_D \approx \omega_{0,z}^2/D$ to pass the light sheet, which is much longer than the time constant for triplet state equilibration. However τ_D is of the same order of magnitude as the redox transition. Therefore the redox rates determined from our measurements are results of a coupled dynamic of diffusion and dark state population and are strongly influenced by the parameters of the experimental setup. Absolutely accurate values of k_{ox} and k_{red} should therefore not be expected with the applied fitting model. However, relative changes in redox parameters can be followed, as shown by simulations in (4).

Determination of the beam profile using razor blade edge tests

To determine the beam profile, a razor blade was inserted in a stepwise manner and perpendicularly to the light propagation direction into the illumination beam. The transmitted power P was measured with a power meter (PM100, Thorlabs). As the profile is assumed to have a Gaussian shape, the measured power is expected to be an error function erf when it is related to the edge position. Thus, the beam waist at the particular point x on the propagation axis is obtained by fitting the following error function to the acquired data:

$$P(x, z) = P_{\text{noise}} + \frac{\pi}{4} I_0(x) \omega_y(x) \omega_z(x) \text{erf}\left(\frac{\sqrt{2}z}{\omega_z(x)}\right) \quad (\text{S26})$$

Here, the razor blade edge is moving in the z -direction, and z in Eq. S26 refers to the position of the edge on the z -axis and the coordinate system is chosen as indicated in Fig. 1. This approach gives unbiased access to the beam waist $\omega_z(x)$ at the particular position x of measurements, because the other parameters P_{noise} and $I_0(x)\omega_y(x)$ define the offset and the amplitude of the curve, but only $\omega_z(x)$ sets its shape. In Fig. S4, three representative measurements are depicted. The error bars correspond to the standard deviations over five acquisitions. Furthermore, this figure shows the obtained beam waists for a series of edge tests at different positions. A fit of the data by Eq. 3 yields the minimum beam waist in focus,

Figure S4: Illumination profile along the light propagation axis x . The beam waist at different positions is determined by several knife edge tests (blue). Each individual point results from fitting an error function to the acquired data (inset). The minimum beam diameter in focus is determined by fitting a Gaussian beam behavior to the data (red).

which is found to be $\omega_{0,z} = (1.6 \pm 0.1) \mu\text{m}$. In this case, the error represents the 95% confidence interval of the fit. In this particular setup, the light sheet (vacuum wavelength $\lambda_0 = 491 \text{ nm}$) is focused in water. Thus, the Rayleigh range is $x_{R,z} = (21.8 \pm 2.7) \mu\text{m}$, which provides sheet-like illumination conditions within more than $40 \mu\text{m}$ around the center of focus. This width is large enough to perform single cell imaging, as it is performed in this study on MCF-7 cells, which have diameters around $18 \mu\text{m}$ (11). By similar means, the beam waist of the collimated dimension is found to be $\omega_{0,y} = 40 \mu\text{m}$.

The presented razor blade edge tests provide access to the beam waists in the sample, even though they are performed in air. The beam diameter in focus is neither affected by the refractive medium, nor by the interface when entering the sample chamber. The change due to the different focusing angle is accounted for by including the refractive indices into the calculations.

FLIM measurements

Fluorescence lifetime measurements by time-correlated single photon counting were performed with a typical epi-illuminated confocal microscope equipped with stage scanning capabilities. Cell samples were excited with a 476 nm pulsed (repetition rate: 50 MHz) laser (Becker and Hickl, Berlin Germany in collaboration with LASOS, Jena, Germany) at a power of around $2 \mu\text{W}$ for cell images and around $20 \mu\text{W}$ for solution, as measured at the objective

(Olympus UPLANAPO, 60x/1.20w). The excitation beam was guided to an OLYMPUS IX70 (Olympus, Hamburg, Germany) microscope stand in which imaging was performed via a scanning stage (MadCityLabs, Madison, USA). The detection was performed either by two APDs (SPCM AQR-14; PerkinElmer Inc., Waltham, USA) and/or by two Hybrid PMTs (HPM-100-50; Hamamatsu, Japan). Images (128x128 pixels) over a field of view of 43 μm were obtained. The data sets were analyzed by a software package developed by the group of C. A. M. Seidel, Heinrich-Heine Univ, Düsseldorf (<http://www.mpc.uni-duesseldorf.de/seidel/index.htm>). The experimental fluorescence decay curves, recorded for each pixel and deconvoluted with respect to the laser pulse duration, were fitted to a single exponential decay model.

The fluorescence lifetimes of Atto 495 and Eosin Y were determined in a PBS buffer droplet on the cover glass. The error deviations represent the standard deviations of at least 3 consecutive measurements.

For determination of the fluorescence lifetime of CFDA-SE and CEDA-SE within labeled MCF-7 cells, three cells stained with each dye were measured on the setup described above. For these measurements, the cells were grown on chambered cover glasses and freshly labeled according to labeling steps described in the cell preparation steps in the main text. During these measurements cells were at room temperature and in air atmosphere. Homogenous lifetime images were obtained similar to the one seen in (12). The mean and the standard deviation of the mean lifetimes averaged over three individual cells is given in table 1.

Figure S5: SPIM-TRAST measurements on Eosin Y (A-E), Atto 495 (F-J) and Fluorescein (K-P).

Figure S6: **(A):** The 2D histogram showing the k_{ox} distribution on the y-axis and their uncertainty (95% confidence intervals) on the x-axis. This histogram includes the pixel-values from 88 measurements on 21 different cells, stained with CEDA-SE. The values below a threshold of 1 s^{-1} have large uncertainties, which justifies their omission from the histograms, thereby distinguishing between reasonably fitted and noise governed outcomes. All compiled measurements were taken at the same peak irradiance ($I_0 = 0.8 \text{ kW/cm}^2$). **(B):** The 2D-histogram of the k_t and k_{ox} values of the same measurements as in (A) reveals an inclination with respect to the axes, which may be a hint for a correlation between the parameters. For this graph, the pixels with k_{ox} values below the aforementioned threshold were rejected. **(C)** The 2D histogram of k_t and k_{ox} values, obtained from 27 measurements on 13 different cells stained with CFDA-SE. As for CEDA-SE, this figure reveals an inclination with respect to the axes. Moreover, there may be a subpopulation discernible in the graph, as the distribution is not centered, but has its maximum on the lower border of the two-dimensional histogram. All measurements of the CFDA-SE stained cells were taken with a peak excitation irradiance $I_0 = 8.9 \text{ kW/cm}^2$.

		k_{ox} [ms^{-1}]	k_{red} [ms^{-1}]
Rhodamine 110	SPIM-TRAST	1.1 ± 0.3	0.2 ± 0.1
	Confocal TRAST	1.7 ± 0.3	1.1 ± 0.3
Fluorescein	SPIM-TRAST	4.0 ± 0.3	0.3 ± 0.1
	Confocal TRAST	3.4 ± 0.2	1.4 ± 0.1
Atto 495	SPIM-TRAST	4.4 ± 0.7	1.6 ± 0.6
	Confocal TRAST	4.7 ± 0.3	2.4 ± 0.3
Eosin Y	SPIM-TRAST	0.9 ± 0.1	0.5 ± 0.1
	Confocal TRAST	1.2 ± 0.2	1.2 ± 0.3

Table S2: Redox transition rates for Rhodamine 110, Fluorescein, Atto 495 and Eosin Y in PBS. The values refer to the same measurements as tab. 2. They are not universal values, but rather represent diffusion and redox build-up, which cannot be separated within the conducted analysis.

Appendix

The propagator of the STR model is found to be:

$$U(t, t_0) = \frac{1}{\lambda_2 \lambda_3} \mathbf{U}_{\text{eq}} + \frac{e^{\lambda_2(t-t_0)}}{\lambda_2(\lambda_2 - \lambda_3)} \mathbf{U}_2 - \frac{e^{\lambda_3(t-t_0)}}{\lambda_3(\lambda_2 - \lambda_3)} \mathbf{U}_3 \quad (\text{S27})$$

The time evolution is governed by exponentials with the eigenvalues of $\mathbf{M}$ as characteristic decay constants. Here, $\mathbf{U}_{\text{eq}}, \mathbf{U}_2, \mathbf{U}_3$ denote the corresponding matrices of prefactors. They do not reveal any temporal dependence, except for the steps in k'_{isc} when the excitation is switching from on to off and vice versa. In detail, these matrices read:

$$\mathbf{U}_{\text{eq}} = \begin{pmatrix} k_{\text{red}}(k_{\text{t}} + k_{\text{ox}}) & k_{\text{red}}(k_{\text{t}} + k_{\text{ox}}) & k_{\text{red}}(k_{\text{t}} + k_{\text{ox}}) \\ k'_{\text{isc}} k_{\text{red}} & k'_{\text{isc}} k_{\text{red}} & k'_{\text{isc}} k_{\text{red}} \\ k'_{\text{isc}} k_{\text{ox}} & k'_{\text{isc}} k_{\text{ox}} & k'_{\text{isc}} k_{\text{ox}} \end{pmatrix} \quad (\text{S28})$$

$$\mathbf{U}_2 = \begin{pmatrix} k'_{\text{isc}}(k'_{\text{isc}} + k_{\text{t}} + \lambda_3) & (k'_{\text{isc}} + \lambda_2)(k'_{\text{isc}} + k_{\text{t}} + \lambda_3) & \frac{k_{\text{red}}(k_{\text{red}} + \lambda_3)(k'_{\text{isc}} + k_{\text{t}} + \lambda_3)}{k_{\text{ox}}} \\ k'_{\text{isc}}(k_{\text{red}} + \lambda_2) & (k'_{\text{isc}} + \lambda_2)(k_{\text{red}} + \lambda_2) & \frac{k_{\text{red}}(k_{\text{red}} + \lambda_2)(k_{\text{red}} + \lambda_3)}{k_{\text{ox}}} \\ k'_{\text{isc}} k_{\text{ox}} & k_{\text{ox}}(k'_{\text{isc}} + \lambda_2) & k_{\text{red}}(k_{\text{red}} + \lambda_3) \end{pmatrix} \quad (\text{S29})$$

$$\mathbf{U}_3 = \begin{pmatrix} k'_{\text{isc}}(k'_{\text{isc}} + k_{\text{t}} + \lambda_2) & (k'_{\text{isc}} + \lambda_3)(k'_{\text{isc}} + k_{\text{t}} + \lambda_2) & \frac{k_{\text{red}}(k_{\text{red}} + \lambda_2)(k'_{\text{isc}} + k_{\text{t}} + \lambda_2)}{k_{\text{ox}}} \\ k'_{\text{isc}}(k_{\text{red}} + \lambda_3) & (k'_{\text{isc}} + \lambda_3)(k_{\text{red}} + \lambda_3) & \frac{k_{\text{red}}(k_{\text{red}} + \lambda_2)(k_{\text{red}} + \lambda_3)}{k_{\text{ox}}} \\ k'_{\text{isc}} k_{\text{ox}} & k_{\text{ox}}(k'_{\text{isc}} + \lambda_3) & k_{\text{red}}(k_{\text{red}} + \lambda_2) \end{pmatrix} \quad (\text{S30})$$

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