Enhancing Fluorescence Brightness: Effect of Reverse Intersystem Crossing Studied by Fluorescence Fluctuation Spectroscopy

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Experiments based on fluorescence detection are limited by the population of the fluorescence marker’s long-lived dark triplet state, leading to pronounced photobleaching reactions and blinking which reduces the average fluorescence signal obtained per time interval. By irradiation with a second, red-shifted laser line, we initiate reverse intersystem crossing (ReISC) which enhances the fluorescence signal of common fluorophores up to a factor of 14. The reverse intersystem crossing from the triplet state back to the singlet system is achieved by photoexcitation to higher-excited triplet states, which are, however, prone to photobleaching.

1. Introduction

Fluorescence contrast detection is an elegant and versatile method in biology, chemistry and physics, exposing structural details in space and time and, above all, inside living cells. Because many applications impose an intrinsic limit on the markers concentration, an important issue in fluorescence experiments is to realize high signal levels by optimizing the amount of fluorescence detected from each single fluorophore within the observation time. One possible approach is to use high excitation irradiances, however a natural limit is encountered when the ground state is depleted and faster photobleaching occurs. Therefore, in many applications, observation time, signal yield and, in the case of imaging, resolution are limited by the brightness of the label’s fluorescence.

The aforementioned depletion of the ground state when increasing the irradiance is usually not due to a saturation of the transition to the excited singlet state, but rather a consequence of intersystem crossing (ISC) to the triplet state, which occurs with a certain probability after each excitation. With its lifetime of micro- to milliseconds (as compared to nanoseconds of the first excited singlet state), the triplet state elicits dark periods, where the dye is dislodged from the fluorescence excitation cycle. Especially at large excitation irradiances, this leads to accumulation of the triplet state and saturation of fluorescence emission. The emitted fluorescence rate is then no longer proportional to the applied irradiance but approaches a maximum beyond which it cannot be increased. To make things worse, the long-lived triplet state is vulnerable to destructive bleaching reactions such as diffusion-controlled biomolecular reactions. In particular, photoreactions following the absorption of additional photons with subsequent efficient bleaching from higher excited electronic states result in efficient photo-destruction of dye molecules.

It is therefore essential to keep triplet population as low as possible and major efforts are made to minimize it. While the utilization of triplet quenchers can effectively reduce triplet build-up, they change the experimental environment and preclude certain applications like live-cell imaging. The optimal solution would thus be to use a triplet depopulation pathway intrinsic to the dye’s photophysics. One possibility is to switch to pulsed excitation and chose a sufficiently low repetition rate so that the fluorescent marker’s triplet state has relaxed between subsequent excitation events. Introduced as dark-state relaxation (D-Rex) microscopy, this method has led to a major improvement in photostability and up to a 20-fold increase of the total fluorescence detected. However, at an assumed triplet lifetime of ~1 μs, low repetition rates of <1 MHz are chosen to ensure a large enough pulse separation.

Unfortunately, lower repetition rates generally result in increased measurement times and therefore, a light-driven pathway, which can be boosted by irradiation with an appropriate

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wavelength, would be more desirable. A promising candidate is photoinduced reverse ISC (ReISC), where the dye in its lowest-excited triplet state $T_1$, is further excited to higher excited triplet states $T_n$ (Figure 1). From $T_n$ the dye can efficiently cross back to the singlet system, where fluorescence emission occurs.\textsuperscript{[17–20]}

ReISC has previously been shown for dyes with high ISC yields such as bipyridien,\textsuperscript{[17]} erythrosine B,\textsuperscript{[18,21,22]} rose bengal,\textsuperscript{[18,21–25]} cyanine dyes,\textsuperscript{[19,20]} anthracene,\textsuperscript{[26]} eosin Y,\textsuperscript{[21,22]} and propiophene.\textsuperscript{[27]}

ReISC has further been included in fluorescence statistics of single-molecule experiments of sulforhodamine 101 or DiI in polyvinyl alcohol (PVA),\textsuperscript{[31]} on air-glass interfaces,\textsuperscript{[32]} under nitrogen atmosphere\textsuperscript{[33]} or in Förster resonance energy transfer (FRET) experiments.\textsuperscript{[34]}

In these experiments, an unexpected change or even increase of fluorescence signal was observed when irradiating with light of a second color, or the saturation level measured was too high to be explained with the known triplet lifetime. In all cases, efficient triplet depopulation due to ReISC offered a consistent explanation of the data.

However, using ReISC for the depopulation of the triplet has an important drawback. Due to the enhanced reactivity of the molecule in the higher excited singlet ($S_n$) and triplet ($T_n$) states,\textsuperscript{[8–12,35]} ReISC not only boosts the brightness of the dye but, depending on the states involved and their bleaching rates, may also result in faster photobleaching. To date, no detailed analysis of these competing phenomena is available for popular marker molecules.

Herein, we conduct a general survey in which, under certain conditions and for several commonly used dyes, ReISC leads to an increase in the fluorescence detected from each molecule. We influence the triplet state population of the investigated organic dyes and fluorescent proteins by irradiating with a second laser line at 568 or 671 nm, whilst the fluorescence excitation wavelengths are 488 or 532 nm. The main objective is to assess whether the additional light, which does not directly elicit fluorescence emission, boosts the fluorophore’s brightness through ReISC and how it influences photobleaching. To this end, we employed fluorescence correlation spectroscopy (FCS)\textsuperscript{[36]} and fluorescence intensity distribution analysis (FIDA)\textsuperscript{[37,38]} to monitor fluorescence brightness, triplet-state population, stimulated emission and photobleaching kinetics at different irradiance levels of the excitation and red-shifted laser light. This allowed us to analyze the competing pathways of ReISC and photobleaching. As an example, upon addition of 671 nm laser light, the dye fluorescein–isothiocyanate (FITC) shows an increase in fluorescence signal by a factor of 5 in PVA and of 10% in aqueous solution. We are therefore confident that optimizing fluorescence brightness using ReISC helps to improve the quality in fluorescence experiments such as confocal imaging, correlation spectroscopy and single-molecule detection.

1.1 Photokinetic Model

Our photokinetic model including characteristics such as ReISC and photobleaching from higher excited states is based on five electronic levels; singlet ground ($S_0$) and first ($S_1$) excited states, lowest excited triplet state ($T_1$), and higher excited singlet ($S_n$) and triplet ($T_n$) states (Figure 1c). Vibration substates can be disregarded due to their comparatively short lifetimes $< 1$ ps. The kinetic rate constants of the underlying transitions define the rate system of Equation (1) of the time ($t$) dependent relative populations of the different states as well as the photobleaching kinetics.

\[
\frac{d}{dt}egin{pmatrix}
S_0 \\
S_1 \\
T_1 \\
S_n \\
T_n
end{pmatrix} = \begin{pmatrix}
-k_{ISC} & k_{ISC} & 0 & 0 & 0 \\
k_{ISC} & -k_{ISC} - k_{ISC} - k_{ISC} & 0 & 0 & 0 \\
0 & k_{ISC} & -k_{ISC} & k_{ISC} & 0 \\
0 & 0 & k_{ISC} & -k_{ISC} & k_{ISC} \\
0 & 0 & 0 & k_{ISC} & -k_{ISC} - k_{ISC}
\end{pmatrix}
\begin{pmatrix}
S_0 \\
S_1 \\
T_1 \\
S_n \\
T_n
end{pmatrix}
\]

where $k_{ISC}$, $k_{ISC}$, and $k_{ISC}$ determine the rate constants for excitation from $S_0$ to $S_1$, $S_n$ to $S_n$, and $T_1$ to $T_n$, respectively. If we

![Figure 1. Intramolecular pathways of photoinduced ReISC.](image)
denote the excitation irradiance by \( I_{\text{exc}} \) and the irradiance of the additional red-shifted light as \( I_{\text{red}} \). The rate constants are given by \( k_{\text{exc}} = \alpha_{\text{exc}} \gamma_{\text{exc}} I_{\text{exc}} + \alpha_{\text{exc}}^* \gamma_{\text{red}} I_{\text{red}} \) \((\lambda = 01, S_1 \text{ or } T_1)\) with the absorption cross sections \( \alpha_{\text{exc}} \) and \( \alpha_{\text{exc}}^* \) and the reciprocal photon energies \( \gamma_{\text{exc}} \) and \( \gamma_{\text{red}} \) of the excitation and red light, respectively. Excitation to \( S_1 \) or \( T_1 \) follows a previous absorption step to the first excited states \( S_1 \) or \( T_1 \) and is thus a multi-step absorption. The reciprocal fluorescence lifetime \( k_0 = k_{10} + k_{1T} + k_{\text{StimEm}} \) includes the de-excitation rate constant \( k_{10} \) from \( S_1 \) to \( S_0 \), the ISC rate constant \( k_{1T} \) denoting the \( S_1 \) to \( T_1 \) transition, and the rate constant \( k_{\text{StimEm}} \) for stimulated emission. At the applied excitation irradiances (\( I_{\text{exc}} \approx 1-600 \text{ kW cm}^{-2} \)), we can neglect stimulated emission by the excitation light, but with \( I_{\text{red}} > \text{MW cm}^{-2} \) the red light may induce stimulated emission with \( k_{\text{StimEm}} = \alpha_{\text{StimEm}} \gamma_{\text{red}} I_{\text{red}} \) given by the cross section of stimulated emission \( \alpha_{\text{StimEm}} \) at the red wavelength. \( k_1 \) is the transition rate from the triplet \( T_1 \) back to the singlet ground state \( S_0 \). The higher excited states either relax back to the first excited states with the rate constants \( k_{1S_n} \) and \( k_{1T_n} \) or cross to the respective other excited states with the rate constants \( k_{nS_n} \) for multi-step absorption ISC, or \( k_{nT_n} \), for reverse ISC (ReISC). Photobleaching is introduced by the microscopic rate constants \( k_{1S_1} \) and \( k_{1T_1} \) from the first excited states \( S_1 \) and \( T_1 \) and by \( k_{nS_n} \) and \( k_{nT_n} \) from the higher excited states \( S_n \) and \( T_n \) respectively. As shown in the Supporting Information, the photokinetic model can be cut down to an electronic three-state model of the ground and first excited states \( S_0 \), \( S_1 \) and \( T_1 \). This significant simplification is allowed despite the involvement of the higher excited states \( S_n \) and \( T_n \) and the application of pulsed excitation, because the kinetics of the higher excited states is much faster and bleaching much slower than the kinetics of \( S_0 \), \( S_1 \) and \( T_1 \). Following an additional irradiance-dependent absorption step, the effective cross sections \( \alpha_{\text{StimCn}} \) and \( \alpha_{\text{StimScn}}^* \) for the ReISC induced by the excitation and red-shifted light, respectively, can be introduced, and in the absence of bleaching the respective steady-state populations \( S_{0\text{ss}}, S_{1\text{ss}} \) and \( T_{1\text{ss}} \) of the \( S_0, S_1 \) and \( T_1 \) are given by Equation (2).

\[
S_{1\text{eq}} = \frac{k_{1S_0}}{k_{1S_1}} S_{1\text{ss}}
\]

\[
T_{1\text{eq}} = \frac{k_{1S_0}}{k_{1T_1}} S_{1\text{ss}}
\]

\[
S_{0\text{eq}} = \frac{k_{10}}{k_{1S_0}} S_{1\text{ss}}
\]

Here, \( k_{1S_1} = k_{1T_1} + k_{\text{StimCn}} \) denotes the overall triplet depopulation constant, comprised of the rate constants \( k_{1T_1} \) for \( S_1 \) to \( S_0 \) recovery and the effective rate \( k_{\text{StimCn}} \) of ReISC through the higher excited states \( S_n \) and \( T_n \) as shown in Equation (3).

\[
k_{\text{StimCn}} = \alpha_{\text{StimCn}} \gamma_{\text{exc}} I_{\text{exc}} + \alpha_{\text{StimCn}}^* \gamma_{\text{red}} I_{\text{red}}
\]

Analogously, the effective cross sections \( \alpha_{\text{Stim}} \) and \( \alpha_{\text{Stim}}^* \) can be defined for multi-step photobleaching by the excitation and red-shifted light, respectively. Due to the photobleaching being much slower than the kinetics of the three (five) electronic states, bleaching does not influence their equilibrium formation. Consequently, the photobleaching can be described as a quasi-unimolecular reaction on a much longer timescale with a microscopic rate constant \( k_r \) calculated using the average populations of the electronic states as shown in Equation (4).

\[
k_r = (k_{10} + \alpha_{\text{Stim}} \gamma_{\text{exc}} I_{\text{exc}} + \alpha_{\text{Stim}}^* \gamma_{\text{red}} I_{\text{red}})(S_{1\text{eq}} + T_{1\text{eq}})
\]

As an approximation, we assumed the effective cross sections \( \alpha_{\text{bb}} \) and \( \alpha_{\text{bb}}^* \) for multi-step photobleaching and the microscopic rate constants \( k_{1S_1} = k_{1T_1} = k_{\text{Stim}} \) for bleaching from the first excited states to be equal for the singlet and triplet system (see the Supporting Information for details).

In experiments based on the detection of single diffusing molecules such as FCS or FiDiA, the most crucial parameter determining the signal strength is the number of photons \( N \) detected per burst, that is, those within the transit time, \( dT \), of the molecules through the detection volume. \( N = q \times dT \) where \( q \) is the molecular brightness. The brightness \( q = \Psi \phi_{\text{exc}} S_{1\text{eq}} \) in the absence of bleaching is solely determined by the population probability \( S_{1\text{eq}} \) of the \( S_1 \) state, the \( S_1 \) decay rate \( k_r \), the detection efficiency \( \Psi \) and the fluorescence quantum yield \( (\Phi) \). However, if there is a non-negligible probability of bleaching the molecules within the transit time, \( N \) is reduced and the apparent brightness is given by Equation (5)

\[
q = N/dT = \Psi \phi_{\text{exc}} S_{1\text{eq}} \frac{1}{k_r dT} [1 - \exp(-k_r dT)]
\]

where the additional factor is merely the average over all possible moments at which the dye could bleach\(^{11,35} \) and becomes equal to 1 for short transit times or in the absence of bleaching \( (k_r dT \rightarrow 0) \).

**Data Analysis**

We applied fluorescence correlation spectroscopy (FCS)\(^{36} \) and fluorescence intensity distribution analysis (FiDA)\(^{37,38} \) to experimentally analyze the competing pathways of ReISC and multi-step photobleaching.

FCS analyses characteristic fluctuations \( \Delta F(t) \) in the fluorescence signal \( F(t) \) in time \( t \) about an average value \( \langle F(t) \rangle = F(t) > + \Delta F(t) \) by calculating the second-order auto-correlation function \( G(t_1) \) as shown in Equation (6)

\[
G(t_1) = 1 + \left( \frac{\langle \Delta F(t_1) \Delta F(t_1 + t_c) \rangle}{\langle F(t) \rangle^2} \right)
\]

where \( t_c \) represents the correlation time. Triangular brackets indicate averaging over the measurement time \( t \). The average fluorescence signal detected from a solution of fluorescent molecules is given by the mean number \( c \) of molecules in the observation volume and the average fluorescence brightness \( q \) of each single molecule; \( F = c \times q \). Characteristic variations in \( F \) are for example caused by diffusion of a dye molecule in and out of the confocal detection volume or by transition into and
out of the dark fluorescence state. Following earlier work, the autocorrelation function taking diffusion dynamics and dark triplet state population into account can be approximated by Equation (7) [5,11,35]

\[
G(t_c) = 1 + \frac{1}{2} \left[ G_D(t_c) - G_1(t_c) \right]
\]

(7)

with

\[
G_D(t_c) = \left( \frac{1}{1 + \frac{t_c}{\tau_D}} \right) \left( 1 + \frac{1}{1 + \left( \frac{t_c}{\alpha_D^2} \right)^{\frac{1}{2}}} \right)^{1/2}
\]

(diffusion)

\[
G_1(t_c) = 1 + \frac{T_{1eq}}{1 - T_{1eq}} \exp(-t_c/\tau_t) \quad \text{(triplet state)}
\]

The characteristic diffusion time \( \tau_D = \alpha_D^{-2}/(4D) \) is given by the diffusion coefficient \( D \) and the lateral \( 1/e^2 \) radius \( \alpha_D \) of the Gaussian-like confocal detection volume. \( \alpha_D \) is the axial \( 1/e^2 \) radius of the detection volume. \( T_{1eq} \) is the equilibrium fraction of molecules in the dark triplet state, and \( \tau_t \) is the triplet correlation time, characterized by the triplet population and depopulation kinetics. Other fluctuations arising from the intermolecular relaxation kinetics of the singlet \( S_0 \rightarrow S_1 \) system as well as from higher excited states dynamics can be neglected, since they all occur on a timescale much faster than the time resolution of \( \approx 100 \text{ ns} \) of our FCS setup.

For the description of the triplet state kinetics in the context of FCS, the three-state model can be further simplified by noting that the relaxation kinetics of the \( S_0 \rightarrow S_1 \) system as well as of the higher excited states are much faster (ps to ns) than the population and depopulation kinetics of the triplet state (usually ms to s) such that \( k_{S0} k_{S1} \gg k_{ISC}^{[5,30]} \). This characteristic allows the simplification to a two-state model comprising the \( S_0 \rightarrow S_1 \) singlet system and the triplet state \( T \) with interconversion rate constants \( k_{ST} \) and \( k_{TS} \), respectively. While the triplet depopulation rate \( k_{TS} = k_t + \sigma_{ISC} \gamma_{ISC} \gamma_{ISC} \gamma_{ISC} \gamma_{ISC} \) has been defined in Equations (2) and (3), the transition rate \( k_{ST} = k_s k_{ISC} \) to the triplet system, depends on (besides the ISC rate \( k_{ISC} \) for \( S_0 \rightarrow S_1 \)) the average relative population \( S_1 = \frac{k_{ISC}}{k_{ISC} + k_{TS}} \) of the first excited state \( S_1 \) within the singlet system, given by the excitation rate \( k_{exc} \) (proportional to \( I_{exc} \)) and the \( S_1 \rightarrow S_0 \) decay rate \( k_{S0} \) (Eq. (1)). This simple two-state system relaxes to its steady-state equilibrium with the triplet correlation time \( \tau_T = 1/(k_{ST} + k_{TS}) \) and has an equilibrium triplet population \( \frac{T_{1eq}}{T} = k_{ST}/(k_{ST} + k_{TS}) \). When \( k_{S0} \gg k_{ISC} \), we can now determine the rate constants \( k_{ISC} \) and \( k_{TS} \) from the values of \( \tau_T \) and \( T_{1eq} \) applying Equations (8a) and (8b)

\[
k_{ISC} = \frac{1}{\tau_T} T_{1eq} \left( 1 + \frac{\sigma_{ISC} \gamma_{ISC} \gamma_{ISC} \gamma_{ISC} \gamma_{ISC}}{k_{exc}} \right)
\]

(8a)

\[
k_{TS} = \frac{1}{\tau_T} (1 - T_{1eq})
\]

(8b)

which include the stimulated emission with the cross section \( \sigma_{ISC} \) by the red light with irradiance \( I_{red} \).

In order to deduce the rate constants \( k_{ISC} \) and \( k_{TS} \) and the effective cross sections \( \sigma_{ISC} \) and \( \sigma_{ISC} \) for ReISC and multi-step photobleaching, several other parameters must be known. The rate constant \( k_{ISC} \) for excitation can be determined from the excitation irradiance \( I_{exc} \) if the absorption cross section \( \sigma_{ISC} \) is known. We used values derived from the literature [5] and from the absorption spectra in Figure 2a. The rate \( k_{ISC} \) was measured using time-correlated single-photon counting (TCSPC) (Table 1). \( k_{CS} \) increases linearly

![Figure 2. Fluorescence signals of FITC and Rh110 under 568 or 671 nm addition. a) Absorption (——) and fluorescence (-----) spectra of FITC (black) and Rh110 (grey) in aqueous solution. Fluorescence excitation is strong at 488 nm, but negligible at 568 or 671 nm. b) Total fluorescence count rate (CR) over time detected for FITC and Rh110 in PVA under periodic addition of 671 nm irradiation \( I_{lam} \) and excitation at 488 nm with constant irradiance \( I_{exc} = 5.5 \text{ kW cm}^{-2} \). A gain in fluorescence up to a factor of 5 is detected for FITC, but not for Rh110.](image)

Table 1. Photophysical parameters of FITC and Rh110 in aqueous solution determined from FCS and brightness analysis.

<table>
<thead>
<tr>
<th>Dye</th>
<th>( \sigma_{ISC} )</th>
<th>( k_{ISC} )</th>
<th>( k_{S0} )</th>
<th>( k_{TS} )</th>
<th>( k_{TS} )</th>
<th>( \sigma_{ISC} )</th>
<th>( \sigma_{ISC} )</th>
</tr>
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<tbody>
<tr>
<td>FITC</td>
<td>3.0</td>
<td>2.63</td>
<td>5.0</td>
<td>0.45</td>
<td>3</td>
<td>0.05</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td>488 nm</td>
<td>568 nm</td>
<td>671 nm</td>
<td>488 nm</td>
<td>568 nm</td>
<td>671 nm</td>
<td>568 nm</td>
</tr>
<tr>
<td>Rh110</td>
<td>2.6</td>
<td>2.5</td>
<td>1.0</td>
<td>0.25</td>
<td>1.7</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>488 nm</td>
<td>568 nm</td>
<td>671 nm</td>
<td>488 nm</td>
<td>568 nm</td>
<td>671 nm</td>
<td>568 nm</td>
</tr>
</tbody>
</table>

[a] Cross section of \( S_0 \rightarrow S_1 \) \( 10^{-19} \text{ cm}^2 \) and rate of \( S_0 \rightarrow S_1 \) \( 10^8 \text{ s}^{-1} \) determined from Figure 2a and TCSPC, respectively. [b] Rate constants of ISC and \( S_0 \rightarrow S_1 \) recovery, respectively \( 10^8 \text{ s}^{-1} \). [c] Bleaching rate constant from \( S_1 \) and \( T_1 \) \( 10^2 \text{ s}^{-1} \). [d] Cross section of ReISC and multi-step photobleaching \( \sigma_{ISC} \) and \( \sigma_{ISC} \) for 488 and \( \sigma_{ISC} \) and \( \sigma_{ISC} \) for 568 and 671 nm, respectively \( 10^{-19} \text{ cm}^2 \). [e] Cross section of stimulated emission for 568 and 671 nm light \( 10^{-19} \text{ cm}^2 \). Standard deviations of all parameters are \( \pm 20\% \) of the absolute value.

with the irradiances \( I_{\text{exc}} \) and \( I_{\text{red}} \) of both the excitation and red-shifted light, with slopes \( \sigma_{\text{ISC}c} \) and \( \sigma_{\text{ISC}r} \), and intercept \( k_F \). Therefore measuring at different irradiances of the excitation light yields \( \sigma_{\text{ISC}c} \), while additional red-shifted irradiance reveals the effective cross section \( \sigma_{\text{ISC}r} \). When neglecting stimulated emission, our measurements yield apparent values of \( k_{\text{ISC}} \) decreasing with increasing \( I_{\text{exc}} \). Accordingly, \( \sigma_{\text{stem}} \) is set to the value yielding constant \( k_{\text{ISC}} \).

For the confocal volume formed by an objective lens of high numerical aperture as in this case, the irradiance and thus some of the rate constants strongly depend on the point in space. For example, the peak focal peak irradiance. A more rigorous approach is possible for short timescales where a weighted average over the volume can be used to obtain a good approximation of the correlation function, but fortunately our assumption of an average excitation rate constant \( k_{\text{exc0}} \equiv \sigma_{\text{exc0}} I_{\text{exc,peak}}/2 \) over the whole excitation volume (with \( I_{\text{exc,peak}} \) denoting the peak focal irradiance) works well for Gaussian-like volumes and moderate saturation of fluorescence emission.\(^{[11,35]}\) However, as depletion of the singlet ground state \( S_0 \) becomes more pronounced at large \( I_{\text{exc}} \), the fluorescence emission is no longer proportional to \( I_{\text{exc}} \); it is saturated. This saturation effect mainly affects the focal center, that is, the point of highest irradiance and results in a distorted, more square-like effective fluorescence detection volume, which deviates significantly from the assumed Gaussian-like FCS volume. This leads to a bias, for example, in the determination of kinetic parameters by FCS.\(^{[5,10]}\)

In our case, saturation leads to an overestimation of the excitation rate \( k_{\text{exc0}} \), and thus of the average population of the \( S_1 \) state. As can be seen from Equation (8a), this underestimates the apparent rate constant \( k_{\text{ISC}} \) (app). Herein, this is solved by recognizing that saturation becomes negligible at very low \( I_{\text{exc0}} \) and the true value of \( k_{\text{ISC}} \) is therefore determined either at low excitation intensities or obtained from an extrapolation. In contrast, the determination of the rate constant \( k_F \) for depopulation of the triplet state to \( S_0 \) is not influenced by this saturation, as shown by simulations.\(^{[12]}\) The additional red-shifted light does not induce direct \( S_0 \rightarrow S_1 \) excitation and thus does not introduce any saturation-biased features.

Photobleaching leads to a shortening of the observation time when the single dye molecules are irreversibly destroyed before leaving the detection volume. Previous FCS experiments\(^{[11,35,41]}\) used an expanded confocal volume to prolong the molecular transit time and to accurately determine the photobleaching rate constant \( k_z \) [Eq. (4)] from the observed shift of the correlation data to shorter correlation times. However, in our experience such expanded volumes increase inaccuracies in the determination of the triplet parameters due to the saturation effects. Therefore, we recorded the correlation data for an almost diffusion-limited spot featuring a focal \( 1/e^2 \) radius \( \sigma_0 \approx 340 \text{ nm} \), that is, an average transit time of \( \tau_{\text{diff}} \approx 100 \mu s \) for common organic dyes in aqueous solution. Consequently, the shortening of the decay time of the correlation data due to photobleaching is not significant enough to accurately determine the rate constant of photobleaching \( k_z \). We therefore determine \( k_z \) by fitting the dependence of the apparent fluorescence brightness \( q(I_{\text{exc}}, I_{\text{red}}) \) to \( I_{\text{exc}} \) and \( I_{\text{red}} \) as given by Equation (5). While \( q \) could be determined from the concentration and average signal determined in FCS experiments or from fluorescence bursts of single-molecule transits recorded in experiments of very low dye concentration,\(^{[11,35]}\) this proved to be less reliable than using FIDA to determine the apparent brightness.

In contrast to FCS, FIDA\(^{[37,38]}\) analyzes the frequency histogram of the fluctuating fluorescence signal \( F(t) \) over time \( t \). \( F(t) \) is observed as the number of photon counts \( n \) recorded in successive time intervals of fixed duration \( T \). A theoretical probability distribution of photon count numbers is fitted to the frequency histogram of \( n, P(n) \), yielding a specific fluorescence brightness value \( q \) and the number of molecules in the observation volume \( c \). The overall average signal count rate \( < F(t) > \) is then given by Equation (9) taking into account the background signal \( B \) due to scattered light or impurities.

\[
< F(t) > = c \cdot q + B
\]  

(9)

Further, FIDA uses three adjustment parameters to describe the inhomogeneous fluorescence detection profile of the focal volume.\(^{[42]}\) By adapting these adjustment parameters for each combination of \( I_{\text{exc}} \) and \( I_{\text{red}} \), FIDA allows us to determine \( q(I_{\text{exc}}, I_{\text{red}}) \) with least bias due to saturation effects on the fluorescence detection profile. By choosing the time interval \( T = 40 \mu s \) close to the transit time \( \tau_{\text{diff}} \) we measure the apparent brightness given in Equation (5).

We now use Equation (5) to theoretically describe the observed irradiance dependence of the fluorescence brightness \( q(I_{\text{exc}}, I_{\text{red}}) \) and establish the photobleaching rate constant \( k_z \) of Equation (4). In the case of freely diffusing molecules, the average transit time \( \tau_{\text{diff}} \) of a single dye molecule through the confocal detection volume can be approximated by \( \tau_{\text{diff}} = 4/3 \tau_{\text{conv}} \) with the characteristic diffusion time \( \tau_{\text{conv}} \) determined from FCS [Eq. (7)].\(^{[43]}\) We can then estimate the constant \( k_{\text{ISC}} \) for bleaching from the first excited states and the effective cross sections \( \sigma_{\text{ISC}r} \) and \( \sigma_{\text{ISC}c} \) for multi-step photobleaching from the higher excited states.

2. Results and Discussion

Initial experiments are performed on the organic dyes fluorescein-isothiocyanate (FITC) and rhodamine110 (Rh110), which are frequently used as labels in fluorescence detection experiments. In both cases, fluorescence was excited close to the maximum of the excitation spectrum at 488 nm, and ReISC by the addition of laser light at 568 or 671 nm. Irradiation with these red-shifted wavelengths alone does not result in direct excitation and produces negligible fluorescence emission (cf. Figure 2a).

Figure 2b presents the fluorescence count rates detected from an ensemble of FITC and Rh110 dye molecules embedded in PVA. With sole illumination at 488 nm, the count rate of both samples decreased (exponentially) over time, indicating photobleaching. The bleaching rate increased at higher irrad-
ances (data not shown) indicating the involvement of excited-state reactions in the process.\[^{[11]}\] To directly observe the effect of the red-shifted light, we periodically switched it on and off using a mechanical shutter. For red light at 671 nm both dyes react differently. FITC shows an increase in fluorescence signal during on-periods which becomes more pronounced when increasing the intensity $I_{\text{red}}$ and reaches 5-times larger values at $I_{\text{red}} = 15 \text{ MW cm}^{-2}$. In contrast, the addition of 671 nm irradiation hardly changes the fluorescence of Rh110; only a slight acceleration of the photobleaching process is observed for a very large irradiance of $I_{\text{red}} = 15 \text{ MW cm}^{-2}$. Before bleaching to 5% of the initial signal, periodic intermittence of 671 nm light increases the total fluorescence signal detected for FITC up to a factor of 2 and hardly influences that of Rh110.

### 2.1 Photoinduced Reverse Intersystem Crossing and Multi-Step Photobleaching

We applied FCS and FIDA on single diffusing FITC and Rh110 molecules in PBS buffered solution to gain more insights into the photophysical pathways that may be responsible for the fluorescence changes induced by the red light. Examination of both dyes diffusing in aqueous solution excludes light-induced thermal effects.\[^{[5]}\] Further, rather than monitoring the total fluorescence count rate, FCS and FIDA access concentration-independent, dye-specific molecular parameters such as the molecular brightness or triplet population kinetics\[^{[5,37,38]}\] avoiding influences due to (potentially light-induced) alteration of the dye’s concentration.

#### 2.1.1 Excitation at 488 nm

Figure 3a depicts exemplary FCS data of FITC recorded for increasing excitation irradiance $I_{\text{exc}}$ of the 488 nm light. The FCS data shows two decays, one in the 100 µs range given by the average observation time of a single fluorophore, and another one in the 1 µs range revealing the triplet population kinetics.\[^{[5]}\] The triplet part increases with $I_{\text{exc}}$ (arrow). As outlined in the Data Analysis Section, the amplitude of this decay reflects the average triplet population $T_{\text{eq}}$, which is plotted in the inset of Figure 3a for both FITC and Rh110 following a fit of Equation (7) to the respective FCS data. Up to ~60% of all FITC and ~25% of all Rh110 fluorophores are trapped in the dark triplet state at one time at irradiances $I_{\text{exc}} > 150 \text{ kW cm}^{-2}$. Equation (8) allows the simultaneous determination of triplet depopulation rate constant $k_{\text{TS}}$, which increases with $I_{\text{exc}}$, and the apparent rate constant $k_{\text{ISC(app)}}$ for ISC from the first excited singlet $S_1$ to the lowest excited triplet state $T_1$, which decreases with $I_{\text{exc}}$ (Figure 3b). Both $k_{\text{TS}}$ and $k_{\text{ISC(app)}}$ are larger for FITC. In the simplest case, triplet depopulation takes place only through spontaneous decay and the constant $k_{\text{TS}}$ is given by the inverse triplet lifetime: $\tau_{\text{TS}} = 1/k_{\text{TS}}$. Both, $k_{\text{ISC}}$ and $k_1$ are intrinsic molecular parameters and do not depend in the light intensity. Therefore, this simple model fails and should include additional photophysical pathways.

Before postulating additional pathways, artifacts should be ruled out. For example, at large irradiances, fluorescence saturation effects may interfere with correct FCS analysis.\[^{[59]}\] In particular, these effects bias the determination of $k_{\text{ISC}}$ and lead to an apparently decreasing $k_{\text{ISC(app)}}$ when increasing $I_{\text{exc}}$ (see Data Analysis Section). While it is possible to correct this bias, this requires sophisticated numerical calculations. In order to determine its true value we therefore extrapolated the measured values to low irradiances, where the determination of $k_{\text{ISC(app)}}$ is unbiased obtaining true values of $k_{\text{ISC}} = 6 \times 10^8 \text{ s}^{-1}$ for FITC and $0.9 \times 10^7 \text{ s}^{-1}$ for Rh110. These values agree well with values of $k_{\text{ISC}}$ determined in previous experiments for FITC and rhodamines similar to Rh110.\[^{[5,11]}\]
However, saturation effects cannot explain the apparent increase in $k_{TS}$ and the kinetic model has to be extended to allow for an additional depopulation pathway. We can exclude stimulated de-excitation of $T_1$ to $S_0$ by the excitation light, since internal conversion and not phosphorescence emission dominates $T_1 \rightarrow S_0$ in aqueous solution at room temperature. The only alternative light-induced intramolecular pathway vacating the triplet state and thus increasing $k_{TS}$ is ReISC taking place through higher-excited triplet states $T_n$ as illustrated in Figure 1. While ReISC is usually observed when irradiating with additional, red-shifted light, changes in the fluorescence emission attributed to ReISC have previously also been reported for strong excitation cycling.\[18,25,29,31,32\] In this context, fluorescence excitation induces both population of $T_1$ via ISC and depopulation via ReISC. ReISC however, since coupled to an additional absorption step, only becomes prominent at very large irradiances $I_{exc}$. We can establish the cross sections $\sigma_r(\text{ReISC}) = 4 \times 10^{-19} \text{cm}^2$ and $2 \times 10^{-19} \text{cm}^2$ of ReISC of FITC and Rh110, respectively, by a linear fit of $k_{TS} = k_t + \sigma_r(\text{ReISC}) \cdot I_{exc}$ to the experimental data of $k_{TS}$ [Eq. (3)]. Further, this fit yields values for the inverse triplet lifetime $k_t = 4.5 \times 10^5 \text{s}^{-1}$ and $2.5 \times 10^5 \text{s}^{-1}$ for FITC and Rh110, respectively, which, again, agree well with values reported previously.\[5,11\]

The quantification of photobleaching is of particular importance in the context of ReISC, because it is known that the population of higher excited states such as $T_n$ opens up new and efficient reaction pathways.\[8,12,16,35,41\] While capable of quantifying photobleaching, FCS does not reveal details of photobleaching kinetics under our experimental conditions due to the (chosen) limited observation time in the confocal volume (Data Analysis Section). FIDA, on the other hand, allows us to observe the dependence of the apparent fluorescence brightness $q$ on the excitation irradiance $I_{exc}$ as depicted in Figure 3c. The brightness parameter $q(N, t)$ is of particular importance in experiments studying single diffusing molecules, since it determines the number of photons $N$ detected during the transit time $t$ through the focal area [Eq. (5)]. While $t \approx 100 \mu\text{s}$ is constant throughout our experiments, $N$ is limited both by photobleaching and triplet population.\[6,11,12,35\]

The dependence of $q$ on $I_{exc}$ shows three characteristic regimes:\[12,11,13\]
1) a linear increase of fluorescence emission for low irradiances $I_{exc} < 50 - 100 \text{ kW cm}^{-2}$ following $S_0 \rightarrow S_1$ excitation,
2) saturation to a maximum value stemming from pronounced triplet population and from photobleaching, and
3) a decrease for large irradiances $I_{exc} > 100 - 300 \text{ kW cm}^{-2}$ due to multi-step photobleaching from electronically higher-excited singlets $S_n$ and triplet states $T_n$. From this characteristic dependence rate constants and cross sections of all involved photophysical and photochemical processes and in particular of photobleaching by nonlinear curve fitting are determined.\[13,35\]

Setting the photophysical constants of the triplet population and depopulation to the values obtained from the FCS analysis, we obtain estimates for the combined cross section of photobleaching from $S_n$ and $T_n$ by 488 nm illumination; $\sigma_{\text{obs}} = 1.5 \times 10^{-19} \text{cm}^2$ and $0.7 \times 10^{-19} \text{cm}^2$ for FITC and Rh110, respectively, from a fit of Equation (5) to the brightness data of Figure 3c.

2.1.2 Irradiation with Red-Shifted Light

Figure 4 presents results of the FCS and FIDA analysis of FITC and Rh110 data in aqueous solution under the addition of 568 or 671 nm light with fluorescence excitation under 488 nm. Figure 4a depicts exemplary FCS data of FITC recorded at an excitation irradiance $I_{exc} = 85 \text{ kW cm}^{-2}$ and for increasing irradiance $I_{red}$ of 671 nm light. Again, we did not observe a significant change in the average observation time with changing...
I_{\text{red}}. However, the triplet state is depopulated by the red light (arrow). Fitting Equation (7) to the data of both FITC and Rh110 reveals that the decrease in average triplet population $I_{\text{tr}}$ (inset Figure 4a) depends on an increase of the triplet depopulation rate constant $k_{\text{tr}}$ and a decrease of the apparent rate constant $k_{\text{ISC}}$ (app) for ISC from $S_1$ to $T_1$. [Eq. (8)] (Figure 4b). (Data of $k_{\text{tr}}$ of Rh110 adding 671 nm of $k_{\text{ISC}}$ of FITC adding 568 nm, and of $k_{\text{ISC}}$ of Rh110 adding 568 nm or 671 nm are not plotted in Figure 4b, but are similar to the corresponding counterparts at the other wavelength). Since the red light does not elicit direct excitation and thus saturation of the dyes’ fluorescence emission, we can exclude bias due to saturation effects. Rather, a combination of stimulated emission and ReISC induced by the 671 nm light offers a consistent explanation of this decrease of $k_{\text{ISC}}$ and increase of $k_{\text{tr}}$ observed for increasing irradiance $I_{\text{exc}}$.

With typical cross sections of $-10^{-16}$–$10^{-18}$ cm$^2$, stimulated fluorescence emission by the red-shifted light can compete with the natural decay $<\text{ns}$ state at irradiances $I_{\text{red}} >$ MW cm$^{-2}$. Stimulated emission increases the de-excitation rate $S_1 \rightarrow S_0$ and reduces the probability of ISC, and, for both FITC and Rh110, we can describe the experimental data well with cross sections of $\sigma_{\text{stim}}$ = 1 and $0.2 \times 10^{-17}$ cm$^2$ of stimulated emission by the 568 and 671 nm light, respectively. [Eq. (8)]. Consequently, we determine the true values of $k_{\text{ISC}}$ = 5 and $1 \times 10^3$s$^{-1}$ for FITC and Rh110, respectively, which are independent of $I_{\text{red}}$ and coincide with the values observed in Figure 3b.

Apart from the 488 nm light, the triplet state $T_1$ may also absorb 568 or 671 nm light leading to the population of $T_0$ from where a return to the singlet system induces an additional depopulation channel of the triplet system. Using Equation (3), effective cross sections $\sigma_{\text{ISC}}^*$ of ReISC induced by 568 or 671 nm light can be obtained from a linear fit of $k_{\text{tr}} = k_T + \sigma_{\text{ISC}}^* I_{\text{exc}} + \sigma_{\text{ISC}}^* I_{\text{red}}$ to the experimental data of $k_{\text{tr}}$. The different values obtained by this analysis are listed in Table 1; $\sigma_{\text{ISC}}^* = 0.2$ and $0.05 \times 10^{-18}$ cm$^2$ for FITC and 0 cm$^2$ for Rh110 at 568 and 671 nm, respectively. Simultaneously, such a fit also yields values for the triplet lifetimes $\tau_{\text{tr}}$ which are similar to those determined in Figure 3b.

Figure 4c shows the apparent fluorescence brightness $q$ detected for Rh110 and FITC at constant excitation irradiance $I_{\text{exc}} = 240$ and 85 kW cm$^{-2}$, respectively, and for increasing irradiance $I_{\text{red}}$ of 568 or 671 nm light (left). Strong irradiation of 568 or 671 nm light results in a decrease of brightness $q$, and only in the case of FITC at 671 nm, an increase of $q$ of up to 10% for irradiances $I_{\text{red}} < 50$ MW cm$^{-2}$, are observed. The Figure 4c (right) compares the dependence of $q$ on the excitation irradiance $I_{\text{exc}}$ at 488 nm with and without addition of 568 or 671 nm light. The fluorescence brightness can be manipulated in a similar way (increase for FITC with 671 nm and decreases for FITC with 568 nm and for Rh110 with 568 and 671 nm) over a wide range of excitation irradiances $I_{\text{exc}}$ and particularly at very large excitation irradiances $I_{\text{exc}} > 50$ kW cm$^{-2}$, where triplet population is largest. Based on the photophysical rate constants determined from the FCS analysis (Table 1) we can fit our photophysical model [Eq. (5)] to the dependencies of $q$ on $I_{\text{exc}}$ and $I_{\text{red}}$ involving the cross section $\sigma_{\text{ISC}}^*$ for multi-step photobleaching by the red light; $\sigma_{\text{ISC}}^* = 0.07$ and $0.006 \times 10^{-18}$ cm$^2$ for FITC and 0.05 and 0.0002 $\times 10^{-18}$ cm$^2$ for Rh110 at 568 and 671 nm, respectively (Table 1).

2.1.3 Comparison of FITC and Rh110

Table 1 lists all photophysical and photochemical parameters obtained from the FCS and brightness data for FITC and Rh110 in aqueous solution. Both independent data sets are consistent with our photophysical model and with the same set of rate constants. Further, several characteristics can only be explained if ReISC and stimulated emission are considered. All kinetic rate constants and importantly, all the effective cross sections determined for ReISC, multi-step photobleaching and stimulated emission are in good agreement with values determined in previous works for the same or other organic dyes.[46] Indeed, our data for the cross sections $\sigma_{\text{ISC}}^*$ shows that it is more pronounced for 568 as compared to 671 nm. Due to some approximations such as underestimating saturation effects of the detection volume or assuming equal bleaching rate constants from the singlet and triplet states ($k_{\text{ISC}}$ and $\sigma_{\text{ISC}}^*$) in Equation (4), the values determined for the photokinetic constants might be somewhat inaccurate but they are certainly a good measure for their order of magnitude. Most importantly the data cannot be explained consistently when excluding ReISC and stimulated emission proving the existence of these disclosed pathways.

The maximum fluorescence brightness $q$, and thus number of detected fluorescence photons $N$ in (single-molecule diffusion) experiments following sole 488 nm excitation is limited by photophysical and photochemical processes, namely population of the dark triplet state and pronounced multi-step photobleaching from higher excited electronic states. A central parameter in these processes is the relative population of the dyes’ dark triplet state. It is mainly given by the ratio $S_0/I_T$ which is approximately 2.5 times larger for FITC, resulting in more pronounced trapping in the dark triplet state ($<60\%$) as compared to Rh110 ($<25\%$). Consequently, FITC is more susceptible to photobleaching reactions from $T_0$, and consequently $T_1$. This characteristic results in a more pronounced decrease in fluorescence brightness towards large excitation irradiances $I_{\text{exc}} > 100–300$ kW cm$^{-2}$ (Figure 3c). Most importantly, since the absorption cross section $\sigma_{\text{abs}}$ (Table 1) and fluorescence quantum yield[47] are comparable for Rh110 and FITC, the larger triplet population results in a maximum achievable fluorescence brightness of FITC that is over a factor of 2 lower than for the rhodamine (Figure 3c). In fact, the fluorescence brightness

would be lowered even further in the absence of ReISC, as exemplified in Figure 3c for the case of FITC (–ReISC).

The cross sections \( \sigma_{\text{exc}}^{\text{s}} \) and \( \sigma_{\text{ISC}}^{\text{n}} \) for multi-step photobleaching and ReISC, respectively, are generally larger for FITC as compared to Rh110. Two reasons may be responsible for these effects; 1) lower absorption cross sections \( \sigma_{\text{exc}}^{\text{s}} \) for excitation into \( S_n \) and \( T_n \) respectively, or 2) less reactive \( S_n \) and \( T_n \) states in Rh110. From literature ref. [48], \( T_1 \rightarrow T_n \) absorption cross sections of \( \sigma_{\text{exc}}^{\text{n}} \sim 2 \times 10^{-16} \) \( \text{cm}^2 \) for Rh110 and \( 0.5 \times 10^{-15} \) \( \text{cm}^2 \) for FITC are known, ruling out (1) and highlighting (2). For example, the increased values of \( \sigma_{\text{ISC}}^{\text{n}} \) for FITC correlate with the larger values of the rate constants \( k_{\text{ISC}} \) and \( k_n \) observed for FITC. The increased rate constants indicate a much better probability of singlet–triplet state crossing. Among other characteristics, the probability of singlet–triplet state crossing scales with the energy gap \( \Delta E_{\text{ST}} \) between the first excited singlet \( S_1 \) and the lowest excited triplet state \( T_1 \), which is slightly larger for Rh110 (\( \Delta E_{\text{ST}} = 4080 \) \( \text{cm}^{-1} \)) than for FITC (\( \Delta E_{\text{ST}} = 2700 \) \( \text{cm}^{-1} \)), at least at the experimental conditions applied for the determination of \( \Delta E_{\text{ST}} \). Consequently, in the case of Rh110 we assume that while the energy of the 488 nm light may be large enough to excite into high enough \( T_n \) states with a sufficiently large density of sub-states in \( T_n \) and \( S_n \), the barrier \( \Delta E_{\text{ST}} \) may be too large for the lower energy of the 568 or 671 nm light, providing a density of sub-states which is too small.

2.1.4 Tradeoff between Reverse ISC and Multi-Step Photobleaching

Our observations highlight the tradeoff between a gain in fluorescence brightness due to ReISC and a loss of fluorescence due to multi-step photobleaching. Indeed, it seems to be daring to boost fluorescence via the population of the higher excited states \( S_n \) and \( T_n \), which are rather fragile states, and the feasibility and effectiveness of this approach is ultimately determined by the ratio of the effective cross sections for ReISC and multi-step photobleaching \( \sigma_{\text{ISC}}^{\text{n}}/\sigma_{\text{bn}}^{\text{exc}} \). For example, in the case of FITC, ReISC is promoted for both 568 and 671 nm light, but an enhancement in fluorescence brightness is only observed in the case of the 671 nm light which is readily explained as follows: When moving from 671 to 568 nm the cross section of multi-step photobleaching \( \sigma_{\text{bn}}^{\text{exc}} \) is increased by a factor of 12, while that of ReISC \( \sigma_{\text{ISC}}^{\text{n}} \) is increased by only a factor of 4. Consequently, the ratio \( \sigma_{\text{ISC}}^{\text{n}}/\sigma_{\text{bn}}^{\text{exc}} \) which determines the relative effectiveness of both pathways is \( \sim -8 \) for the 671 nm and only \( \sim -3 \) for the 568 nm light. Therefore, ReISC is more efficient in the first case and leads to an increase in the fluorescence signal while 568 nm light induces so much additional multi-step photobleaching that the signal actually decreases.

It is insightful to simulate the gain in fluorescence brightness \( q \) of single diffusing dye molecules expected from additional red light assuming different pairs of effective cross sections of ReISC (\( \sigma_{\text{ISC}}^{\text{n}} \)) and multi-step photobleaching (\( \sigma_{\text{bn}}^{\text{exc}} \)). The results of such an analysis are shown in Figure 5 which depicts the ratio \( Q = q_{\text{exc}}/q_{\text{exc}}^\ast \) of fluorescence brightness with and without the addition of the red light. We calculated the values of \( Q \) for different pairs of \( \sigma_{\text{ISC}}^{\text{n}} \) and \( \sigma_{\text{bn}}^{\text{exc}} \) using Equation (5), an excitation irradiance \( I_{\text{exc}} = 200 \) kW cm\(^{-2} \) at 488 nm, additional light of 671 nm with irradiance \( I_{\text{red}} = 30 \) MW cm\(^{-2} \), and values of the parameters of \( k_{\text{ISC}} \), \( k_{\text{ISC}} \), \( k_n \), and \( k_{\text{ISC}} \) as listed in Table 1 for our dyes Rh110 (Figure 5a) and FITC (Figure 5b). We also simulated conditions leading to increased \( k_{\text{ISC}} \) (Figure 5c) and increased triplet lifetime, that is, decreased rate \( k_n \) in the dye FITC (Figure 5d). In all cases, the black line marks the barrier between a gain (\( Q > 1 \)) and a loss (\( Q < 1 \)) of fluorescence brightness due to the addition of the red light. The actual experimentally determined values of \( \sigma_{\text{ISC}}^{\text{n}} \) and \( \sigma_{\text{bn}}^{\text{exc}} \) are marked as dots and highlight why a gain in fluorescence in aqueous solution seems only possible for FITC and 671 nm irradiation.

In our simulations, we have neither regarded dependencies on the irradiances \( I_{\text{exc}} \) of the excitation light nor on the irradiance \( I_{\text{red}} \) of the red-shifted light. While \( I_{\text{exc}} \) influences both the level of triplet population and of fluorescence brightness, \( I_{\text{red}} \) determines the degree of excitation into \( T_n \). Consequently, we have chosen \( I_{\text{exc}} = 200 \) kW cm\(^{-2} \) and \( I_{\text{red}} = 30 \) MW cm\(^{-2} \) for our simulations. At this excitation irradiance the experimentally determined triplet populations and fluorescence brightness values are largest for both Rh110 and FITC, while in the case of adding 671 nm light an irradiance of \( I_{\text{red}} = 30 \) MW cm\(^{-2} \) for FITC evoked the largest increase and for Rh110 a remarkable decrease of fluorescence brightness. Further, the choice of the wavelength of the red-shifted light of no importance in our simulations. A change in wavelength only results in different values of \( \sigma_{\text{ISC}}^{\text{n}} \) and \( \sigma_{\text{bn}}^{\text{exc}} \).
Despite these simplifications, our simulations highlight that a gain in fluorescence brightness is not to be expected 1) for \( \sigma_{\text{ISC}} > 10^{-28} \text{cm}^2 \), that is, too efficient multi-step photobleaching, and 2) for ratios \( \sigma_{\text{ISC}}/\sigma_{\text{em}} \) of approximately < 6 for Rh110 and < 8 for FITC (white dashed line). These restrictions on the photosynthetic cross sections change slightly for other values of \( k_{\text{ISC}} \) and \( k_1 \). Both an increase of the ISC rate \( k_{\text{ISC}} \) and a decrease of \( k_1 \) elicit enhanced triplet population and thus different initial situations of ReISC and multi-step photobleaching. Taking the parameters of FITC as an example, at constant depopulation rate \( k_1 \) but increased population rate \( k_{\text{ISC}} \) a gain in fluorescence is still not to be expected for smaller ratios \( \sigma_{\text{ISC}}/\sigma_{\text{em}} \)
< 8. However, an increased rate \( k_{\text{ISC}} \) strongly boosts the gain \( Q \) for pairs \((\sigma_{\text{ISC}},\sigma_{\text{em}}) \) with initial positive net effect \( Q > 1 \) (arrow in Figure 5c). In contrast, a longer triplet lifetime \( 1/k_1 = 10 \mu \text{s} \) allows to gain fluorescence under the addition of red light already for lower ratios \( \sigma_{\text{ISC}}/\sigma_{\text{em}} \) < 1.5 (arrow in Figure 5d).

Even more red-shifted wavelengths than 671 nm may, because ionization reactions from higher excited states (i.e. \( \sigma_{\text{em}} \)) become less pronounced for lower energetic levels, \( \text{eventually improve } \sigma_{\text{ISC}}/\sigma_{\text{em}} \) and thus the effectiveness of ReISC. In the case of Rh110, photon energies of up to 2000 nm light and in the case of FITC of up to 3500 nm light should still be sufficient to cross the triplet–singlet energy gap \( \Delta E_{ST} \approx 4080 \text{ cm}^{-1} \) and 2700 \( \text{cm}^{-1} \), respectively. However, the effective cross section for ReISC \( \sigma_{\text{ISC}}(\lambda) \) decreases by a factor of \( \approx 4 \) when moving from 568 to 671 nm and even by a factor of up to \( \approx 100 \) when comparing 488 and 671 nm (Table 1). As a consequence, irradiances \( I_{\text{red}} \) needed to influence the triplet state population by ReISC are about one order of magnitude larger for 671 nm (> 10 MW cm\(^{-2}\)) than for 568 nm (> 1 MW cm\(^{-2}\)) light, and even several orders above the excitation irradiances \( I_{\text{exc}} = 1–600 \text{ kW cm}^{-2} \) at 488 nm. Therefore it is foreseeable that even longer wavelengths would require too high intensities to be practicable.

### 2.2 Other Dyes and Environment Factors

All parameters involved in the ReISC pathway vary between different fluorescent labels and are very sensitive on the environment. Thus, the effect of the red light may dramatically change from dye to dye, between different environmental conditions, or when adding chemical compounds. A significant increase of single-molecule fluorescence following ReISC has, for example, been shown previously for several different cyanine dyes. However, this increase was highly dependent on the dye’s environment. The fluorescence of single Cy5 dye molecules could efficiently be boosted by ReISC when attached to a peptide and spin-coated in PVA, \(^{[31]}\) bound to DNA, \(^{[31]}\) or immobilized on an air-glass interface, \(^{[31]}\) but ReISC was absent for the free dye in solution. \(^{[31]}\)

We tested our experimental approach on several other dyes by periodically adding red light and observed for changes in the fluorescence signal, as performed in Figure 1. Excited at 488 nm, the organic dyes rhodamine green, Alexa488, oregon green, Atto565, di-chloro-fluorescein and Fluo4, as well as the green fluorescent protein (GFP) did not show an instantaneous increase in fluorescence signal in aqueous solution upon addition of 568 or 671 nm light; rather a decrease was observed similar to Rh110, accounting for excessive multi-step photobleaching.

Using 532 nm excitation, we examined the more red-shifted fluorophores Atto532, rhodamine6G (Rh6G), EosinY, 2-bromo-fluorescein and the enhanced yellow fluorescent protein (EYFP) in aqueous solution. As exemplarily shown in Figure 6 and summarized in Table 2, the organic dyes Atto532, Rh6G (Figure 6a), EosinY (Figure 6b) and 2-bromo-fluorescein showed an instantaneous decrease in fluorescence signal upon the addition of the 671 nm light, which we attributed to multi-
step photobleaching. Multi-step photobleaching is extremely pronounced for EosinY and 2-bromo-fluorescein, which are known for their large ISC yield. In contrast, we observed a slight instantaneous increase in fluorescence signal for EYFP (Figure 6c). Again, this increase in fluorescence signal was not due to direct excitation by 671 nm as sole excitation by 671 nm did not elicit a noticeable signal.

Figure 6 and Table 2 also present the fluorescence signal characteristics of the same dyes in PVA. As for FITC and Rh110 in PVA (Figure 2b), we observed an (exponential) decrease in fluorescence signal over time upon sole fluorescence excitation at 532 nm. (Despite a transient and reversible depletion of intact fluorophores inside and around the focal area during strong illumination and photobleaching, an overall decrease in fluorescence signal is absent in aqueous solution, since photobleached molecules are recovered by diffusion. Depletion of the pool of fluorophores of the whole sample is unlikely, since the sample volume of ~0.1–1 mL is almost infinite compared to the observation volume of ~πf11). Addition of 671 nm light caused an immediate increase in fluorescence signal up to 30% for Atto532 and Rh6G (Figure 6a), but also an acceleration of the overall bleaching rate. For EosinY and 2-bromo-fluorescein we perceived a dramatic boost in instantaneous fluorescence of a factor of up to 14 (Figure 6b). Further, fluorescence was again instantaneously increased for EYFP, even slowing down the overall bleaching rate (c). Consequently, in PVA the total signal detected before photobleaching down to 75% of the initial fluorescence signal is only slightly increased by ~10–20% for the dyes Atto532 and Rh6G, stronger increased by a factor of 2 for the fluorescent protein EYFP, and boosted by a factor of up to 3.5–5 for EosinY and 2-bromo-fluorescein (Table 2).

The data recorded for EosinY and 2-bromo-fluorescein in PVA is in major contrast to their data recorded in aqueous solution. In all cases but EYFP, the effect of adding red-shifted light changes with environment, increasing the fluorescence signal in PVA and decreasing it in water. The slight fluorescence enhancement observed for EYFP in PVA is similar to that observed in water. Compared to organic dyes, the dependence on environment is less pronounced for fluorescent proteins such as EYFP, where the protein barrel shields the chromophore to a certain extent.

Summarized, the effect of the red-shifted light is dramatically altered by changes of the environment which influences both the triplet parameters and in particular the ratio of efficiency of ReISC and multi-step photobleaching (σ_{ReISC}/σ_{ISC}). For example moving from PVA to aqueous solution results in changes of the mobility of the triplet quencher oxygen and changes of the reactivity of the excited states, potentially increasing the lifetime of the triplet or other dark states and affecting photobleaching. While the addition of red light probably raises the fluorescence signal under conditions of prolonged triplet lifetimes, one has to keep in mind that an increased triplet lifetime 1/kT results in worse fluorescence brightness q_{flu}. For example, at constant kISC a 10-fold increase of 1/kT readily leads to an approximately 10-fold increase in triplet population and hence to a 10-fold decrease in single population and concomitantly fluorescence signal [compare Eq. (2)].

Because the red light increases the population of the (more photolabile) higher excited states, our approach benefits from the addition of certain antioxidants known to enhance the fluorophore’s photostability and to protect against ionization reactions from these states. Figure 7 depicts the fluorescence brightness q of single diffusing FITC molecules in aqueous solution gained under addition of 671 nm light with and without the presence of 1 mM of the antioxidant ascorbic acid (ACA). Ascorbic acid leads to a decrease of multi-step photobleaching and a larger increase in fluorescence due to ReISC.

$$q = \frac{\text{fluorescence brightness}}{\text{irradiance}}$$

Figure 7. Influence of ascorbic acid. Fluorescence brightness q of FITC for increasing irradiance I_{red} of 671 nm light and at constant irradiance I_{blue} = 85 kW cm^{-2} of the 488 nm excitation light with (+) and without (-) the addition of 1 mM ascorbic acid (+ ACA). Ascorbic acid leads to a decrease of multi-step photobleaching and a larger increase in fluorescence due to ReISC.
3. Conclusions

The population of the long-lived dark triplet state of a fluorescence marker poses a distinct limit on fluorescence detection. We show that the triplet population can be minimized by adding light, that is red-shifted with respect to the excitation light and does not elicit fluorescence emission on its own. The obtained enhancement in fluorescence brightness stems from the efficient depopulation of the triplet state by photoinduced RefSC. RefSC follows an additional excitation step from the lowest excited triplet state \( T_1 \) to higher excited triplet states \( T_n \) and subsequent recovery into the fluorescent singlet system via higher excited singlet states \( S_n \). Our results show that it is important to include photoinduced RefSC into the molecular photokinetic model and we have laid out a theoretical model that allows quantitative analysis of data obtained by fluorescence fluctuation techniques such as FCS and FIDA. Stimulated emission and most important an increase in photobleaching are concomitant with RefSC. As a consequence, the success of our experimental approach depends on the ratio of the efficiency of RefSC and photobleaching from \( S_n \) and \( T_n \). This ratio is highly sensitive to the wavelength chosen for inducing RefSC, on the environmental conditions and on the properties of the dye selected as fluorescence marker. As an example, adding 671 nm light we achieved fluorescence enhancement for FITC and EYFP in aqueous solution and PVA, but not with 568 nm light, and for Rh6G, Atto532, Eosin Y and 2-bromo-flourescein in PVA, but not in aqueous solution. Important molecular parameters for a positive net effect in fluorescence signal are a sufficiently large absorption cross section of the triplet state \( \sigma_T \) and the yield of energy transfer from \( S_n \) to \( T_n \). Since the photophysics of most fluorophores is influenced by ISC to the triplet system, a gain in signal may be inducible in many common fluorescence experiments. If at least some kinetic parameters of the dye under consideration are known, it may be possible to approximate the expected gain (or loss) in fluorescence upon adding red-shifted light by using Equation (S) or the graphs outlined in Figure S. Success is more probable in systems where the triplet population is significant and that are, therefore, severely limited by ISC. By selecting an appropriate laser wavelength inducing RefSC and carefully choosing the chemical environment to limit multi-step photobleaching a significant increase in brightness should be possible in such cases without changing other experimental parameters. Light-induced RefSC thus introduces a possible non-invasive approach to optimize fluorescence experiments where the fluorescence brightness is critical.

Experimental Section

Confocal Microscope: We carried out the fluorescence experiments at a confocal epi-illuminated microscope (DM IRB, Leica Microsystems, Germany) equipped with a water immersion objective (60× UPLSAPO, NA 1.2, Olympus, Japan). We excited fluorescence emission by 488 or 532 nm laser light (PicoTA, Picoquant, Berlin, Germany), pulsed excitation with \( \approx 100 \) ps pulse width and 80 MHz repetition rate) and induced RefSC using either 568 nm laser light (Krypton laser, Innova Sabre, Coherent, CA) or 671 nm laser light (OPSS5 Monolas-671-300MM, Alphalas, Germany). All lasers were linearly polarized, overlaid and coupled into the microscope using appropriate dichroic mirrors (AHF Analysetechnik, Tübingen, Germany). We spatially and spectrally filtered and 50:50 split up the fluorescence emission from the focal laser spot by the confocal pinhole (~0.8 times the Airy disc of the image laser spot) and by bandpass filters prior to detection by two single-photon avalanche photodiodes (SPCM-AQR-13-FC, Perkin–Elmer Optoelectronics, CA). We further analyzed the single-photon counting detection events using either a hardware correlator card (Flexo02-01D, Correlator.com, NJ) or a single-photon based PC card (SPC 830, Becker & Hickl GmbH, Germany) also offering the possibility to measure fluorescence lifetimes by time-correlated single-photon counting (TCSPC). We determined the size of the excitation volume from correlation data of Rh110 diffusion at low excitation irradiances (Eq. (7)) or from direct point-spread function (PSF) measurements at a scattering gold bead of sub-diffraction diameter (80 nm gold colloid, En.GC80, BBInternational, UK) on a non-confocal detector (MP 963 Photon Counting Module, Perkin–Elmer). The full-width-at-half-maximum (FWHM) of the confocal spot (FWHM \( \approx 400 \) nm for the 488 and 532 nm light and \( \approx 480 \) nm for the 568 and 671 nm light) together with the measured laser power \( P \) at the sample allowed us to calculate the applied irradiance \( I = P/(\pi(FWHM/2)^2) \). The time of illumination and the laser power was controlled by a fast shutter (Uniblitz, BFI Optilas, Germany) and a laser power controller (Brockton Electro-Optics, Brockton, MA), respectively. We determined the background signal due to scattering (<2 kHz) prior to each measurement.

Spectral Measurements: The absorption and fluorescence emission spectra were recorded using a UV/VIS (Cary 4000, Varian, Darmstadt, Germany) and a fluorescence spectrometer (Cary Eclipse, Varian), respectively, employing a 1 cm × 2 mm quartz cuvette (Hellma, Germany). For determination of the energy gap \( \Delta E_{515} \) between the first excited singlet \( S_1 \) and the lowest excited triplet state \( T_1 \), we recorded fluorescence and phosphorescence emission spectra at liquid nitrogen temperature using a home-build cryostat and calculated \( \Delta E_{515} \) from the difference in the maxima of the two emission spectra. In this way determined values of \( \Delta E_{515} \) might change for different environments such as different polarity solvents.

Sample Preparation: All samples were freshly prepared and measured at room temperature of –22 °C. We diluted all dyes Rh110, Rh6G, Atto532, Atto565, rhodamine green, Alex488, Oregon green, di-chloro-fluorescein, Eosin Y, 2-bromo-fluorescein (MoBiTec, Göttingen, Germany; AttoTec, Siegen, Germany; or Radiant Dyes, Wermelskirchen, Germany) and the GFP and EYFP in PBS buffer pH 7 except the fluorescein-isothiocyanate (FITC, Sigma–Aldrich), which we diluted in PBS buffer pH 8. The final dye concentrations were \( 10^{-4} \) M for FCS and FIDA measurements and \( 10^{-3} - 10^{-10} \) M for ensemble measurements. We performed measurements of the free diffusing dyes in 100 µL samples sealed on microscope cover glass. For ensemble measurements in poly(vinyl-alcohol) (PVA) we diluted the buffered stock solution of the dyes in PVA to a final dye concentration of \( 10^{-6} - 10^{-10} \) M and spin-coated the PVA samples on microscope cover glass. The ascorbic acid (ACA, Fluka) was added to aqueous solution at a final concentration of 1 mM.
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