Intramolecular photostabilization via triple-state quenching was recently revived as a tool to impart synthetic organic fluorophores with “self-healing” properties. To date, utilization of such fluorophore derivatives is rare due to their elaborate multi-step synthesis. Here we present a general strategy to covalently link a synthetic organic fluorophore simultaneously to a photostabilizer and biomolecular target via unnatural amino acids. The modular approach uses commercially available starting materials and simple chemical transformations. The resulting photostabilizer-dye conjugates are based on rhodamines, carbopyronines and cyanines with excellent photophysical properties, that is, high photostability and minimal signal fluctuations. Their versatile use is demonstrated by single-step labelling of DNA, antibodies and proteins, as well as applications in single-molecule and super-resolution fluorescence microscopy. We are convinced that the presented scaffolding strategy and the improved characteristics of the conjugates in applications will trigger the broader use of intramolecular photostabilization and help to emerge this approach as a new gold standard.
Introduction

Organic fluorophores are a major driving force for the recent success of fluorescence-based methods, but they intrinsically suffer from transient excursions to dark states (blinking) and irreversible destruction (photobleaching). Both processes fundamentally limit their applicability and have, for a long time, hampered the development of advanced microscopy techniques with single-molecule sensitivity or optical super-resolution <250nm. Lütke and colleagues introduced covalent binding of triplet-state quenchers and singlet-oxygen scavengers to organic fluorophores as a strategy to reduce the above mentioned effects. Such photostabilizer-dye conjugates with intramolecular triplet-state quenching have “self-healing” or “self-protecting” properties, preventing photodamage without the use of solution additives. This non-invasive strategy has clear advantages compared with commonly used approaches, where micro- to millimolar concentrations of organic compounds are added to the buffer system.

Intramolecular photostabilization was recently revived independently by two groups, to reduce photobleaching and blinking even in demanding applications such as single-molecule fluorescence microscopy or in vivo imaging. It was also shown that efficient intramolecular photostabilization can be achieved without additives in the buffer system, minimizing potential influences on the biological system of interest. Applications of intramolecular photostabilization in biophysical or microscopy research are, however, still limited to proof-of-principle studies. This is mainly due to the synthetic effort of photostabilizer-dye conjugates, which thus far requires a multi-step synthesis route. These synthetic challenges represent a fundamental hurdle for researchers with limited organic chemistry experience to use this concept. Moreover, only a small number of bifunctional cyanine derivatives are currently available to synthesize photostabilizer-dye conjugates on specific biomolecular targets (DNA, RNA, proteins and antibodies), strongly restricting the choice of fluorophore type (chemical structure, redox potential, water solubility and so on) and photophysical properties (colour, brightness, fluorescence lifetime and so on). Especially the available cyanine fluorophores suffer from limited brightness and signal-to-noise ratio (SNR) due to cis/trans isomerization, a fact that emphasizes the urgent need for a synthetic strategy to study and use other classes of organic fluorophores via intramolecular photostabilization.

Here we introduce a versatile and simple design concept to synthesize photostabilizer-dye conjugates on a specific bio-molecular target using unnatural amino acids (UAAs). UAAs were chosen as a scaffold that links multiple chemical units, that is, the fluorophore and photostabilizer to a specific target. The presented conjugation strategy is based on well-known chemical reactions with (commercially available derivatives of) synthetic organic fluorophores, photostabilizers and UAAs, which can be bound as a single moiety to a biomolecular target. Depending on the UAA scaffold, the chemical nature of the functional groups can be N-hydroxysuccinimimid esters (NHS), alkynes, azides or other bio-orthogonal reactive functionalities. Two different UAAs were used to bind different rhodamine, carbopyronine and cyanine fluorophores (Alexa555, RhodamineB, KK114, ATTO647N and Cy5) covalently to a photostabilizer on distinct biomolecular targets.
The synthesized fluorophore derivatives comprise of either a reducing or oxidizing photostabilizer in the form of the antioxidant Trolox (TX) or a nitrophenyl group. We characterized their photophysical properties with single-molecule fluorescence microscopy on the biomolecular target DNA and observed significant increases in photostability for all compounds including suppression of triplet-based blinking. Secondly, photostabilizer-dye conjugates were synthesized, which allow labelling of biomolecules (DNA, antibodies and proteins) in a single step as with commercially available fluorophores. Finally, we demonstrate state-of-the-art applications of photostabilizer-dye conjugates in single-molecule Förster resonance energy transfer (smFRET) and super-resolution stimulated emission depletion (STED) microscopy, with significantly increased sensitivity and photostability of the conjugates compared to their non-stabilized parent fluorophores.

**Results**

**Amino acids for intramolecular photostabilization.**

Figure 4.1 shows the central idea to use UAAs as a scaffold that links multiple chemical units, that is, fluorophore and photostabilizer onto a specific biomolecular target. The conjugation strategy is based on established chemical reactions (amide bond formation or click chemistry) using commercially available derivatives of synthetic organic fluorophores (F), photostabilizers (P) and UAAs.

As a proof-of-concept, we synthesized photostabilizer-dye conjugates of fluorophores that could thus far not be tested for intramolecular photostabilization due to limited scaffolding options. We focused on rhodamines and carbopyronines, that is, fluorophores from the ATTO and Alexa series, which are extremely popular for (life science) applications. (S)-Nitrophenylalanine, NPA (Scheme 4.1), was used as a scaffold for the first generation of compounds, which consists of single-stranded DNA (ssDNA) as the biomolecular target, a commercially available organic fluorophore (Alexa555, ATTO647N and Cy5) and the p-nitrophenyl group of the known photostabilizer NPA. (Scheme 4.1 compounds 5, 6 and 7). The cyanine fluorophore Cy5 served as a “positive control” experiment that permits to benchmark the effects of intramolecular photostabilization for different fluorophore classes with respect to published studies. The used DNA and its specific base sequence was selected because of the detailed photophysical characterization of various fluorophores on this target.

![Figure 4.1](image_url) Design concept for photostabilizer-dye conjugates. UAAs are used to combine an organic fluorophore covalently with a photostabilizer on a biomolecular target or linker structure.
Scheme 4.1 | Synthesis route towards photostabilizer-dye conjugates on nucleic acids. NPA-based fluorophores of Alexa555, ATTO647N and Cy5 on ssDNA. Fmoc, fluorenylmethyloxycarbonyl; DCC, N-N’-dicyclohexylcarbodiimide; Su, succinimide; DMF, dimethylformamide; NaHCO₃ buffer, 200 mM sodium hydrogen carbonate buffer pH 8.35; TBTA, tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine; TEAA buffer, 50 mM triethylammonium acetate buffer.

The synthesis shown in Scheme 4.1 was conducted starting from commercially available fluorenylmethyloxycarbonyl (Fmoc)-protected NPA (1), which was converted into the corresponding NHS-ester derivative (2) to react with a 5’-aminoalkyl functionalized ssDNA, yielding 3. Subsequent Fmoc deprotection of 3 was followed by a reaction of the resulting primary amine (4) with the commercially available NHS-ester derivative of Alexa555, ATTO647N or Cy5, to yield 5, 6 and 7, respectively. All compounds comprise an organic fluorophore and a p-nitrophenyl group for intramolecular photostabilization linked to the biomolecular ssDNA target (Figure 4.1 and Scheme 4.1) and are abbreviated “NPA fluorophore” throughout the text. The non-stabilized control compounds were obtained via a direct reaction of the NHS-ester-activated fluorophores with the respective amino-modified ssDNA-NH₂. The DNA-photostabilizer-dye conjugates were isolated by high-performance liquid chromatography (HPLC; see Materials and Methods section) and characterized by ultraviolet-visible absorption spectroscopy and mass spectrometry (see Materials and Methods section). After purification, all compounds elute as single peaks in the HPLC chromatogram (see Materials and Methods section) and display ultraviolet-visible absorption maxima characteristic for DNA (~260 nm) and the respective chromophore (~550/~650nm) (see Materials and Methods section). In addition, matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry reveals a significant mass increase of the DNA-dye conjugates compared with the non-modified DNA (see Materials and Methods section). See the Materials and Methods section and the Materials and Methods section for further details on chemical synthesis and characterization of functionalized oligonucleotides and reactive precursor molecules.

Next, single-molecule fluorescence microscopy was used to benchmark the potential of the scaffolding approach with respect to photophysical parameters. Confocal scanning microscopy was used to investigate signal fluctuations and fluorescence lifetime, whereas total-internal-reflection fluorescence (TIRF) microscopy was employed to obtain quantitative pho-
to physical values such as bleaching lifetime, fluorescence count rate, total number of photons and SNR ratio. The different fluorophore derivatives were immobilized according to published procedures on a streptavidin-coated microscope coverslip by hybridization to form double-stranded DNA (dsDNA) comprising a 3’-terminal biotin unit (Appendix Figure 4.A.1-4.A.9). All experiments described in this section were performed in the absence of oxygen, to minimize the convolution of triplet-state quenching by molecular oxygen and intramolecular photostabilization by NPA.

Using confocal microscopy, single immobilized fluorophores on DNA were identified as spots in the images and fluorescence time traces were recorded for each of the spots using single-photon counting detectors. The length of the fluorescence time traces gave an estimate of the photostability (the longer, the more stable), whereas fluctuations in the time traces indicated blinking due to transient population of dark states (such as the triplet state). The calculation of the autocorrelation function of the individual fluorescence time traces revealed the underlying time constants of the blinking kinetics (Figure 4.2-4.4) and time-correlated single-photon counting was used to determine the fluorescence lifetime. The non-stabilized fluorophores, showed fast photobleaching and pronounced blinking on the corresponding dsDNA in deoxygenated PBS buffer (Figure 4.2a, 4.3a and 4.4a), whereas the NPA derivatives showed a stable fluorescence signal over extended periods of time (Figure 4.2b,c, 4.3b,c and 4.4b,c).

Fluorescence time traces of Alexa555, a rhodamine-based fluorophore, were characterized by short observation times and pronounced blinking on the timescale of 22 ± 6 ms in deoxygenated PBS buffer (Figure 4.2a and Appendix Figure 4.A.1). This is a typical behaviour for rhodamines and is caused by population of the triplet state. The fluorescence lifetime of the sample was found to be 1.2 ± 0.3 ns (Appendix Figure 4.A.1). On conjugation of Alexa555 to the NPA scaffold (NPA-Alexa555, compound 5), the triplet-induced blinking diminished (negligible autocorrelation) and the overall fluorophore photostability and count rate increased to several seconds and 5-10 kHz, respectively, at ~0.3 kW-cm⁻² irradiance (Figure 4.2b and Appendix Figure 4.A.2). Despite the overall increase in count rate (mainly due to abolishment of dark state transitions), the fluorescence lifetime of NPA-Alexa555 decreased to 0.9 ± 0.1 ns compared with the parent fluorophore, indicating the presence of singlet quenching by the NPA scaffold (Appendix Figure 4.A.2).

The photophysical behaviour of ATTO647N in deoxygenated PBS buffer was characterized by a mixture of short and long photobleaching times ranging from seconds to minutes with pronounced blinking on the millisecond timescale (Figure 4.3c and Appendix Figure 4.A.3). The off-state lifetime associated with the blinking events was found to be 29 ± 5 ms and is attributed to the triplet state (Figure 4.3c and Appendix Figure 4.A.3). This and the observed fluorescence lifetime of 4.2 ± 0.4 ns are in agreement with literature. Covalent binding of NPA to ATTO647N resulted in a homogeneous, bright, non-blinking and prolonged fluorescence emission (Figure 4.3b). The fluorescence lifetime was reduced to 3.4 ± 0.2 ns for the majority of traces, again indicating dynamic singlet quenching by the NPA scaffold. Notably, >50% of the observed NPA-ATTO647N molecules did not photobleach within the 2 min observation period.
Figure 4.2 | Photophysical characterization of photostabilizer-Alexa555 conjugates with confocal microscopy. All data was recorded with a home-built confocal microscope with a sample in aqueous PBS buffer at pH 7.4 in the absence of oxygen. Each panel shows a representative overview image (10 × 10 μm, 50 nm pixel size, 2 ms per pixel) with spots from individual immobilized fluorophores (left), a fluorescence time trace (middle) and the corresponding autocorrelation decay in black, with according fits in grey (right). (a,b) Alexa555 and NPA-Alexa555 (image intensity scale from 3 to 60 counts, excitation intensity of ∼0.3 kW·cm⁻² at 532 nm). (c) Additional fluorescence time traces for NPA-Alexa555.

at excitation intensities of ∼0.66 kW·cm⁻² (see Figure 4.3b and Appendix Figure 4.A.4). In addition, a small fraction (<30%) of NPA-ATTO647N molecules showed a reduced lifetime of around 2.6 ns in combination with a lower count rate pointing to stronger singlet quenching (Appendix Figure 4.A.4). Direct switching of a single NPA-ATTO647N molecule between the two states was observed and will be a topic of future research.¹¹

Results for Cy5 (Figure 4.4e and f) were obtained at higher excitation intensities (4 kW·cm⁻²), to allow for a direct comparison with previously published results using different approaches towards intramolecular photostabilization.¹⁰ In agreement with earlier findings, the autocorrelation analysis of Cy5 fluorescent time traces revealed the presence of two different photophysical processes that were attributed to triplet blinking (11 ± 4 ms) and cis/trans isomerization (54 ± 12 μs).⁴,¹⁸,²⁷,²⁸ The fluorescence lifetime of Cy5 was found to be 1.65 ± 0.15 ns (compare also Appendix Figure 4.A.5). NPA-Cy5 (7) under identical conditions revealed bright and prolonged
fluorescence emission with typical observation times of several seconds and, as revealed by the autocorrelation analysis, with negligible triplet-state blinking for ~75% of the observed emitters (Figure 4.4). The remaining autocorrelation decay revealed the on-off transition due to cis/trans isomerization, with a lifetime in the order of ~50-70 µs (see Appendix Figure 4.A.5 and 4.A.6), which therefore still restricts the overall achievable count rate for the cyanine fluorophores. In addition, a mono-exponential fit does not fully describe the experimental autocorrelation decay (see the deviations between the obtained data and the grey exponential fit function in Figure 4.4), suggesting a remaining small triplet population with a lifetime <100 µs. A smaller population of molecules (<25%) showed intensity fluctuations on completely different timescales (Appendix Figure 4.A.6), which is consistent with earlier reports, and its origin remains to be explained mechanistically. The fluorescence lifetime of NPA-Cy5 was found to be 1.60 ± 0.15 ns and is similar to the parent fluorophore, indicating that any reduction in the signal brightness

**Figure 4.3** | Photophysical characterization of photostabilizer-dye conjugates with confocal microscopy. All data was recorded with a home-built confocal microscope with a sample in aqueous PBS buffer at pH 7.4 in the absence of oxygen. Each panel shows a representative overview image (10 × 10 µm, 50 nm pixel size, 2 ms per pixel) with spots from individual immobilized fluorophores (left), a fluorescence time trace (middle) and the corresponding autocorrelation decay in black, with according fits in grey (right). (a,b) ATTO647N and NPA-ATTO647N (image intensity scale from 5 to 100 counts, excitation intensity of ~0.66 kW·cm⁻² at 640 nm). (c) Additional fluorescence time traces for NPA-ATTO647N.
Figure 4.4 | Photophysical characterization of photostabilizer-dye conjugates with confocal microscopy. All data was recorded with a home-built confocal microscope with a sample in aqueous PBS buffer at pH 7.4 in the absence of oxygen. Each panel shows a representative overview image (10 × 10 µm, 50 nm pixel size, 2 ms per pixel) with spots from individual immobilized fluorophores (left), a fluorescence time trace (middle) and the corresponding autocorrelation decay in black, with according fits in grey (right). (a, b) Cy5 and NPA-Cy5 image intensity scale from 10 to 300 counts, excitation intensity of 4 kW·cm⁻² at 640 nm. (c) Additional fluorescence time traces for NPA-Cy5.

of NPA-Cy5 is due to static quenching, that is, transient formation of non-fluorescent complexes between photostabilizer and fluorophore rather than dynamic singlet quenching. These findings are consistent with a subtle blue shift in the Cy5-absorption spectrum when bound to NPA.

To further quantify the improved performance of the photostabilizer-dye conjugates, we used single-molecule TIRF microscopy to determine various parameters with high statistics. Movies with 100 ms integration time were recorded at laser excitation intensities of ~50-100 W·cm⁻², that is, a significantly lower excitation intensity than in the confocal scanning microscopy experiments, yielding different count rates and SNR ratios. Figure 4.5a shows a typical example of a camera frame with single Cy5 fluorophores. The number of fluorescent molecules per video frame was determined and the decay in number of molecules over subsequent image frames was fitted with an exponential decay \( y(t) = B + Ae^{-t/\tau_{\text{bleach}}} \), to obtain the photobleaching lifetime \( \tau_{\text{bleach}} \) (Figure 4.5a Fig. 4a). Background-corrected single-molecule time traces were extracted and

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used to determine the fluorescence count rate in kHz (Figure 4.5a, brightness), the SNR ratio (Figure 4.5a) and the total number of detected photons before photobleaching (Figure 4.5a, \( N_{\text{total}} = \text{brightness} \times \tau_{\text{bleach}} \)). The mean and s.d. of all values was derived from multiple \((n > 3)\) independent experiments. We benchmarked the performance of the photostabilizer-dye conjugates against the antioxidant TX as a solution additive in the deoxygenated imaging buffer (2 mM after 20 min ultraviolet treatment), which is a common standard for photostabilization in single-molecule experiments\(^{10,12}\).

The data of the non-stabilized rhodamine Alexa555 revealed a brightness of \(1.5 \pm 0.2\) kHz, \(N_{\text{total}} = 5.1 \pm 0.4 \times 10^4\) and an SNR = 3.5 \(\pm 0.3\), whereas the photobleaching could be described by a mono-exponential decay with a time constant \(\tau_{\text{bleach}} = 34 \pm 26\) s (Figure 4.5b). An improvement for most of the photophysical parameters was found for both adding 2 mM TX or Alexa555-NPA with up to a 20-fold increase in photostability, 25-fold increase in \(N_{\text{total}}\) and a 3-fold increase in SNR, whereas the brightness was found to be comparable.

Similar results were obtained for both the carbopyronine ATTO647N and the cyanine dye Cy5 (Figure 4.5c and Supplementary Movie 1). The photobleaching time increased from \(\tau_{\text{bleach}} = 138 \pm 93\) s (ATTO647N) to 212 \(\pm 52\) s (NPA-ATTO647N) and 298 \(\pm 45\) s (ATTO647N + 2 mM TX), and from \(\tau_{\text{bleach}} = 7.0 \pm 1.5\) s (Cy5) to 139 \(\pm 55\) s (NPA-Cy5) and 384 \(\pm 60\) s (Cy5 + 2 mM TX). This results in reduced blinking and an increase in the total number of detected photons \((N_{\text{total}})\) and SNR from \(N_{\text{total}} = 1.9 \pm 0.7 \times 10^5\) and SNR = 1.6 \(\pm 0.2\) (ATTO647N) to \(N_{\text{total}} = 8.6 \pm 0.4 \times 10^5\) and SNR = 11.6 \(\pm 1.7\) (NPA-ATTO647N), and \(N_{\text{total}} = 2.0 \pm 0.7 \times 10^6\) and SNR = 17.4 \(\pm 2.4\) (ATTO647N + 2 mM TX), as well as \(N_{\text{total}} = 3.1 \pm 0.9 \times 10^4\) and SNR = 3.0 \(\pm 0.7\) (Cy5) to \(N_{\text{total}} = 6.4 \pm 1.6 \times 10^5\) and SNR = 11.6 \(\pm 1.7\) (NPA-Cy5), and \(N_{\text{total}} = 2.4 \pm 0.6 \times 10^6\) and SNR = 9.5 \(\pm 1.2\) (ATTO647N + 2 mM TX), that is, 20- to 50-fold increases.

The investigations in Figure 4.2-4.4 and Figure 4.5 regarding the photophysical properties of rhodamines, carbopyronines and cyanines with intramolecular photostabilization revealed the following: (i) photobleaching and blinking could be efficiently removed using UAA scaffolding and (ii) all photophysical parameters were improved substantially compared with the parent fluorophore. (iii) Despite showing no decrease in brightness, rhodamines and carbopyronines showed singlet quenching, whereas cyanines show no reduction of the excited state lifetime. (iv) Solution-based healing using 2 mM TX remained more efficient than intramolecular photostabilization.

Despite the slightly lower photostability of compounds with intramolecular photostabilization compared with solution additives such as TX, this approach has unique advantages that compensates for these shortcomings. The UAA approach is thus far the only possible method for photostabilization when organic fluorophores are used under live-cell conditions, in experiments where the addition of a diffusion-based photostabilizer is not tolerated due to its toxicity or when the photostabilizer has an unwanted influence on properties of the system of interest\(^{32}\). It is also the only viable option when diffusion-based photostabilization remains ineffective which could be caused by lack of collisions between the photostabilizer and the fluorophore\(^{33,34}\).
Figure 4.5 | Photophysical characterization of photostabilizer-dye conjugates and their parent fluorophores with single-molecule TIRF microscopy. Data was recorded in aqueous PBS buffer at pH 7.4 in the absence of oxygen under continuous 640 nm excitation with \( \sim 50 \, \text{W/cm}^2 \) or 532 nm excitation with \( \sim 20 \, \text{W/cm}^2 \). (a) Representative image frame (left panel) showing single fluorescent molecules (10 × 10 \( \mu \text{m} \), exemplarily for Cy5). Subsequent images recorded over a period of 500 s showed an exponential decrease in the number of fluorescing molecules with a photobleaching lifetime \( \tau_{\text{bleach}} \), as shown for Cy5 (black), Cy5 with 2 mM TX (blue) and NPA-Cy5 (red) (middle panel). The curves shown were obtained by averaging over >5 TIRF movies. (b,c,d) Right panel: chemical structures (left panels) and respective photophysical parameters obtained from background-corrected fluorescence traces for Alexa555 (b, rhodamines), ATTO647N (c, carbopyronines) and Cy5 (d, cyanines). Values and error bars (s.d.) in bar graphs obtained from \( N > 4500 \) molecules.

UAAs as a general scaffold for photostabilizer-dye conjugates.
The scaffolding strategy presented in Scheme 4.1 is restricted by the availability of commercial
UAAs with different (photostabilizing) residues and does not allow to use custom-made photostabilizers. We hence set out to generalize the approach and to allow linkage of three arbitrary moieties. The amino acid propargylglycine (PG (8); Scheme 4.2) represents a more versatile scaffold that can link three chemical groups via NHS and clickchemistry (here, a [3+2]-Huysgens cycloaddition between alkyne and azide derivatives). Hence, PG (8) provides the flexibility needed to combine any photostabilizer and fluorophore on a (bio)molecular target, assuming their availability as NHS- or click-reactive derivatives.

In the first synthetic step, racemic 8 was reacted with the NHS-ester derivative of TX (9). The resulting adduct 10 was converted into the corresponding NHS-ester derivative 11 and reacted with the 5’-aminoalkyl functionalized oligonucleotide to yield 12. Finally, the fluorophore Cy5 was bound using a copper-catalysed click reaction yielding 13, called TX-PG-Cy5 throughout the manuscript.

TX-PG-Cy5 (13) showed a very similar photophysical behaviour compared with NPA-Cy5,
Scheme 4.2 | DCC, *N*-N’-dicyclohexylcarbodiimide; Su, succinimide; DMF, dimethylformamide; NaHCO₃ buffer, 200 mM sodium hydrogen carbonate buffer pH 8.35; TBTA, tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine; TEAA buffer, 50 mM triethylammonium acetate buffer, pH 7.0.

Figure 4.7 | Photophysical characterization of TX-PG-Cy5 (13) with confocal microscopy. All data was recorded with a home-built confocal microscope with a sample in aqueous PBS buffer at pH 7.4 in the absence of oxygen. Here a representative overview image (10 × 10 µm, 50 nm pixel size, 2 ms per pixel) with spots from individual TX-PG-Cy5 (13) fluorophores (top-left), a fluorescence time trace (top-middle) and the corresponding autocorrelation decay in black, with according fits in grey (top-right) and additional fluorescence time traces. Intensity scale from 10 to 300 counts, excitation intensity of 4 kW cm⁻² at 640 nm.

that is, removal of blinking and an increased photobleaching lifetime (Figure 4.5d) with significant improvement factors (up to 11-fold). However, a higher heterogeneity was observed with ~60% of the molecules showing fluorescent traces with little intensity fluctuations (shorter bleaching lifetime; Figure 4.7). The remaining ~40% exhibited longer observation times with
an increased amount of blinking events (Figure 4.7). A unifying fluorescence lifetime of $1.65 \pm 0.15$ ns was observed, which is equal to the parent Cy5. Several TX-PG-Cy5 molecules showed a typical behaviour for fluorophores with intramolecular photostabilization, where “bleaching” or irreversible destruction of the stabilizer occurred before photobleaching of the fluorophore. Here the fluorophore drastically changed its emission pattern from stable to a blinking emission pattern that closely resembles the behaviour in the absence of photostabilizer (Figure 4.7, bottom row, middle panel: stable: 0-8.5 s; blinking: 8.5-10 s).

The PG-based fluorophore system showed to be a versatile scaffold where the photostabilizer and fluorophore can be varied independently of each other. This has the advantage that the fluorophore and photostabilizer can be matched, to obtain maximum photostabilization as was shown above for the fluorophore Cy5 and the photostabilizer TX.

**Direct labelling of biomolecules.**

The synthesis and photophysical characterization of the photostabilizer-dye conjugates in the previous sections demonstrated the principle improvements in fluorescence emission properties by the UAAs scaffolding approach. Yet, our overall goal is to show the potential of the new fluorophores in biological research. For this, the most important step is labelling biomolecules such as proteins, nucleic acids or antibodies. As shown in “UAAs as a general scaffold for photostabilizer-dye conjugates”, this usually requires a complex (multi-step) synthesis process, demanding larger amounts of substances, that is, both fluorophore and biomolecular target. However, the amount of biomolecules is often limited. We hence reduced the chemical steps needed for labelling to a minimum. For this we altered the synthesis strategy of the photostabilizer-dye conjugates, introducing a functionalized group such as a NHS ester or a maleimide, which allow using the fluorophore derivative straightforward for labelling of a biomolecular target via primary amines or cysteine residues (as is done conventionally, Figure 4.8). NPA was again used as a scaffold for this second generation of photostabilizer-dye conjugates.

Owing to its ease and cost-effective accessibility, we first used the NHS-ester derivative of the dye RhodamineB (14), which was subjected to NPA (15) under basic conditions to yield 16 (Scheme 4.3). Subsequent activation with NHS/N-N’-dicyclohexylcarbodiimide in dimethylformamide (DMF) gave the NHS-ester derivative of the NPA-RhodamineB conjugate (17). In a
Scheme 4.3 | Synthesis of reactive photostabilizer-dye conjugates of RhodamineB for direct labelling of primary amines. The strategy can be extended to other biomolecular targets by a reaction of 17 to change or add a complementary functionality, such as 2-maleimidoethylamine to yield a maleimide derivative for labeling of proteins via solvent exposed cysteines.

Figure 4.9 | Photophysical characterization of RhodamineB and NPA-RhodamineB with confocal microscopy. All data was recorded with a home-built confocal microscope with a sample in aqueous PBS buffer at pH 7.4 in the absence of oxygen. Each panel shows a representative overview image (10 × 10 μm, 50 nm pixel size, 2 ms per pixel) with spots from individual immobilized fluorophores (left), a fluorescence time trace (middle) and the corresponding autocorrelation decay in black, with according fits in grey (right). (a) RhodamineB; (b) NPA-RhodamineB (image intensity scale from 3 to 60 counts, excitation intensity of ~0.3 kW·cm⁻² at 532 nm). (c) Additional fluorescence time traces for NPA-RhodamineB.

first step, we (as for the previous fluorophores) targeted this conjugate to ssDNA (18), which was done straightforward from 17 without further purification. The photophysical characteristics of the resulting compound were characterized when immobilized on glass in deoxygenated buffer. Similar to the fluorophores studied before (see Figure 4.2-4.4), RhodamineB showed strong
Figure 4.10 | (a) Average and standard deviation of the mean (error bars) of photophysical parameters of RhodamineB without and with Trolox (2 mM TX) and of NPA-RhodamineB, as determined from the TIRF data of Appendix Figure 4.A.6 and 4.A.9: photobleaching time $\tau$ (left panel), total number of detected photons $N_{\text{total}}$ (middle left), brightness (middle right), and signal-to-noise ratio (SNR, right panel). Recorded in aqueous PBS buffer at pH 7.4 in the absence of oxygen (continuous 532 nm excitation at $\sim$50 W cm$^{-2}$). Values in bar graphs were derived from $N > 1000$ molecules. (b) Representative single molecule time traces measured with TIRF microscopy. RhodamineB (left) and NPA-RhodamineB (right) measured at 532 nm excitation with 50 W cm$^{-2}$ (detection with HC582/75).

Blinking in deoxygenated PBS buffer with typical observation times until photobleaching on the timescale of 10–20 s (Figure 4.9b and Appendix Figure 4.A.8). In contrast, RhodamineB displayed strongly heterogeneous blinking characteristics with very short or longer off times within one trace (Figure 4.9a and Appendix Figure 4.A.8). Consequently, the autocorrelation function of this fluctuating signal could only be described by a bi-exponential decay with average off times peaking at 7 ± 4 ms and a significant fraction of values >20 ms, deviating from a normal distribution (Figure 4.9a and Appendix Figure 4.A.8). This indicates the presence of multiple dark states or heterogeneous dye environments. Still, the blinking off times were in the same range as determined before for the triplet state of the structurally related rhodamine fluorophores, for example, Alexa555 (22 ± 6 ms, Appendix Figure 4.A.1) or ATTO565 (6 ± 2 ms) and are hence considered to be triplet related. Yet, the fluorescence lifetime of RhodamineB was found to be rather homogeneously distributed with an average of 3.1 ± 0.4 ns (Figure 4.9a and Appendix Figure 4.A.8). Strikingly, NPA-RhodamineB on ssDNA (18) showed strongly reduced blinking, an increased photostability resulting in a stable and non-blinking emission pattern with observation times of up to minutes (Figure 4.9b and Appendix Figure 4.A.9) and an increased brightness (Figure 4.10).

Next, we intended to use our NPA-based photostabilizer-dye conjugates for direct labelling of proteins. For this purpose, different synthesis strategies were developed, accounting for the available quantity of the fluorophore. In the most straightforward case, that is, larger amounts
of amine-reactive photostabilizer-dye conjugate are available (for example, >50 mg of compound 17), the NHS ester of the fluorophore (17) can be coupled directly with for example 2-maleimidodithyline, to yield a maleimide derivative of, for example, NPA-RhodamineB. Note that variations to 2-maleimidodithyline can result in photostabilizer-dye conjugates with longer linkers or other biocompatible reactive linking groups. As shown in “Biomolecular FRET study with photostabilizer-dye conjugates”, maleimide derivatives of photostabilizer-dye conjugates can covalently bind to recombinant proteins via solvent-exposed cysteine residues (see Materials and Methods section).

**Biomolecular FRET study with photostabilizer-dye conjugates.**

To show the benefits of intramolecular photostabilization in fluorescence applications with proteins, we studied the substrate-binding domain 2 (SBD2) of the Lactococcus lactis ABC transporter GlnPQ36. Using smFRET and alternating laser excitation (ALEX) spectroscopy, the conformational states of the protein were monitored (Figure 4.11a, open unliganded and closed liganded). The structural rearrangements of SBD2 on ligand binding causes a change of ∼0.9 nm regarding the distance between two selected amino acids in the protein36. As the FRET donor and acceptor are attached via maleimide chemistry at these positions in the protein (mutant of SBD2: T369C/S451C), the transfer efficiency $E^*$ reports on the conformational state of the protein. As described previously36, SBD2 was labelled stochastically using appropriate mixtures of donor and acceptor fluorophores (details see Materials and Methods section). To understand the effects of intramolecular photostabilization in FRET-based assays, we used different fluorophore combinations: Cy3B or RhodamineB as donor fluorophores and ATTO647N as the acceptor. In experiments described below, either the donor (RhodamineB) or the acceptor (ATTO647N) was stabilized via covalent linkage to NPA (synthesis see Scheme 4.3 and the Materials and Methods section).

We used smFRET and ALEX spectroscopy, as described in literature34,36,37, to investigate the photophysical properties of the fluorophores when bound covalently to the protein and the biomolecular function of SBD2. ALEX is a valuable tool for both purposes, as it allows to distinguish the desired protein molecules containing both fluorophores, thus monitoring protein conformation (donor-acceptor species; Figure 4.11b, 0.9 < S < 0.4), from those labelled with only one species not providing distance information (donor only, S > 0.9; acceptor only, S < 0.4). Although S relates to the relative fluorophore brightness and labelling stoichiometry, $E^*$ indicates the FRET efficiency and thus distance between the donor and acceptor (with larger values indicating small donor-acceptor distances), which is the final read out of the protein conformation. In addition, ALEX reveals photophysical artefacts such as blinking of donor or acceptor in the form of bridges between the three different subpopulations38. For such measurements, the fluorescence emission of the donor F(DD) under green excitation, that of the acceptor F(DA) when excited via FRET from the donor and that of the acceptor via direct red excitation light F(AA) was determined (see the Materials and Methods section and literature34,36,37). In our experiments, individual biomolecules were studied for short time periods of a few milliseconds,
while diffusing through the excitation volume of a confocal microscope. The challenge of such an experiment is to acquire intense fluorescent bursts during the short observation time under the high excitation intensities of >10 kW·cm\(^{-2}\).

The combination of Cy3B and ATTO647N is known for excellent photophysical performance resulting in fast data acquisition and superior histogram quality in smFRET with little bleaching artefacts and narrow distributions (Figure 4.11c). For apo-SBD2 we find mean E* and S of 0.48 ± 0.08 and 0.59 ± 0.07, respectively, with these labels. These values are in good agreement with our published work and the mean E* correlates with the expected interprobe distance of ∼4.9 nm derived from the crystal structure.

Results of such quality are, however, only available when using 2 mM TX as a photostabilizer in solution, seen from comparison of Figure 4.11c and d. Here we show data of apo-SBD2 (labelled with Cy3B/ATTO647N) in the presence and absence of TX. In agreement with Kong et al., the high excitation intensities used in our experiments promote acceptor signal fluctuations, that is, blinking and/or bleaching. Cy3B and ATTO647N can hence be seen as a FRET couple where the acceptor photostability is limiting. This appears as a prominent bridge between the donor- only and donor-acceptor population (Figure 4.11d), altering both E*/S- values substantially. Closer inspection of the histogram reveals that a significant portion of the molecules show these unwanted photophysical effects. Under these conditions, neither mean E* nor correction factors for accurate FRET determination are directly accessible. Besides the complete loss of information, the overall acquisition time has also increased in the absence of photostabilizer to obtain sufficient statistics from the relevant donor-acceptor species. It should be noted that such photophysical artefacts of the acceptor (Figure 4.11d) are extremely problematic for data interpretation, as they suggest the existence of (non-biological) species in between the donor only and the actual FRET species (see 1D-E* in Figure 4.11d that can only be fitted by the sum of two Gaussians with E* = 0.19 ± 0.07 and 0.43 ± 0.1). Furthermore, it remains challenging to identify those when performing smFRET with green excitation in continuous-wave mode.

Strikingly, the bridge population can be removed by sole photostabilization of the acceptor fluorophore via scaffolding of ATTO647N to NPA. The ALEX data of apo-SBD2 with Cy3B/NPA-ATTO647N is shown in Figure 4.11e. Here the bridge between the donor-acceptor and donor-only population is fully removed without the addition of stabilizer to the solution (Figure 4.11d versus Figure 4.11e). The resulting mean E*/S values are changed compared to apo-SBD2 with Cy3B/ATTO647N (2 mM TX in solution, Figure 4.11b) accounting for decreased acceptor brightness; mean E* is now found at 0.34 ± 0.09 and mean S at 0.67 ± 0.08 (Figure 4.11e). These differences are, however, expected considering the results from ATTO647N on DNA, where a decrease in the overall brightness is observed on conjugation to NPA (Figure 4.5c). It should be mentioned that differences in the donor-acceptor population relative to donor and acceptor only comparing the samples Cy3B/ATTO647N and Cy3B/NPA-ATTO647N are not solely due to photophysics but also due to different labelling ratios of the protein.

Next, we used Cy3B/NPA-ATTO647N to study the biomolecular function of SBD2 (Figure 4.11b). On addition of the ligand glutamine, the mean E* changes from 0.36 ± 0.1 (open, Results | 93
Figure 4.11 | Improving smFRET-ALEX measurements by using NPA-based fluorophore as FRET acceptor on the protein GlnPQ-SBD2. (a) Crystal structures of the SBD2 (T369C/S451C) open (left panel, PDB: 4KR5) and closed state (right panel, PDB:4KQP, after binding of the ligand glutamine shown in red) with label positions of donor (D) and acceptor (A). (b) Corresponding one-dimensional histograms of E*-values for increasing amounts of ligand glutamine where an increased E = 0.55 ± 0.1 (dashed line, closed conformation) becomes visible (as opposed to the open conformation, E = 0.36 ± 0.1, dashed line). Excitation intensities of 30 kW·cm⁻² at 532 nm and 20 kW·cm⁻² at 640 nm; data evaluated with dual colour burst search and displayed with additional per-bin thresholds of F(DD) + F(DA) + F(AA) >75 and minimal number of counts per bin in ALEX histogram of 3. (c), (d), (e) 2D histograms of joint pair values of S (labelling stoichiometry) and E* (FRET efficiency, that is, interprobe distance) of Cy3B/ATTO647N in the presence (c) and absence (d) of 2 mM TX and Cy3B/NPA-ATTO647N (e) without ligand glutamine. Figure caption continues on the next page.
Figure 4.11 | (Continued) Excitation intensities of 30 kW·cm⁻² at 532nm and 20 kW·cm⁻² at 640 nm; data evaluated with all photon burst search and displayed with additional per-bin thresholds of F(DD) + F(DA) + F(AA) >100 and minimal number of counts per bin in ALEX histogram of 3. (f, g, h) Histogram of fluorophore brightness values as determined from photon-counting histograms (PCHs) on single-molecule transits of labelled SBD2 diffusing through the observation volume, comparison of donor brightness F(DD) (f), acceptor brightness when excited via FRET, F(DA) (g) and acceptor brightness via direct red excitation F(AA) (h). Excitation intensities of 30 kW·cm⁻² at 532nm and 20 kW·cm⁻² at 640 nm. (i, j, k) Dependence of the mean count rate of F(DD), F(DA) and F(AA) of the different samples for increasing excitation intensity, respectively.

interprobe distance of ~4.9 nm) to 0.55 ± 0.1 (closed, with a decreased interprobe distance of ~4.0 nm). A concentration of 200 mM saturates ligand binding and therefore results in a 100% population of the closed state (Figure 4.11b). A ligand concentration of 1 mM, which is close to the Kd-value of the protein, consequently results in a mix of open and closed states (Figure 4.11b).

As fluorophore brightness and the resulting photon budget ultimately determine the quality of the final histograms including the necessary measurement time, we quantitatively analysed Cy3B/ATTO647N and Cy3B/NPA-ATTO647N by means of photon-counting histograms. Figure 4.11f, g, and h shows the three relevant photon streams used to determine E* and S of Cy3B/ATTO647N (donor-acceptor: 0.9 < S < 0.4; bridge: 0.9 < S < 0.7) and Cy3B/NPA-ATTO647N (donor-acceptor: 0.9 < S < 0.4). F(DD) shows that the strongest donor quenching and hence the most efficient FRET is found for the addition of TX to Cy3B/ATTO647N, whereas molecules in the bridge (Cy3B/ATTO647N, no TX) show inefficient donor quenching due to a non-functional acceptor (Figure 4.11f). Cy3B/NPA-ATTO647N and healthy molecules from the Cy3B/ATTO647N population with no TX show a similar performance of the acceptor-based donor quenching. F(DA) correlates directly with quenching in F(DD) as seen in Figure 4.11g. Again, the best performance is observed from Cy3B/ATTO647N in the presence of TX, while molecules in the bridge show the lowest counts. A striking difference between the bridge and all other conditions is seen in F(AA), that is, direct excitation of the acceptor (Figure 4.11h). The overall comparison suggest that NPA-based acceptor stabilization is sufficient to remove photophysical artefacts and hence make the smFRET useful for biomolecular studies. Further optimization of the data quality could, however, be obtained by additional donor stabilization of, for example, Cy3B in this case. This interpretation is further supported by excitation intensity-dependent count rates of Cy3B/ATTO647N and Cy3B/NPA-ATTO647N (Figure 4.11i, j and k). NPA-based acceptor stabilization improves the saturation characteristics in all three channels but the addition of TX to the solution (resulting in stabilization of both donor and acceptor at the same time) remains superior in terms of achievable count rates.

To study the donor dependence in more detail, we repeated the above described experiments using RhodamineB/ATTO647N and NPA-RhodamineB/ATTO647N. Here, the photostability of the donor is the limiting factor for smFRET data quality. For RhodamineB/ATTO647N we find prominent donor blinking in the absence of photostabilizer (Figure 4.12b). The bridge between the donor-acceptor and acceptor-only population can be removed by addition of TX in solution.
Figure 4.12 | (a, b, c) Two-dimensional histograms (2D) of joint pair values of S (labeling stoichiometry, dashed lines $0.42 < S < 0.75$ donor-acceptor population DA) and $E^*$ (FRET-efficiency, i.e., interprobe distance) of Cy3B/ATTO647N in the presence (a) and absence (b) of 2 mM TX, and Cy3B/NPA-ATTO647N (c) without ligand glutamine. Excitation intensities of 30 kW·cm$^{-2}$ at 532 nm and 10 kW·cm$^{-2}$ at 640 nm; data evaluated with all photon burst search and displayed with additional per-bin thresholds of $F(DD + F(DA) + F(AA)) > 150$ and minimal number of counts per bin in ALEX histogram of 3. (d, e, f) Histogram of fluorophore brightness values as determined from PCH on single-molecule transits of labeled SBD2 diffusing through the observation volume of our confocal microscope: comparison of donor-brightness $F(DD)$ (d) acceptor-brightness when excited via FRET, $F(DA)$ (e), and acceptor-brightness via direct red excitation, $F(AA)$ (e). Excitation intensities of 30 kW·cm$^{-2}$ at 532 nm and 20 kW·cm$^{-2}$ at 640 nm. Note the varying S-ranges used for data evaluation using only bursts for the PCH corresponding to $0.75 < S < 0.42$ for Cy3B/ATTO647N (± 2 mM TX) and Cy3B/NPA-ATTO647N, but $0.42 < S < 0.32$ for the bridge in Cy3B/ATTO647N. (g, h, i) Dependence of the mean count rate of $F(DD)$, $F(DA)$ and $F(AA)$ of the different samples for increasing excitation intensity.

(Figure 4.12a) or conjugation of RhodamineB to NPA (Figure 4.12c). The overall magnitude of the observed effects is lower than for the case of acceptor bleaching/bleinking in Figure 4.11. Photon-counting histograms and the intensity dependence show a similar trend as before, that is, correlation between $F(DD)$ and $F(DA)$ with the wish to increase donor photostability as much as possible. Our data makes clear that NPA-RhodamineB can be used at significantly higher excitation intensities than RhodamineB, as triplet-state population was minimized and would allow for faster and better data acquisition (Figure 4.12g, h, and i).
Our results show that the simple addition of the NPA unit to the acceptor fluorophore is sufficient to gather reliable results from solution-based smFRET (Figure 4.11 and Figure 4.12). Although the addition of a stabilizer on the donor fluorophore can increase the overall available photon budget (which should always be maximized), the acceptor strictly requires a solution-based or covalently linked photostabilizer. We consequently suggest to stabilize the acceptor as a minimum and (if possible) also to stabilize the donor to maximize available count rates and hence data quality. We finally note that all smFRET results of SBD2 with the different pairs of fluorophores shown here are in good agreement with biochemical data from isothermal calorimetry and with our previous results, supporting our hypothesis of an induced-fit-type mechanism in GlnPQ\textsuperscript{36}. In addition, it confirms successful labelling of the protein with the custom-made NPA derivative, and that the results from the established FRET assay are indeed independent of the fluorophore pair that is used to monitor the protein conformation\textsuperscript{35}. It is noteworthy that spectrally uncorrected apparent FRET is reported here accounting for absolute differences in mean E*-values from varying Förster radius $R_0$ of the fluorophore pairs used or variations in the setup alignment\textsuperscript{46}.

**Conclusion and Discussion**

Our results show that effective intramolecular photostabilization and removal of (triplet induced) dark states can be achieved for NPA- and PG-based fluorophore derivatives with similar or better photostabilization effects as previously introduced photostabilizer-dye conjugates. This shows that the UAA scaffold is a simple and useful tool to covalently link fluorophore, photostabilizer and biomolecular target in a modular manner. The presented approach has many advantages over existing strategies, as it is cost effective, requires low amounts of material, uses only well-known chemical transformations such as amide bond formation or click chemistry, requires standard purification procedures and has the potential to address various important biomolecular targets.

Moreover, our conjugation strategy opens the possibility to apply intramolecular photostabilization to fluorophore classes that could not be studied before. This possibility broadens our understanding of intramolecular photostabilization and might provide a basis for further improvement of the photostabilizer-dye conjugates by studying the effects of fluorophore properties such as charge, redox potential or absorption/emission wavelength. For certain types of fluorophores, for example, oxazines and perylenes, conjugation to NPA is expected to show effects different from those described here considering their interaction with antioxidants\textsuperscript{4,25,26}, DNA bases and amino acids such as tryptophane\textsuperscript{40–42}. Intramolecular quenching of Alexa fluorophores observed with natural amino acids\textsuperscript{43} even suggests that labelling of proteins at strategic locations, for example, in the vicinity of aromatic amino acids, could be used for fluorophore stabilization without the use of UAAs, provided that a suitable combination of fluorophore and amino acids can be found.

Intramolecular photostabilization remains, however, less effective compared to the use of diffusion-based photostabilization (see Figure 4.5) as reported before in Chapter 2\textsuperscript{18}. This
shortcoming of intramolecular photostabilization is compensated by the fact that it is often the only viable option for applications such as live-cell imaging, for assays where the addition of photostabilizer remains ineffective or is not tolerated by the biological system. We are convinced that future work - also facilitated by the presented synthesis strategy - will allow solving this problem. We hypothesize that the properties of photostabilizer-dye conjugates can be optimized by parameters such as the linking geometry between fluorophores and photostabilizer. This is supported by published data of Cy5 derivatives, where we linked the aromatic nitro group to the fluorophore in a different way as shown in Chapter 19, compared with the compounds shown in this study (compound 7). These architectures show a significantly higher photostability using basically the same photostabilizer moiety. We are in the process of varying the linker length of NPA-based fluorophore derivatives, to optimize the effects of intramolecular photostabilization and to become fully competitive with solution additives as shown in Chapter 19.

We finally suggest that UAA scaffolding could be extended beyond photostabilization, to provide a general framework for the manipulation of fluorophore properties. Potentially interesting UAs feature antioxidants, triplet sensitizers, photoswitchable molecules to induce blinking for localization-based super-resolution microscopy, alter water solubility or the affinity to membranes and residues of natural amino acids such as thiols (see Lui et al. for an overview of UAA residues). UAs with “clickable” functionalities could be used to link the fluorophore to a biomolecular target and an affinity tag (strep-/his-tag) to simplify purification of labelled protein species.

Materials and Methods

Sample preparation and surface immobilization of oligonucleotides. Immobilization and investigation of single fluorophores was achieved using a dsDNA scaffold comprising two 40-mer oligonucleotides, that is, ssDNA fluorophore and ssDNA biotin. Sequences of both oligomers were adapted from literature. As a non-stabilized control we used ssDNA fluorophore (Cy5-C6-5'-TAA TAT TCG ATT CCT TAC ACT TAT ATT GCA TAG CTA TAC G-3' ; as received from Eurofins and IBA). Single immobilized fluorophore molecules were studied in Lab-Tek eight-well-chambered cover slides (Nunc/VWR, The Netherlands) with a volume of 750 ml as described in literature. After cleaning with 0.1% hydrofluoric acid (HF) and washing with PBS buffer (one PBS tablet was dissolved in deionized water yielding a 10 mM phosphate buffer with 2.7 mM potassium chloride and 137 mM sodium chloride at pH 7.4; Sigma Aldrich, The Netherlands), each chamber was incubated with a mixture of 5 mg per 800 ml BSA and 1 mg per 800 ml BSA/biotin (Sigma Aldrich) at 4°C in PBS buffer overnight. After rinsing with PBS buffer, a 0.2-mg·ml⁻¹ solution of streptavidin was incubated for 10 min and subsequently rinsed with PBS buffer.

The immobilization of dsDNA was achieved via a biotin-streptavidin interaction using pre-annealed dsDNA with the aim to observe single emitters for prolonged time periods and to guaranty free rotation of fluorophores. For this, 5-50 ml of a 1 μM solution of ssDNA fluorophore,
ssDNA-NPA-fluorophore or ssDNA-TX-PG-Cy5 was mixed with the complementary ssDNA-biotin at the same concentration (Biotin-5’-CGT ATA GCT ATG CAA TAT AAG TGT AAG GAA TCG AAT ATT A-3’, used as received from IBA). The respective mixtures of two oligomers were heated to 98°C for 4 min and cooled down to 4°C with a rate of 1°C-min⁻¹ in annealing buffer (500 mM sodium chloride, 20 mM TRIS-HCL and 1 mM EDTA at pH 8). The treated LabTek coverslides were incubated with a 50-100 pM solution of pre-annealed dsDNA for 1–2 min, leading to a typical surface coverage of fluorophore-labelled dsDNA as shown in Figure 4.2–4.4, 4.5 and 4.7 and Appendix Figure 4.A.1–4.A.9.

All single-molecule experiments were carried out at room temperature (22 ± 1°C). Oxygen was removed from the buffer system using an oxygenscavenging system (PBS, pH 7.4, containing 10% (wt-vol⁻¹) glucose and 10% (vol-vol⁻¹) glycerine, 50 mg·ml⁻¹ glucose oxidase, 100-200 mg·ml⁻¹ catalase and 0.1 mM Tris(2-carboxyethyl)phosphine hydrochloride (TCEP)). As shown before, such low concentrations of the reducer TCEP has no noticeable effect on the photo-physics of organic fluorophores and hence do not convolute with effects from intramolecular stabilization. Glucose oxidase catalase was used instead of a combination of protocatechuic acid and protocatechuate-3,4-dioxygenase to avoid convolution of inter- and intramolecular photostabilization with protocatechuic acid. When adding TX to the imaging buffer (PBS with 2 mM TX), the latter was irradiated with ultraviolet light for 30 min before adding 10% (wt-vol⁻¹) glucose and 10% (vol-vol⁻¹) glycerine, 50 mg·ml⁻¹ glucose oxidase, 100-200 mg·ml⁻¹ catalase and 0.1 mM TCEP.

Confocal scanning microscopy and data analysis. A custom-built confocal microscope, described in Chapter 2, was used to study fluorescence properties of organic fluorophores on the level of single molecules. Briefly, excitation was achieved with a spectrally filtered laser beam of a pulsed supercontinuum source (SuperK Extreme, NKT Photonics, Denmark) with an acousto-optical tunable filter (AOTFnc-VIS, EQ Photonics, Germany), leading to ~2 nm broad excitation pulses centred at 640 nm or 532 nm. The spatially filtered beam was coupled into an oil-immersion objective (60×, numerical aperture (NA) 1.35, UPLSAPO 60XO mounted on an IX71 microscope body, both from Olympus, Germany) by a dichroic beam splitter (zt532/642rpc, AHF Analysentechnik, Tuebingen, Germany). Surface scanning was performed using a XYZ-piezo stage with 100 x 100 x 20 μm range (P-517-3CD with E-725.3CDA, Physik Instrumente, Germany). Fluorescence was collected by the same objective, focused onto a 50-μm pinhole and detected by two avalanche photodiodes (τ-spad, <50 dark counts per second, Picoquant, Germany) with appropriate spectral filtering (green: HC582/75, red: ET700/75 AHF, both from Analysentechnik). The detector signal was registered using a Hydra Harp 400 ps event timer and a module for time-correlated single-photon counting (both from Picoquant). The data were evaluated using custom-made LabVIEW software. Blinking kinetics were extracted from fluorescent time traces in the form of ON and OFF times according to established procedures. Fluorescence lifetimes were determined using time-correlated single-photon counting as described before in Chapter 2.
TIRF microscopy including data analysis. Widefield fluorescence imaging was conducted on an inverted microscope (Olympus IX-71 with UPlanSApo × 100, NA 1.49, Olympus, Germany) in objective type TIRF) configuration as described before in Chapter 2. Images were collected with a back-illuminated electron multiplying charge-coupled device camera (512 × 512 pixel, C9100-13 (Hamamatsu, Japan), in combination with either ET585/50 or ET700/75 (AHF Analysentechnik)). Excitation from a diode laser (Sapphire and Cube (Coherent, Germany), filtered either by ET535/70 or ZET640/10 (Chroma, USA)) was at 532 nm and 640 nm with ~50 W cm⁻² at the sample location. To quantitatively characterize photostability, we imaged areas with the size of ~25 × 35 mm containing >100 molecules. A movie was typically recorded for 300-600 s with an integration time of 100 ms. Fluorescent time traces were extracted by identifying pixels, which showed at least 2-3 s.d. above background noise (s.d. of all pixels over all frames of the movie) and summing the intensity in a 3 × 3 pixel area. Neighbouring peaks closer than 5 pixels were not taken into account (see typical examples of fluorophore density and fluorescent time traces in Appendix Figure 4.A.1-4.A.9). The number of fluorescent spots in each frame image was determined using an absolute threshold criterion. The number of fluorescent emitters per image were than plotted over time and fitted to a mono-exponential decay (Equation 4.1):

\[ y(t) = B + Ae^{-ct} \] (4.1)

with \( c = 1/\tau_{\text{bleach}} \) and \( \tau_{\text{bleach}} \) being the characteristic bleaching time constant. For some samples with a more complicated behaviour, a double exponential decay of similar form was used and \( \tau_{\text{bleach}} \) was calculated according to \( \tau_{\text{bleach}} = A_1 \cdot \tau_1 + A_2 \cdot \tau_2 \) with amplitude normalization to 1. Bleaching times and associated s.d. were derived from multiple repeats of the same experiment on different days, where each compound was tested in >5 movies. The SNR ratio was determined using fluorescent time traces, by dividing the s.d. of the signal before photobleaching with the average fluorescence intensity during that period. The total number of detected photons before photobleaching was calculated by multiplying the obtained count rate and photobleaching lifetime.

Single-molecule FRET and ALEX spectroscopy. For data acquisition we used the same home-built confocal microscope as described above. Fluorescence photons arriving at the two detection channels (donor detection channel D or acceptor detection channel A) were assigned to either donor- or acceptor-based excitation, based on their photon arrival time as described previously. Three relevant photon streams were extracted from the data corresponding to donor-based donor emission \( F(DD) \), donor-based acceptor emission \( F(DA) \) and acceptor-based acceptor emission \( F(AA) \). During diffusion, stoichiometry \( S \) and apparent FRET efficiencies \( E^* \) were calculated for each fluorescent burst above a certain threshold yielding a two-dimensional (2D) histogram. Uncorrected FRET efficiency \( E^* \) monitors the proximity between the two fluorophores and is calculated according to Equation 4.2:

\[ E^* = \frac{F(DA)}{F(DD) + F(DA)} \] (4.2)
Stoichiometry $S$ is defined as the ratio between the overall fluorescence intensity during the green excitation period over the total fluorescence intensity during both green and red periods, and describes the ratio of donor-to-acceptor fluorophores in the sample (Equation 4.3):

$$S = \frac{F(DA) + F(DD)}{F(DD) + F(DA) + F(AA)}$$  \hspace{1cm} (4.3)

Using published procedures to identify bursts corresponding to single molecules, we obtained bursts characterized by three parameters ($M$, $T$ and $L$). A fluorescent signal is considered a burst provided it meets the following criteria: a total of $L$ photons are collected, having $M$ neighbouring photons within a time interval of $T$ microseconds. We applied different burst search algorithms on the data sets: the sum of all three detection channels (all photons) using parameters $M = 15$, $T = 500 \mu s$ and $L = 50$ or a dual-colour burst search with $M = 12$, $T = 500 \mu s$ and $L = 30$; additional per-bin thresholding removed spurious changes in fluorescence intensity and selected for intense single-molecule bursts; precise treatment of each data set is given in each figure caption. Binning of the detected bursts into a 2D $E^*/S$ histogram yielded subpopulations that can be separated according to their $S$-values. Photon-counting histograms were obtained using similar thresholds as for the 2D $E^*/S$ histograms. ALEX experiments were carried out at room temperature using 25-50 pM of double-labelled protein in imaging buffer: 50mM KPi, pH 7.4, 150mM KCl, pH 7.4, containing 10% (wt-vol$^{-1}$) glucose and 10% (vol-vol$^{-1}$) glycerine, 50 mg·ml$^{-1}$ glucose oxidase, 100-200 mg·ml$^{-1}$ catalase and 0.1 mM TCEP. For experiments with TX added to the imaging buffer, 2 mM TX, 50 mM KPi, pH 7.4, and 150 mM KCl were irradiated with ultraviolet light for 30 min before adding 10% (wt-vol$^{-1}$) glucose and 10% (vol-vol$^{-1}$) glycerine, 50 mg·ml$^{-1}$ glucose oxidase, 100-200 mg·ml$^{-1}$ catalase and 0.1 mM TCEP. Titration experiments were done via adding increasing concentrations of ligand to the imaging buffer (0, 1.0 and 200 mM of glutamine).

**Count rate versus excitation intensity.** The count rate per molecule dependence on the excitation intensity was recorded using 20-100 nM of labelled SBD2 protein in imaging buffer (10 mM KPi, 2.7 mM KCl, 137 mM NaCl, pH 7.4, 10% (wt-vol$^{-1}$) glucose, 50 mg·ml$^{-1}$ glucose oxidase, 100-200 mg·ml$^{-1}$ catalase, and 0.1 mM TCEP, both in the presence and the absence of 2 mM aged TX. The count rate per molecule was acquired via excitation from the 543 and 633 nm laser on a LSM710 confocal laser scanning microscope (Carl Zeiss, Jena, Germany) through a C-apochromat × 401.20 w Korr M27 water-immersion objective (NA = 1.2). Excitation light from both lasers (at different intensities) was coupled into the objective by a dichroic beam splitter (MBS 458/543/633). Fluorescence was collected through the same objective with appropriate spectral filtering (NFT 635 VIS and LP 580).

**STED and confocal microscopy.** STED microscopy and the corresponding confocal microscopy was performed on an Abberior Instruments Resolf microscope (Abberior Instruments, Goettingen, Germany) to which a Ti:Sa STED laser was added (MaiTai, Newport-Spectra...
Physics; as outlined in detail in literature[49]. Excitation was performed at 640 nm (LDH-D-C-640P laser diode, Picoquant) and STED at 780 nm, at 80 MHz. Pulsing of the excitation laser was triggered by the Ti:Sa laser using a photodiode (APS-100-01), an amplifier (CON-TTL, both from Becker & Hickl, Berlin, Germany) along with a ps-delay unit (either Abberior Instruments or LAS-015617, MPD/Laser2000 UK). A donut-shaped intensity distribution of the STED laser focus was realized by the incorporation of a vortex phase plate (VPP-1a, RPC Photonics, Rochester, NY) into the collimated STED beam. Laser focusing and fluorescence collection was done by an oil-immersion microscope objective (UPlanSapo x 100/1.4 oil, Olympus, Japan). The collected fluorescence was detected by an avalanche photodiode (SPCM-AQRH-13, Excelitas Technologies) with a 650-690 nm bandpass filter (670/40, AHF Analysentechnik).

Immunolabelling. Gm5756T human fibroblast were fixed with 3% paraformaldehyde for 10 min, rinsed several times in PBS, permeabilized in 0.1% Triton X-100 for 10 min, rinsed again several times in PBS and blocked in 2% BSA/5% FCS in PBS for 1 h. Samples were incubated with primary mouse antibodies, to stain nuclear pore diluted 1:500 in blocking buffer for 1 h at room temperature. Coverslips were washed five times in 1% BSA in PBS and then incubated for 30 min with goat anti-mouse antibodies (Abberior GmbH, Göttingen, Germany) diluted 1:250 in blocking buffer. Samples were washed five times in 1% BSA in PBS and mounted in the buffer system using an oxygen-scavenging system as used before in the single-molecule experiments.

Synthesis of photostabilizer-dye conjugates and precursors. All reagents were purchased from commercial suppliers and used without further purification, unless stated otherwise. Cy5 fluorophores were obtained from Lumiprobe (Germany), Alexa555-NHS was obtained from Life Technologies (USA) and ATTO647N-NHS from ATTOTEC (Germany). Synthetic oligomers (NH$_2$-C6-5’-TAA TAT TCG ATT CCT TAC ACT TAT ATT GCA TAG CTA TAC G-3’) were received in HPLC-purified quality from IBA or Eurofins (Germany). TX-NHS 9 was synthesized following a published procedure[50]. A Varian 400 (400 and 100 MHz) and Varian 500 MHz were used to record $^1$H-NMR and $^{13}$C-NMR spectra. Chemical shifts ($\delta$) are denoted in p.p.m. using residual solvent peaks as internal standard ($\delta$H$ = 7.26$ and $\delta$C$ = 77.0$ for CDCl$_3$; $\delta$H$ = 3.31$, 4.78 and $\delta$C$ = 49.15$ for CD$_3$OD). High-resolution mass spectra were recorded on an Orbitrap XL (Thermo Fisher Scientific; ESI pos. or neg. mode). Liquid chromatography-mass spectra were recorded on Waters Acquity H-class UPLC equipped with TUV detector and a Xevo G2 TOF mass detector from Waters Chromatography BV. Flash chromatography was performed using a Grace Revelesis Flash System (40 µm silica column).

Purification of functionalized oligonucleotides. The functionalized oligonucleotides were purified on an Illustra NAP 5 column loaded with Sephadex G-25 DNA Grade material obtained from GE Healthcare. Illustra NAP 5 columns were equilibrated with 3 x 5 ml of eluent before use (50 mM triethylammonium acetate (TEAA) buffer, pH 7.0, or water for Fmoc-protected oligonucleotide 3 and 12). After the reaction, the oligonucleotide sample was diluted with eluent to a final volume of 0.5 ml and added to the column. After the sample was loaded, 1 ml of eluent
was added and the purified oligonucleotide was collected in one portion. The oligonucleotide solution was lyophilized (Christ Alpha 2-4 LD plus freeze dryer) directly after collection.

Isolation and characterization of functionalized oligonucleotides. Reversed-phase HPLC (rp-HPLC) analysis and preparative purifications (isolation) was performed on a Shimadzu LC-10AD VP machine equipped with Waters Xterra MS C18 column (3.0 × 150 mm, particle size 3.5 µm) and Waters Xterra MS C18 prep column (7.8 × 150 mm, particle size 10 µm) using a gradient of acetonitrile/TEAA buffer (50 mM, pH 7.0). Gradient 1:05/95, 0-10 min, to 65/35 at 60 min, to 75/25 at 65 min, to 05/95 at 75 min for 15 min. Gradient 2: 05/95, 0-10 min, to 35/65 at 60 min, to 65/35 at 65 min, to 05/95 at 70 min for 20 min. Flow 0.5 ml·min⁻¹ analytical run or 1.0 ml·min⁻¹ preparative run. The DNA was isolated by collecting the major peak of interest. The resulting compounds were characterized by ultraviolet-visible absorption spectroscopy and MALDI-TOF mass spectrometry. Spectra were recorded on an ABI Voyager DE-PRO MALDI-TOF (delayed extraction reflector) Biospectrometry Workstation mass spectrometer.

2: ("NPA-NHS") (S)-2,5-dioxopyrrolidin-1-yl-2-(((9H-fluoren-9-yl)methoxy)carbo-nyl)amino)-3-(4-nitro-phenyl)propanoate To 1 (173 mg, 0.4 mmol) and N-hydroxsuccinimide (NHS) (46 mg, 0.4 mmol), in 1.5 ml anhydrous 1,4-dioxane, N,N’-dicyclohexylcarbodiimide (DCC) (83 mg, 0.4 mmol) was added at 0 °C. After stirring at ambient temperature overnight, the mixture was cooled to 10 °C and the precipitate was filtered. The filtrate was evaporated in vacuo. Residual 1,4-dioxane was removed by subsequent addition and evaporation of anhydrous ethanol. NPA-NHS was used for coupling without further purification. For analysis NPA-NHS (2) was purified by column chromatography (SiO2, chloroform) and isolated as colorless oil. Yield: 85 mg (0.160 mmol, 40%). 1H NMR (400 MHz, CDCl₃) δ = 8.14 (d, J=8.2, 2H), 7.78 (d, J=7.4, 2H), 7.55 (d, J=7.3, 2H), 7.37 (m, 6H), 5.19 - 5.03 (m, 2H), 4.58 (dd, J=10.6, 6.5, 1H), 4.45 (dd, J=11.0, 6.2, 1H), 4.20 (t, J=6.4, 1H), 3.40 (dd, J=14.3, 5.4, 1H), 3.31 (dd, J=14.1, 5.1, 1H), 2.87 (s, 4H). 13C NMR (101 MHz, CDCl₃) δ = 168.4, 166.8, 155.1, 147.4, 143.6, 143.3, 141.9, 141.4, 141.3, 130.7, 127.9, 127.1, 124.9, 124.8, 123.8, 120.1, 120.0, 67.0, 52.7, 47.1, 38.0, 25.6. HRMS (positive): (C₂₈H₂₃N₃NaO₈⁺) 552.1355 (found M+Na), 552.1377 (calc.).

10: ("TX-P") 2-(6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxamido)pent-4-ynoic acid A solution of DL-propargylglycine 8 (136 mg, 1.20 mmol) and K₂CO₃ (498 mg, 3.60 mmol) in 4 ml of H₂O was added to a solution of Trolox-NHS 9 (417 mg, 1.20 mmol) in 2 ml of 1,4-dioxane and stirred at room temperature until full conversion of the activated ester (~3 hours). 5 ml of H₂O was added and the mixture was acidified to pH 1. The aqueous solution was extracted by EtOAc (3 × 10 ml). The combined organic phases were dried over Na₂SO₄ and evaporated under reduced pressure to give the crude product. After flash column chromatography (SiO₂, DCM/EtOH: 100/0 to 80/20) the product was isolated as yellow sticky oil. Yield: 360 mg (1.043 mmol, 87%). Mixture of diastereomers: 1H NMR (400 MHz, CDCl₃) δ = 7.44 (d, J=2.8, 1H), 7.42 (d, J=2.5, 1H), 4.69 - 4.61 (m, 2H), 2.85 - 2.79 (m, 2H), 2.74 - 2.64 (m,
11: ("TX-PG-NHS") 2,5-dioxopyrrolidin-1-yl 2-(6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxamido) pent-4-ynoate To 10 (266 mg, 0.771 mmol) and N-hydroxysuccinimide (NHS) (89 mg, 0.771 mmol), in 1.0 l anhydrous 1,4-dioxane, N,N'-dicyclohexyl carbodiimide (DCC) (159 mg, 0.771 mmol) was added at 0°C. After stirring at ambient temperature overnight, the mixture was cooled to 10°C and the precipitate was filtered. The filtrate was evaporated in vacuo. The crude was purified by flash column chromatography (SiO₂, Pentane/EtOAc: 100/0 to 0/100) and the product was isolated as white solid. Yield: 172 mg (0.385 mmol, 50%). Mp: 60 - 61°C. Mixture of diastereomers: ¹H NMR (400 MHz, CDCl₃) δ = 7.30 (s, 1H), 7.28 (s, 1H), 5.08 - 4.98 (m, 2H), 4.45 (br, 1H), 4.43 (br, 1H), 2.97 - 2.72 (m, 4H), 2.84 (s, 4H), 2.78 (s, 4H), 2.69 - 2.53 (m, 4H), 2.40 - 2.29 (m, 2H), 2.19 (s, 6H), 2.16 (s, 3H), 2.15 - 2.10 (m, 4H), 2.08 (s, 3H), 2.05 (s, 3H), 1.98 - 1.86 (m, 3H), 1.55 (s, 3H), 1.54 (s, 3H). ¹³C NMR (101 MHz, CDCl₃) δ = 174.5, 174.3, 168.3, 168.0, 166.1, 165.8, 145.7, 145.5, 144.1, 144.1, 122.1, 122.1, 121.4, 121.4, 119.0, 118.9, 78.0, 76.7, 76.3, 72.3, 48.6, 29.3, 29.3, 25.5, 25.5, 24.1, 24.0, 22.5, 22.4, 20.3, 20.3, 12.2, 12.2, 11.9, 11.8, 11.3, 11.2. HRMS (negative): (C₁₉H₂₂NO₅⁻) 344.1493 (found, M-H), 344.1504 (calc.).

16: ("NPA-RhodamineB") (S)-Rhodamine B 4-(4-(1-Carboxy-2-(4-nitrophenyl)-ethyl)amino)-4-oxo-butyryl) piperazine amide Rhodamine B 4-(3-Carboxypropionyl) piperazine amide was prepared as described previously in literature. To rhodamine B 4-(3-Carboxypropionyl) piperazine amide (200 mg, 0.3 mmol) and N-hydroxysuccinimide (45 mg, 0.4 mmol), in 20 ml anhydrous DMF, N,N'-dicyclohexyl carbodiimide (79 mg, 0.4 mmol) was added. After stirring for 12 h under inert conditions at room temperature, the precipitated dicyclohexylurea (DCU) was removed by filtration. RhodamineB-NHS (14) was used for coupling without further purification. To RhodamineB-NHS (14), in 30 ml DMF, (S)-Nitrophenylalanine (NPA) (344 mg, 1.6 mmol) was added, followed by 50 µL triethylamine. After stirring for 12 h at room temperature under inert conditions the solvent was evaporated in vacuo. The product (NPA-RhodamineB (16)) was purified by column chromatography (SiO₂, DCM/Methanol: 10/1) and isolated as pink solid. Yield: 210.2 mg (0.262 mmol, 80%). ¹H NMR (400 MHz, CDCl₃) δ 1.28-1.32 (t, 12H, J=6.8), 2.41 (br s, 2H), 2.47 (br s, 2H), 3.01 (dd, 1H, J=14.1, 5.1), 3.34 (m, 10H), 3.66-3.71 (q, 8H, J=7.2), 4.48 (dd, J=11.0, 6.2, 1H), 6.96-6.97 (br s, 2H), 7.06 (d, 2H, J=2.0), 7.24-7.27 (d, 2H, J=9.6), 7.41 (d, 2H, J=7.3), 7.49-7.50 (m, 1H), 7.68-7.69 (m, 1H), 7.74-7.75 (m, 2H), 8.02 (d, J=8.2, 2H). ¹³C NMR(101 MHz, CDCl₃) δ = 175.9, 172.4, 171.2, 168.1, 157.8, 155.8, 155.5, 146.8, 146.5, 135.1, 131.7, 131.0, 129.9, 127.5, 122.7, 114.0,
17: ("NPA-RhodamineB-NHS") To (S)-rhodamine B 4-4-[(1-Carboxy-2-(4-nitrophenyl) ethyl)amino]-4-oxobutyryl) piperazine amide (16) (80.4 mg, 0.1 mmol) and N-hydroxysuccinimide (13.6 mg, 0.12 mmol), in 20 ml anhydrous DMF, N,N’-dicyclohexylcarbodiimide (23.7 mg, 0.12 mmol) was added. After stirring for 12 h under inert conditions at room temperature, the precipitated dicyclohexylurea (DCU) was removed by filtration. The resulting RhoB-NPA-NHS was used further without purification.

**Synthesis of NPA-RhodamineB-Maleimide derivative for labeling of proteins via solvent exposed cysteines** The procedure for synthesis of NPA-RhodamineB-Maleimide is fully described in detail by Cordes and co-workers.\(^\text{52}\)

**Synthesis of NPA-ATTO647N-Maleimide derivative for labeling of proteins via solvent exposed cysteines** The procedure for synthesis of NPA-ATTO647N-Maleimide is fully described in detail by Cordes and co-workers.\(^\text{52}\)

5, 6 and 7: **Synthesis of ssDNA-NPA-Alexa555/ATTO647N/Cy5** Step 1: the lyophilized ssDNA NH\(_2\) was resuspended in MilliQ water and adjusted to 80 mM in 0.2 M NaHCO\(_3\) buffer, pH 8.35. To 100 ml of this solution, the same volume of a 20 mg·ml\(^{-1}\) solution of 2 in DMF was added and the mixture was vortexed thoroughly. If necessary, additional DMF was added in 10 ml portions, to obtain a clear solution. After the reaction at room temperature overnight, 3 was purified on Illustra NAP 5 column (vide supra), MilliQ water was used as eluent for purification on Illustra NAP 5 columns, to prevent partial deprotection of the Fmoc group), and isolated by preparative rp-HPLC (gradient 1, vide supra), to yield ssDNA NPA-Fmoc 3. Step 2: Fmoc deprotection of 3 was performed as follows. The HPLC-purified and lyophilized oligonucleotide was resuspended in 50 ml of 50 mM TEAA buffer, pH 7.0. Deprotection was achieved by addition of 40 ml DMF and 10 ml piperidine. The mixture was vortexed and allowed to react for 2 h at room temperature. The deprotected oligonucleotide was purified on Illustra NAP 5 column (vide supra) and lyophilized to yield 4. Step 3: coupling with fluorophore NHS was achieved as follows. Cy5-NHS as obtained from Lumiprobe, Alexa555-NHS was obtained from Life Technologies and ATTO647N-NHS from ATTOTEC; all samples were evenly distributed into ~300 nmol portions inside a glove box. Each portion was sealed with Parafilm and kept in the dark at -18°C. Lyophilized 4 (~2 nmol) was resuspended into 50 ml of 0.2 M NaHCO\(_3\) buffer, pH 8.35. To this solution, one portion of fluorophore NHS dissolved in 10 ml of dimethyl sulfoxide (DMSO) was added and the mixture was vortexed thoroughly. After incubation overnight the oligonucleotide was purified on Illustra NAP 5 column (vide supra) and isolated by preparative rp-HPLC (gradient 1, vide supra) to yield 5, 6 and 7 (see Scheme 4.1).
13 Synthesis of ssDNA TX-PG-Cy5 Step 1: the lyophilized ssDNA was resuspended in MilliQ water and adjusted to 80 mM in 0.2 M NaHCO$_3$ buffer, pH 8.35. To 100 ml of this solution, the same volume of a 20 mg·ml$^{-1}$ solution of 11 in DMF was added and the mixture was vortexed thoroughly. If necessary, additional DMF was added in 10 ml portions, to obtain a clear solution. After the reaction at room temperature overnight, the oligonucleotide was purified on Illustra NAP 5 column and isolated by preparative rp-HPLC (gradient 2, vide supra) to yield 12. Step 2: coupling with Cy5-N3 was achieved following a modified manufacturer protocol (Lumiprobe). After every addition step the mixture was vortexed briefly. The HPLC-purified and lyophilized oligonucleotide 12 (∼2 nmol) was resuspended in 30 ml of 2 M TEAA buffer, pH 7.0 and added into a 0.5 ml tube. To this solution, 8 ml MilliQ water and 7 ml of DMSO were added. A 15-nmol portion of Cy5-N3 was dissolved in 40 ml of DMSO and added. To this mixture, 10 ml of 5 mM stock solution of ascorbic acid in MilliQ water was added. The solution was degassed by a stream of N$_2$ for 30 s and finally 5 ml of a 10 mM stock solution of Cu(II)-tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine in 55% DMSO/MilliQ water was added. The vial was flushed with N$_2$, closed and sealed with Parafilm. After incubation overnight the oligonucleotide was purified on Illustra NAP 5 column (gradient 2, vide supra) and isolated by preparative rp-HPLC (vide supra) to yield 13.

Synthesis of ssDNA NPA-RhodamineB Step 1: the lyophilized ssDNA-NH$_2$ was resuspended in MilliQ water and adjusted to 20 µM in 0.2 M NaHCO$_3$ buffer, pH 8.35. To this solution, RhoB-NPA-NHS (20) in 100 ml of DMF was added and the mixture was vortexed thoroughly. After incubation overnight the oligonucleotide was purified on Illustra NAP 5 column (vide supra) and isolated by preparative rp-HPLC (gradient 1, vide supra) to yield 18 (Scheme 4.3). The yield was found to be 25% for coupling NPA-RhodamineB-NHS to ssDNA.

20 Synthesis of ssDNA Alexa555/ATTO647N Step 1: Alexa555-NHS was obtained from Life Technologies and ATTO647N-NHS from ATTOTEC; all were evenly distributed into ∼300 nmol portions inside a glove box. Each portion was sealed with Parafilm and kept in the dark at -18°C. ssDNA-NH$_2$ (∼2 nmol) was resuspended into 50 ml of 0.2 M NaHCO$_3$ buffer, pH 8.35. To this solution, one portion of Alexa555-NHS/ATTO647N-NHS dissolved in 10 ml of DMSO was added and the mixture was vortexed thoroughly. In case of RhodamineB, an NHS crude mixture in 100 ml of DMF was added to ssDNA-NH$_2$ and the mixture was vortexed thoroughly. After incubation overnight the oligonucleotide was purified with an Illustra NAP 5 column (vide supra) and isolated by preparative rp-HPLC (gradient 1, vide supra).

Synthesis of ssDNA RhodamineB Step 1: the lyophilized ssDNA-NH$_2$ was resuspended in MilliQ water and adjusted to 20 µM in 0.2 M NaHCO$_3$ buffer, pH 8.35. To this solution, RhodamineB-NHS in 100 ml of DMF was added and the mixture was vortexed thoroughly. After incubation overnight the oligonucleotide was purified on Illustra NAP 5 column (vide supra) and isolated by preparative rp-HPLC (gradient 1, vide supra).
Labelling of GlnPQ-SBD2. SBD2 cysteine-containing derivative was obtained as described in literature and stored at -20°C in 100 µl aliquots of 20-40 mg·ml⁻¹ in 50 mM KPi, pH 7.4, 50 mM KCl and 50% glycerol plus 1 mM dithiothreitol (DTT). Stochastic labelling with maleimide derivatives of donor and acceptor fluorophores was carried out on ~5 nmol of protein; SBD2 derivatives were labelled with RhodamineB-/NPA-RhodamineB-maleimde (donor) and KK114-maleimide (acceptor) in a ratio of protein:donor:acceptor of 1:6:8. Briefly, purified proteins were treated with DTT (10mM, 30 min), to fully reduce oxidized cysteines. After dilution of the protein sample to a DTT concentration of 1mM, the reduced protein was bound to a Ni²⁺-Sepharose resin (GE Healthcare) and washed with ten column volumes of 50 mM KPi, pH 7.4, 150 mM KCl and 5% glycerol (buffer B). Simultaneously, the applied fluorophore stocks (50 nmol (s)) dissolved in 5 ml of anhydrous DMSO were added at appropriate amounts to buffer B and immediately applied to the protein bound to the Ni²⁺-Sepharose resin (keeping the final DMSO concentration below 1%). The resin was incubated overnight and kept at 4°C (under mild agitation). After labelling, unreacted dye was removed by sequential washing with ten column volumes of buffer B, and this was followed by 100 column volumes of 50 mM KPi, pH 7.4, 1 M KCl and 50% glycerol. The protein was eluted in 1 ml of 50 mM KPi, pH 7.4, 150 mM KCl, 5% glycerol and 500 mM imidazole, and was analyzed on a Superdex-200 column (GE Healthcare) equilibrated with 50 mM KPi, pH 7.4, and 200 mM KCl.
Figure 4.A.1 | Photophysical characterization of Alexa555. (a) Schematic view of the photostabilizer-dye conjugate on DNA and the experimental strategy for surface immobilization. Continued next page.
Figure 4.A.1 | Continued (b) Confocal scanning image of immobilized Alexa555 molecules, including the identification of positions of single emitters for further analysis (right panel) (scale bar 5 µm). (c) Histogram of fluorescence lifetimes determined for each of these single emitters, including Gaussian fit (black line) and average lifetime value $\tau_{fl}$. (d) Representative fluorescence time traces of the selected single emitters, indicating blinking characteristics. (e) Histogram of off-state lifetimes, with Gaussian fit (red line) and mean plus standard deviation value. Here all individual fluctuating fluorescence time traces investigated with an autocorrelation analysis, and the autocorrelation decay was fitted to obtain values of the lifetime of the dark off-state. (f) Representative TIRF images of immobilized Alexa555 molecules at two different time points (0 and 2 min, as given) after starting illumination and recording (scale bar 2 µm). (g) Representative fluorescence time traces of single emitters from TIRF recordings, indicating long-term blinking and photobleaching characteristics. Excitation intensities are 0.3 kW·cm$^{-2}$ for confocal and 50 W·cm$^{-2}$ for TIRF-experiments (excitation at 532 nm, detection with HC582/75). Brightness and contrast settings 1950 (low) to 10000 (high) and 3 (low) to 60 (high) for TIRF and confocal images, respectively.
Figure 4.2 | Photophysical characterization of NPA-Alexa555. (a) Schematic view of the photostabilizer-dye conjugate on DNA and the experimental strategy for surface immobilization. (b) Confocal scanning image of immobilized NPA-Alexa555 molecules, including the identification of positions of single emitters for further analysis (right panel) (scale bar 5 µm). (c) Histogram of fluorescence lifetimes determined for each of these single emitters, including Gaussian fit (black line) and average lifetime value τ_fl. (d) Representative TIRF images of immobilized Alexa555 molecules at two different time points (0 and 2 min, as given) after starting illumination and recording (scale bar 2 µm). (e) Representative fluorescence time traces of single emitters from TIRF recordings, indicating long-term blinking and photobleaching characteristics. Excitation intensities are 0.3 kW·cm\(^{-2}\) for confocal and 50 W·cm\(^{-2}\) for TIRF-experiments (excitation at 532 nm, detection with HC582/75). Brightness and contrast settings 1950 (low) to 10000 (high) and 3 (low) to 60 (high) for TIRF and confocal images, respectively.
Figure 4.A.3 | Photophysical characterization of ATTO647N. (a) Schematic view of the photostabilizer-dye conjugate on DNA and the experimental strategy for surface immobilization. (b) Confocal scanning image of immobilized ATTO647N molecules, including the identification of positions of single emitters for further analysis (right panel) (scale bar 5 µm). (c) Histogram of fluorescence lifetimes determined for each of these single emitters, including Gaussian fit (black line) and average lifetime value \( \tau_{fl} \). Continued next page.
Figure 4.A.3 | Continued (d) Representative fluorescence time traces of the selected single emitters, indicating blinking characteristics. (e) Histogram of off-state lifetimes, with Gaussian fit (red line) and mean plus standard deviation value. Here all individual fluctuating fluorescence time traces investigated with an autocorrelation analysis, and the autocorrelation decay was fitted to obtain values of the lifetime of the dark off-state. (f) Representative TIRF images of immobilized ATTO647N molecules at two different time points (0 and 2 min, as given) after starting illumination and recording (scale bar 2 µm). (g) Representative fluorescence time traces of single emitters from TIRF recordings, indicating long-term blinking and photobleaching characteristics. Excitation intensities are 0.66 kW·cm$^{-2}$ for confocal and 50 W·cm$^{-2}$ for TIRF-experiments (excitation at 640 nm, detection with ET700/75). Brightness and contrast settings 3100 (low) to 16000 (high) and 5 (low) to 100 (high) for TIRF and confocal images, respectively.
Figure 4.A.4 | Photophysical characterization of NPA-ATTO647N. (a) Schematic view of the photostabilizer-dye conjugate on DNA and the experimental strategy for surface immobilization. (b) Confocal scanning image of immobilized NPA-ATTO647N molecules, including the identification of positions of single emitters for further analysis (right panel) (scale bar 5 µm). (c) Histogram of fluorescence lifetimes determined for each of these single emitters, including Gaussian fit (black line) and average lifetime value $\tau_{fl}$. (d) Representative TIRF images of immobilized NPA-ATTO647N molecules at two different time points (0 and 2 min, as given) after starting illumination and recording (scale bar 2 µm). (e) Representative fluorescence time traces of single emitters from TIRF recordings, indicating long-term blinking and photobleaching characteristics. Excitation intensities are 0.66 kW·cm$^{-2}$ for confocal and 50 W·cm$^{-2}$ for TIRF-experiments (excitation at 640 nm, detection with ET700/75). Brightness and contrast settings 3100 (low) to 16000 (high) and 5 (low) to 100 (high) for TIRF and confocal images, respectively.
Figure 4.A.5 | Photophysical characterization of Cy5. (a) Schematic view of the photostabilizer-dye conjugate on DNA and the experimental strategy for surface immobilization. (b) Confocal scanning image of immobilized Cy5 molecules, including the identification of positions of single emitters for further analysis (right panel) (scale bar 5 µm). (c) Histogram of fluorescence lifetimes determined for each of these single emitters, including Gaussian fit (black line) and average lifetime value $\tau_{fl}$. Continued next page.
Figure 4.A.5 | Continued (d) Representative fluorescence time traces of the selected single emitters, indicating blinking characteristics. (e) Histogram of off-state lifetimes, with Gaussian fit (red line) and mean plus standard deviation value. Here all individual fluctuating fluorescence time traces investigated with an autocorrelation analysis, and the autocorrelation decay was fitted to obtain values of the lifetime of the dark off-state. (f) Representative TIRF images of immobilized Cy5 molecules at two different time points (0 and 2 min, as given) after starting illumination and recording (scale bar 2 µm). (g) Representative fluorescence time traces of single emitters from TIRF recordings, indicating long-term blinking and photobleaching characteristics. Excitation intensities are 0.66 kW·cm⁻² for confocal and 50 W·cm⁻² for TIRF-experiments (excitation at 640 nm, detection with ET700/75). Brightness and contrast settings 4500 (low) to 22000 (high) and 5 (low) to 100 (high) for TIRF and confocal images, respectively.
Figure 4.A.6 | Photophysical characterization of NPA-Cy5. (a) Schematic view of the photostabilizer-dye conjugate on DNA and the experimental strategy for surface immobilization. (b) Confocal scanning image of immobilized NPA-Cy5 molecules, including the identification of positions of single emitters for further analysis (right panel) (scale bar 5 µm). (c) Histogram of fluorescence lifetimes determined for each of these single emitters, including Gaussian fit (black line) and average lifetime value \( \tau_{fl} \). (d) Histogram of off-state (cis/trans isomerization) lifetimes, with Gaussian fit (red line) and mean plus standard deviation value derived from autocorrelation analysis of multiple molecules. (e) Representative TIRF images of immobilized NPA-Cy5 molecules at two different time points (0 and 2 min, as given) after starting illumination and recording (scale bar 2 µm). (f) Representative fluorescence time traces of single emitters from TIRF recordings, indicating long-term blinking and photobleaching characteristics. Excitation intensities are 0.66 kW·cm\(^{-2}\) for confocal and 50 W·cm\(^{-2}\) for TIRF-experiments (excitation at 640 nm, detection with ET700/75). Brightness and contrast settings 4500 (low) to 22000 (high) and 5 (low) to 100 (high) for TIRF and confocal images, respectively.
Figure 4.A.7 | Photophysical characterization of TX-PG-Cy5. (a) Schematic view of the photostabilizer-dye conjugate on DNA and the experimental strategy for surface immobilization. (b) Confocal scanning image of immobilized TX-PG-Cy5 molecules, including the identification of positions of single emitters for further analysis (right panel) (scale bar 5 µm). (c) Histogram of fluorescence lifetimes determined for each of these single emitters, including Gaussian fit (black line) and average lifetime value $\tau_{fl}$. (d) Histogram of off-state (cis/trans isomerization) lifetimes, with Gaussian fit (red line) and mean plus standard deviation value derived from autocorrelation analysis of multiple molecules. (e) Representative TIRF images of immobilized TX-PG-Cy5 molecules at two different time points (0 and 2 min, as given) after starting illumination and recording (scale bar 2 µm). (f) Representative fluorescence time traces of single emitters from TIRF recordings, indicating long-term blinking and photobleaching characteristics. Excitation intensities are 0.66 kW·cm$^{-2}$ for confocal and 50 W·cm$^{-2}$ for TIRF-experiments (excitation at 640 nm, detection with ET700/75). Brightness and contrast settings 4500 (low) to 22000 (high) and 5 (low) to 100 (high) for TIRF and confocal images, respectively.
Figure 4.A.8 | Photophysical characterization of RhodamineB. (a) Schematic view of the photostabilizer-dye conjugate on DNA and the experimental strategy for surface immobilization. (b) Confocal scanning image of immobilized RhodamineB molecules, including the identification of positions of single emitters for further analysis (right panel) (scale bar 5 µm). (c) Histogram of fluorescence lifetimes determined for each of these single emitters, including Gaussian fit (black line) and average lifetime value $\tau_{fl}$. Continued next page.
Figure 4.A.8 | Continued (d) Representative fluorescence time traces of the selected single emitters, indicating blinking characteristics. (e) Histogram of off-state lifetimes, with Gaussian fit (red line) and mean plus standard deviation value. Here all individual fluctuating fluorescence time traces investigated with an autocorrelation analysis, and the autocorrelation decay was fitted to obtain values of the lifetime of the dark off-state. (f) Representative TIRF images of immobilized RhodamineB molecules at two different time points (0 and 2 min, as given) after starting illumination and recording (scale bar 2 \( \mu \text{m} \)). (g) Representative fluorescence time traces of single emitters from TIRF recordings, indicating long-term blinking and photobleaching characteristics. Excitation intensities are 0.3 kW cm\(^{-2}\) for confocal and 50 W cm\(^{-2}\) for TIRF-experiments (excitation at 532 nm, detection with HC582/75). Brightness and contrast settings 2300 (low) to 6800 (high) and 3 (low) to 50 (high) for TIRF and confocal images, respectively.
Figure 4.A.9 | Photophysical characterization of NPA-RhodamineB. (a) Schematic view of the photostabilizer-dye conjugate on DNA and the experimental strategy for surface immobilization. (b) Confocal scanning image of immobilized NPA-RhodamineB molecules, including the identification of positions of single emitters for further analysis (right panel) (scale bar 5 µm). (c) Histogram of fluorescence lifetimes determined for each of these single emitters, including Gaussian fit (black line) and average lifetime value $\tau_{fl}$. (d) Representative TIRF images of immobilized NPA-RhodamineB molecules at two different time points (0 and 2 min, as given) after starting illumination and recording (scale bar 2 µm). (e) Representative fluorescence time traces of single emitters from TIRF recordings, indicating long-term blinking and photobleaching characteristics. Excitation intensities are 0.66 kW·cm$^{-2}$ for confocal and 50 W·cm$^{-2}$ for TIRF-experiments (excitation at 532 nm, detection with HC582/75). Brightness and contrast settings 2300 (low) to 6800 (high) and 3 (low) to 50 (high) for TIRF and confocal images, respectively.
References


25. Vogelsang, J., Cordes, T., Forthmann, C., Steinhauser, C. & Tinnefeld, P. Controlling the fluorescence of ordinary oxazine dyes for single-molecule switching and superresolution


