A simple and versatile design concept for fluorophore-derivatives with intramolecular photostabilization

Chapter 2

A Simple and Versatile Design Concept for Fluorophore-derivatives with Intramolecular Photostabilization


2.1 Introduction

Fluorescence applications penetrate nearly every field of biological research. Improvement of small organic fluorophores becomes a major driving force to move single-molecule fluorescence spectroscopy and super-resolution microscopy into the mainstream of biological research. Ideal small organic fluorophores should be photostable, bright, non-phototoxic, slow-photobleaching, capable to specifically linkable to a biomolecule of interest, as well as showing little intensity fluctuation (at least in the timescale of interesting biological events under study). However, like most fluorophores, organic dyes are intrinsically compromised by unwanted photophysical properties, including blinking and photobleaching. Blinking is reversible losses of fluorescence leading to “dark” states. In contrast, photobleaching is a permanent damage to the fluorophore, rendering it non-emissive. 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 <250 nm.

The utility of solution-based protective agents (such as Trolox, Nitrophenyl acetic acid, ascorbic acid and methyl viologen) as a strategy for minimizing fluorophore blinking and photobleaching is highlighted over the past decade. Despite the remarkable advancements, simple addition of triplet quenchers to laser dye solutions suffers from severe constraints. The high concentrations (micro- to millimolar) of organic quencher molecules required to provide sufficient collision frequencies with dye molecules are often near the solubility limit of these additives. Correspondingly, their use may cause toxic effects and interfere with the integrity of biological systems. To circumvent these issues, Lüttke and co-workers 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, which automatically prevent 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 by two groups independently to reduce photobleaching and blinking even in demanding applications such as single-molecule fluorescence microscopy or in vivo imaging. It was also shown that intramolecular photostabilization can be achieved without additives in the buffer system, minimizing the 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.\textsuperscript{5,6,16–19} This is mainly due to the synthetic effort of photostabilizer-dye conjugates, which so far require a multi-step synthesis route.\textsuperscript{16,17} 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, antibodies), strongly restricting the choice of fluorophore type (chemical structure, redox potential, water solubility etc.) and photophysical properties (colour, brightness, fluorescence lifetime etc.). Especially the available cyanine fluorophores suffer from limited brightness and signal-to-noise ratio due to cis/trans-isomerization\textsuperscript{21}, a fact that emphasizes the urgent need for a synthetic strategy to study and use other classes of organic fluorophores via intramolecular photostabilization.\textsuperscript{22}

Here, we introduce a versatile and simple design concept to synthesize photostabilizer-dye conjugates on a specific biomolecular target using unnatural amino acids (UAAs).\textsuperscript{23} UAAs were chosen as a scaffold that links multiple chemical units, i.e., fluorophore and photostabilizer to a specific target. The presented conjugation strategy is based on well-known chemical reactions with (commercially available derivatives of) organic fluorophores F, photostabilizers P and UAAs, which can be bound as a single moiety to a biomolecular target B (Figure 2.1). Depending on the UAA-scaffold, the chemical nature of the functional groups can be N-hydroxysuccinimid-esters (NHS), alkynes, azides, or other bio-orthogonal reactive functionalities.

\textbf{Figure 2.1.} Design concept to combine an organic fluorophore covalently with a photostabilizer on a biomolecular target or linker structure using (unnatural) amino acids.

Two different unnatural amino acids were used to bind different rhodamine-, carbopyronine- and cyanine-fluorophores covalently as a photostabilizer on distinct
biomolecular targets. The synthesized fluorophore-derivatives comprise a reducing or oxidizing photostabilizer in the form of the antioxidant Trolox or a nitrophenyl group.\textsuperscript{11,12} 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.

\section*{2.2 RESULTS AND DISCUSSION}

\subsection*{2.2.1 Design and synthesis of amino acids as photostabilizers and building blocks for fluorophore-derivatives}

As a proof-of-concept for the proposed strategy, 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, i.e., fluorophores from the ATTO- and Alexa-series, which are extremely popular for (life-science) applications. (S)-Nitrophenylalanine, NPA (Scheme 2.1), was used as a scaffold for the first generation of compounds, which consist of ssDNA as the biomolecular target, a commercially available organic fluorophore (Alexa 555, ATTO647N and Cy5) and the \( p \)-nitrophenyl group of the known photostabilizer NPA\textsuperscript{16,17,19} (Scheme 2.1, compounds 5, 6 and 7). The cyanine fluorophore Cy5 served as a "positive-control" experiment that allows to benchmark the effects of intramolecular photostabilization for different fluorophore classes with respect to published studies.\textsuperscript{16–20} The used DNA and its specific base-sequence was selected because of the detailed photophysical characterization of various fluorophores on this target.\textsuperscript{25–27}

The synthesis shown in Scheme 2.1 was conducted starting from commercially available Fmoc-protected (S)-nitrophenylalanine (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 target ssDNA (Figure 2.1 & Scheme 2.1) 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-NH\(_2\).
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Scheme 2.1. Synthesis route to obtain 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 (pH 7.0).

2.2.2 UAAs as a general scaffold for photostabilizer-dye conjugates.

The scaffolding strategy presented in Scheme 2.1 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; Scheme 2.2) represents a more versatile scaffold that can link three chemical groups via NHS and click chemistry (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, that is, removal of blinking and an increased photobleaching lifetime (Figure 2.5d) 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 S2.7). The remaining ~40% exhibited longer observation times with an increased amount of blinking events (Figure S2.7). A unifying fluorescence lifetime of 1.65 ± 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 S2.7d, bottom row, left 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.

### 2.2.3 Characterization of DNA-photostabilizer-dye conjugates

The DNA-photostabilizer-dye conjugates were isolated by reversed phase high performance liquid chromatography (HPLC) (Figure 2.2), and characterized by UV-vis absorption spectroscopy and mass spectrometry (Figure 2.3).

As shown in Figure 2.2a, it was observed that the major peak corresponding to the Fmoc-protected NPA conjugate of DNA (peak 3) was retarded in mobility compared to control DNA functionalized with a primary amino group at its 3’-position (peak 1). This is because of the increased interaction between the lipophilic Fmoc-
A simple and versatile design concept for fluorophore-derivatives with intramolecular photostabilization protecting group and the C18-column. By taking this advantage, we were able to purify the NPA-modified DNA easily after Fmoc-deprotection.

Figure 2.2. HPLC-characterization and purification of functionalized oligonucleotides: ssDNA-fluorophore and ssDNA-NPA-fluorophore (5, 6, 7 and 13).
In the next step, purification of DNA-photostabilizer-dye conjugates were monitored at two wavelengths: 260 nm (DNA detection) and λmax of the respective dye. The peaks, which showed absorbance at both wavelengths, were collected.

Once obtained, the purified ssDNA-NPA-dye conjugates were firstly analyzed by analytical HPLC. All the analytical runs gave single main peaks indicating the high purity of the synthesized products (up to 94%). Conjugates with different dyes showed varied retention times from the HPLC elugram: 29 min for ssDNA-NPA-Alex555, 48 min for ssDNA-NPA-ATTON647N at and 44 min for ssDNA-NPA-Cy5 (Figure 2.2b).

As expected, all compounds showed UV-vis absorption characteristic peaks for DNA (~260 nm) and the respective chromophore (~555/~649 nm) (Figure 2.3a). Compared to the absorption spectrum of the corresponding ssDNA-dye conjugates, the introduction of NPA moiety didn't change the absorption maxima of ssDNA-NPA-dye conjugates. For the ssDNA-TX-PG-Cy5 conjugate, a minor (5 nm) bathochromic shifts was observed comparing to the absorption maximum of ssDNA-Cy5 conjugates. Additionally, no spectral broadening and no additional absorption band were found for the ssDNA-NPA- and ssDNA-TX-PG-conjugated dye (Figure 2.3a), compared to that ssDNA-dye conjugates.

Finally, the obtained conjugates were characterized by MALDI-TOF mass spectrometry which revealed certain mass increases compared to the non-modified DNA, further confirming the formation of the desired products. (Figure 2.3b) (See the Materials and Methods section for further details on chemical synthesis and characterization of functionalized oligonucleotides and reactive precursor molecules.)
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Figure 2.3. a) UV-VIS absorption spectra of functionalized oligonucleotides: ssDNA-fluorophore and ssDNANPA-fluorophore (5, 6, 7 and 13) showing the characteristic peak of DNA in the UV-range and the strong visible absorption of the dye. b) Maldi-TOF mass spectra DNA-photostabilizer-dye conjugates. The comparison of the spectra of the non-modified DNA with that of the conjugates clearly indicates an increase of the molecular mass originating from coupling of dye (and stabilizer).
2.2.4 Photophysical characterization

Next, single-molecule fluorescence microscopy was used to benchmark the potential of the scaffolding approach with respect to photophysical parameters. Confocal scanning microscopy\textsuperscript{11,12,18,24} was used to investigate signal fluctuations and fluorescence lifetime, while TIRF-microscopy\textsuperscript{11,18} was employed to obtain quantitative photophysical values such as bleaching lifetime, fluorescence count rate, total number of photons, and signal-to-noise 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 (Figure S2.1a-S2.7a).\textsuperscript{18,19} 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.

2.2.4.1 Confocal microscopy

Using confocal microscopy, single immobilized fluorophores on DNA were identified by 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), 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 2.4), and time-correlated single-photon-counting was used to determine the fluorescence lifetime. The reference compounds, i.e., non-stabilized fluorophores, showed fast photobleaching and pronounced blinking on the corresponding dsDNA in deoxygenated PBS-buffer (Figure 2.4a/c/e and S2.1d/S2.3d/S2.5d), while the NPA-derivatives showed a stable fluorescence signal over extended periods of time (Figure 2.4b/d/f and S2.2d/S2.4d/S2.6d).
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Figure 2.4. Photophysical characterization of rhodamine, carbopyronine and cyanine fluorophores and their respective photostabilizer-conjugates with confocal scanning
microscopy. Each panel shows a representative overview image showing spots from individual immobilized fluorophores (left), a fluorescence time trace (middle) and the corresponding autocorrelation decay (black, with according fits in green and red) (right). 

\textbf{a/b)} Data of Alexa555 and NPA-Alexa555 in aqueous PBS buffer at pH 7.4 in the absence of oxygen (10×10 µm, 50 nm pixel size, 2 ms/pixel, intensity scale from 3-60 counts, recorded at an excitation intensity of ≈0.3 kW/cm² at 532 nm). 

\textbf{c/d)} Data of ATTO647N and NPA-ATTO647N in aqueous PBS buffer at pH 7.4 in the absence of oxygen (10×10 µm, 50 nm pixel size, 2 ms/pixel, intensity scale from 5-100 counts, recorded at an excitation intensity of ≈0.66 kW/cm² at 640 nm). 

\textbf{e/f)} Data of Cy5 and NPA-Cy5 in aqueous PBS buffer at pH 7.4 in the absence of oxygen (10×10 µm, 50 nm pixel size, 2 ms/pixel, intensity scale from 10-300 counts, recorded at an excitation intensity of 4 kW/cm² at 640 nm). The autocorrelation decays in this case reveal blinking kinetics due to triplet transitions and cis-trans isomerization, as labelled. Further experimental data for each fluorophore can be found in the supplementary information.

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 2.4a and S2.1). This is a typical behavior for rhodamines and is caused by population of the triplet-state.\textsuperscript{4} The fluorescence lifetime of the sample was found to be 1.2±0.3 ns (Figure S2.1). Upon 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/cm² irradiance (Figure 2.4b and S2.2). 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 to the parent fluorophore indicating the presence of singlet quenching by the NPA-scaffold (Figure S2.2).

The photophysical behavior of ATTO647N in deoxygenated PBS buffer was characterized by a mix of short and long photobleaching times ranging from seconds to minutes with pronounced blinking on the millisecond timescale (Figure 2.4c and S2.3). The off-state lifetime associated with the blinking events was found to be 29±5 ms and is attributed to the triplet-state (Figure 2.4e and S2.3e).\textsuperscript{4} This and the observed fluorescence lifetime of 4.2±0.4 ns are in agreement with literature.\textsuperscript{11,28} Covalent binding of NPA to ATTO647N resulted in homogeneous, bright, non-blinking and prolonged fluorescence emission (Figure 2.4d). 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 two-minute observation period at excitation intensities of ≈0.66 kW/cm² (see Figure 2.4d and S2.4). In addition, a small fraction (<30 %) of NPA-ATTO647N molecules showed a reduced...
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lifetime of around 2.6 ns in combination with a lower count rate pointing to stronger singlet quenching (Figure S2.4). Direct switching of a single NPA-ATTO647N molecule between the two lifetime/brightness-states was observed for both fractions and will be a topic of future research.\(^\text{11}\)

Results for Cy5 (Figure 2.4e/f) were obtained at a higher excitation intensity (4 kW/cm\(^2\)) to allow for a direct comparison to previous publications using different approaches towards intramolecular photostabilization.\(^\text{28}\) In agreement with earlier findings, the autocorrelation analysis of Cy5 fluorescent time traces\(^\text{11}\) revealed the presence of two different photophysical processes that were attributed to triplet-blinking (11±4 ms) and cis-trans isomerization (54±12 µs).\(^\text{4,18,25,26}\) The fluorescence lifetime of Cy5 was found to be 1.65±0.15 ns (compare also Figure S7).\(^\text{18,29}\) 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 2.4f). 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 Figures S7/S8), 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 red fit and black data in Figures 2.4f), suggesting a remaining small triplet population with a lifetime < 100 µs. A smaller population of molecules (<25%) showed intensity fluctuations on completely different timescales (Figure S2.6), which is consistent with earlier reports\(^\text{18,19}\), and its origin remains to be explained mechanistically. The fluorescence lifetime of NPA-Cy5 was found to be 1.60±0.15 ns and similar to the parent fluorophore, indicating that any reduction in the signal brightness of NPA-Cy5 is due to static quenching, i.e., 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 (Figure 2.3a).

2.2.4.2 TIRF microscopy

To further quantify the improved performance of photostabilizer-dye conjugates, we performed single-molecule TIRF-microscopy to determine various parameters with high statistics. 500 s movies with 100 ms integration time were recorded at laser excitation intensities of ≈50-100 W/cm\(^2\), i.e., a significantly lower excitation intensity than in the confocal scanning microscopy experiments, resulting in differing count rates and signal-to-noise ratios (SNR). Figure 2.5a 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 numbers over subsequent image frames were fitted with an exponential decay \( y(t) = C + A \cdot \exp(t/\tau_{\text{bleach}}) \) to obtain the photobleaching lifetime \( \tau_{\text{bleach}} \) (Figure 2.5a).\(^{18}\) Background-corrected single-molecule time traces were extracted, from which we determined the fluorescence count rate in kHz (Figure 2.5a, brightness), the signal-to-noise ratio (Figure 2.5a, SNR), and the total number of detected photons before photobleaching (Figure 2.5a, \( N_{\text{total}} = \text{brightness} \times \tau_{\text{bleach}} \)). The mean and standard deviation of all values was derived from multiple (\( n \geq 3 \)) independent experiments. We benchmarked the performance of the photostabilizer-dye conjugates against the antioxidant Trolox (TX) as a solution additive in the deoxygenated imaging buffer (2 mM after 20 min UV-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±0.2 kHz, \( N_{\text{total}} = 5.1\pm0.4 \cdot 10^4 \) and a SNR = 3.5±0.3, while the photobleaching could be described by a mono-exponential decay with a time constant \( \tau_{\text{bleach}} = 34\pm26 \) s (Figure 2.5b). An improvement for most of the photophysical parameters was found for both adding 2 mM TX or Alexa555-NPA with an up to a 20-fold increase in photostability, 25-fold increase in \( N_{\text{total}} \) and a 3-fold increase in SNR, while the brightness was found to be comparable.

Similar results were obtained for both the carbopyronine ATTO647N and the cyanine dye Cy5 (Figure 2.5c). The photobleaching time increased from \( \tau_{\text{bleach}} = 138\pm93 \) s (ATTO647N), to 212±52 s (NPA-ATTO647N) and 298±45 s (Atto647N + 2 mM TX); from \( \tau_{\text{bleach}} = 7.0\pm1.5 \) s (Cy5) to 139±55 s (NPA-Cy5) and 384±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 \( N_{\text{total}} = 1.9\pm0.7 \cdot 10^5 \) and SNR = 1.6±0.2 (ATTO647N) to \( N_{\text{total}} = 8.6\pm0.4\cdot10^5 \) and SNR = 11.6±1.7 (NPA-ATTO647N), and \( N_{\text{total}} = 2.0\pm0.7 \cdot 10^6 \) and SNR = 17.4±2.4 (Atto647N + 2 mM TX), as well as \( N_{\text{total}} = 3.1\pm0.9\cdot10^4 \) and SNR = 3.0±0.7 (Cy5) to \( N_{\text{total}} = 6.4\pm1.6\cdot10^5 \) and SNR = 11.6±1.7 (NPA-Cy5) and \( N_{\text{total}} = 2.4\pm0.6\cdot10^6 \) and SNR = 9.5±1.2 (Atto647N + 2 mM TX), i.e. 20-50-fold increases.

The investigations in Figure 2.4 and Figure 2.5 regarding the photophysical properties of rhodamines, carbopyronines and cyanines with intramolecular photostabilization revealed: (i) photobleaching and blinking could be efficiently removed using UAA-scaffolding, (ii) all photophysical parameters were improved...
A simple and versatile design concept for fluorophore-derivatives with intramolecular photostabilization substantially compared to the parent fluorophore. (iii) Despite showing no decrease in brightness, rhodamines and carbopyronines showed singlet-quenching while cyanines show no reduction of the excited state lifetime. (iv) Solution-based healing using 2 mM TX remained more efficient than intramolecular photostabilization.

**Figure 2.5.** Photophysical characterization of photostabilizer-dye conjugates and their parent fluorophore compounds with single-molecule TIRF microscopy. Data was recorded in aqueous PBS buffer at pH 7.4 in the absence of oxygen under continuous either 640 nm excitation with \( \approx 50 \) W/cm\(^2\) or 532 nm excitation with 50 W/cm\(^2\) a) Representative image frame (left panel) showing single fluorescent molecules (10 x 10 m, exemplarily for Cy5). Subsequent images recorded over a period of 500 s showed a decrease in the number of fluorescing molecules, as
shown for Cy5 (black), Cy5 with 2mM TX (blue) and NPA-Cy5 (red) (middle panel). Fitting an exponential (grey) to this decay resulted in the photobleaching lifetime $\tau_{\text{bleach}}$. The curves shown were obtained by averaging over >5 TIRF movies. (Right panel) Background-corrected intensity traces were used to derive the total number of photons ($N_{\text{total}}$), the average signal in kHz (brightness) and the signal-to-noise ratio (SNR). For further details of the experimental techniques, data acquisition and analysis see Materials and Methods. b/c/d) Chemical structures (left panels) and respective photophysical parameters for Alexa555 (b, rhodamines), ATTO647N (c, carbopyronines) and Cy5 (d, cyanines). Values and error bars (s.d.) in bar graphs from $N > 500$ molecules.

Despite the slightly lower photostability of compounds with intramolecular photostabilization compared to solution additives such as TX, this approach has unique advantages that compensate 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.\textsuperscript{31} It is also the only viable option when diffusion-based photostabilization remains ineffective due to the lack of collisions between the photostabilizer and the fluorophore.\textsuperscript{32,33}

### 2.3 Conclusion and Discussion

Our results indicate 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 (bio-)molecular target in a modular fashion. The synthetic strategy has many advantages over existing strategies since it is cost effective, requires low amounts of material, uses only well-known chemical transformations such as amide-bond formation, requires only 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, e.g., oxazines and perylenes, conjugation to NPA is expected to show effects different from those described here considering their interaction with antioxidants,\textsuperscript{4,24,25} DNA bases, and
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amino-acids such as tryptophane. Intramolecular quenching of Alexa-fluorophores observed with natural amino acids even suggests that labeling of proteins at strategic locations, i.e., 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-acid can be found.

Intramolecular photostabilization remains, however, less effective compared to the use of diffusion-based photostabilization (see Figure 2.5) as reported before. This shortcoming of intramolecular photostabilization is, however, 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 – 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 compound to the fluorophore in a different way compared to the structures shown in this manuscript (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 ref.

We finally suggest that UAA-scaffolding could be extended beyond photostabilization to provide a general framework for the manipulation of fluorophore properties. Potentially interesting UAAs feature antioxidants, triplet sensitizers, photoswitchable molecules to induced 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). UAAs 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 labeled protein species.

2.4 Experimental Section

2.4.1 Synthesis of 2 (“NPA-NHS”)
(S)-2,5-dioxopyrrolidin-1-yl-2-(((9H-fluoren-9-yl)methoxy)carbonyl)amino)-3-(4-nitrophenyl)propanoate ("NPA-NHS") : To 1 (173 mg, 0.4 mmol) and N-hydroxysuccinimide (NHS) (46 mg, 0.4 mmol), in 1.5 mL anhydrous 1,4-dioxane, N,N'-dicyclohexyl carbodiimide (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), 3.48 (dd, J=14.3, 4.9, 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-PG") 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 ticky 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, 3H), 2.64 - 2.50 (m, 3H), 2.35 - 2.23 (m, 2H), 2.20 (s, 3H), 2.19 (s, 3H), 2.16 (s, 3H), 2.15 (s, 3H), 2.08 (s, 4H), 2.07 (s, 4H), 2.03 (t, J=2.5, 1H), 2.00 - 1.88 (m, 2H), 1.78 (t, J=2.5, 1H), 1.53 (s, 3H), 1.53 (s, 3H). 

13C NMR (101 MHz, CDCl₃) δ = 175.3, 175.1, 173.7, 173.6, 172.3, 145.6, 145.5,
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144.1, 144.0, 122.1, 122.1, 121.7, 121.6, 78.0, 77.9, 77.7, 77.2, 72.0, 71.7, 50.2, 50.0, 29.4, 29.2, 25.3, 23.8, 23.8, 21.7, 21.7, 20.3, 20.2, 12.2, 11.9, 11.8, 11.3. HRMS (negative): (C_{19}H_{22}NO_{5}) \text{ 344.1493 (found, M-H), 344.1504 (calc.)}.

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 1.5 mL 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 (SiO\textsubscript{2}, Pentane/EtOAc: 100/0 to 0/100) and the product was isolated as white solid. Yield: 172 mg (0.385 mmol, 50%). Mixture of diastereomers: \textsuperscript{1}H NMR (400 MHz, CDCl\textsubscript{3}) \(\delta = 7.30 \text{ (s, 1H)}, 7.28 \text{ (s, 1H)}, 5.08 - 4.98 \text{ (m, 2H)}, 4.45 \text{ (br, 1H)}, 4.43 \text{ (br, 1H)}, 2.97 - 2.72 \text{ (m, 4H)}, 2.84 \text{ (s, 4H)}, 2.78 \text{ (s, 4H)}, 2.69 - 2.53 \text{ (m, 4H)}, 2.40 - 2.29 \text{ (m, 2H)}, 2.19 \text{ (s, 6H)}, 2.16 \text{ (s, 3H)}, 2.15 - 2.10 \text{ (m, 4H)}, 2.08 \text{ (s, 3H)}, 2.05 \text{ (s, 3H)}, 1.98 - 1.86 \text{ (m, 3H)}, 1.55 \text{ (s, 3H)}, 1.54 \text{ (s, 3H)}. \textsuperscript{13}C NMR (101 MHz, CDCl\textsubscript{3}) \(\delta = 174.5, 174.3, 168.3, 168.0, 166.1, 165.8, 145.7, 145.5, 144.1, 144.1, 122.1, 121.1, 121.4, 121.4, 119.0, 118.9, 78.0, 76.7, 76.3, 72.7, 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): (C_{23}H_{25}N_{2}O_{7}) \text{ 441.1662 (found, M-H), 441.1667 (calc.)}.

2.4.2 Synthesis of photostabilizer-dye conjugates and reactive 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 Lifetechnologies (USA) and ATTO647N-NHS from ATTOTECH (Germany). Synthetic oligomers (NH\textsubscript{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). Trolox-NHS 9 was synthesized following a published procedure.\textsuperscript{48} A Varian 400 (400 and 100 MHz) was used to record \textsuperscript{1}H-NMR and \textsuperscript{13}C-NMR spectra. Chemical shifts (\(\delta\)) are denoted in ppm using residual solvent peaks as internal standard (\(\delta_{H}=7.26\) and \(\delta_{C}=77.0\) for CDCl\textsubscript{3}, \(\delta_{H}=3.31, 4.78\) and \(\delta_{C}=49.15\) for CD\textsubscript{3}OD). High-resolution mass spectra (HRMS) were recorded on an Orbitrap XL (Thermo Fisher Scientific; ESI pos. or neg. mode).
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Flash chromatography was performed using a Grace Reveleris® 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 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-dyer) 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 x 150 mm, particle size 3.5 µm) and Waters Xterra MS C18 prep column (7.8 x 150 mm, particle size 10 µm) using a gradient of acetonitrile / triethylammonium acetate buffer (50 mM, pH 7.0). Gradient 1: 05/95 0 to 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 to 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 analytical run or 1.0 ml/min preparative run. The DNA was isolated by collecting the major peak of interest (see Figure 2.2). The resulting compounds were characterized by UV/Vis-absorption spectroscopy (Figure 2.3a) and Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (Figure 2.3b). Spectra were recorded on an ABI Voyager DE-PRO MALDI TOF (delayed extraction reflector) Biospectrometry Workstation mass spectrometer.

**Synthesis of ssDNA-NPA-Alexa555/ATTO647N/Cy5 (5, 6 and 7):** **Step 1:** The lyophilized ssDNA-NH₂ was resuspended in MilliQ water and adjusted to 80 µM in 0.2 M NaHCO₃ buffer (pH 8.35). To 100 µl of this solution, the same volume of a 20 mg/ml solution of 2 in DMF was added and the mixture was vortexed thoroughly. If necessary, additional DMF was added in 10 µl 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 (Figure 2.3, gradient 1, vide supra) to yield ssDNA-NPA-Fmoc 3. **Step 2:** Fmoc deprotection of 3 was performed as follows. The HPLC-purified and
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lyophilized oligonucleotide was resuspended in 50 µl of 50 mM triethylammonium acetate buffer (pH 7.0). Deprotection was achieved by addition of 40 µl DMF and 10 µl piperidine. The mixture was vortexed and allowed to react for 2 hours at room temperature. The deprotected oligonucleotide was purified on illumina 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 Lifetechnologies (USA) and ATTO647N-NHS from ATTOTEC (Germany); 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 µl 0.2 M NaHCO₃ buffer (pH 8.35). To this solution, one portion of fluorophore-NHS dissolved in 10 µl of DMSO was added and the mixture was vortexed thoroughly. After incubation overnight the oligonucleotide was purified on illumina NAP 5 column (vide supra) and isolated by preparative rp-HPLC (gradient 1, vide supra) to yield 5, 6 and 7 (see Scheme 2.1 & Figure S2.2).

Synthesis of ssDNA-TX-PG-Cy5 (13): Step 1: The lyophilized ssDNA was resuspended in MilliQ water and adjusted to 80 µM in 0.2 M NaHCO₃ buffer (pH 8.35). To 100 µl of this solution, the same volume of a 20 mg/ml solution of 11 in DMF was added and the mixture was vortexed thoroughly. If necessary, additional DMF was added in 10 µl portions to obtain a clear solution. After the reaction at room temperature overnight, the oligonucleotide was purified on illumina NAP 5 column and isolated by preparative rp-HPLC (Figure 2.2, gradient 2, vide supra) to yield 12. Step 2: Coupling with Cy5-N₃ was achieved following a modified manufacturer protocol (Lumiprobe, Germany). After every addition step the mixture was vortexed briefly. The HPLC-purified and lyophilized oligonucleotide 12 (~2 nmol) was resuspended in 30 µl of 2 M triethylammonium acetate buffer (pH 7.0) and added into a 0.5 ml tube. To this solution 8 µl MilliQ water and 7 µl of DMSO were added. A 15 nmol portion of Cy5-N₃ was dissolved in 40 µl of DMSO and added. To this mixture 10 µl of 5 mM stock solution of ascorbic acid in MilliQ water was added. The solution was degassed by a stream of N₂ for 30 sec. and finally 5 µl of a 10 mM stock solution of Cu(II)-TBTA in 55% DMSO/MilliQ water was added. The vial was flushed with N₂, closed and sealed with Parafilm®. After incubation overnight the oligonucleotide was purified on illumina NAP 5 column (gradient 2, vide supra) and isolated by preparative rp-HPLC (vide supra) to yield 13 (Figure 2.2).

Synthesis of ssDNA-Alexa555/ATTO647N: Step 1: Alexa555-NHS was obtained from Lifetechnologies (USA) and ATTO647N-NHS from ATTOTEC (Germany); 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-NH₂ (~2 nmol) was resuspended into 50 µl 0.2 M NaHCO₃ buffer (pH 8.35). To this solution, one portion of Alexa555-NHS/ATTO647N-NHS dissolved in 10 µl of DMSO was added and the mixture was vortexed thoroughly. After incubation overnight the oligonucleotide was purified with an illlustra NAP 5 column (vide supra) and isolated by preparative rp-HPLC (gradient 1, vide supra). (Figure 2.2)

2.4.3 Sample preparation and surface immobilization of oligonucleotides

Immobilization and investigation of single-fluorophores was achieved using a dsDNA scaffold comprising two 40-mer oligonucleotides, i.e., ssDNA-fluorophore and ssDNA-biotin. Sequences of both oligomers were adapted from refs.¹²,²⁴,²⁵. 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, Germany). Single immobilized fluorophore-molecules were studied in Lab-Tek 8-well chambered cover slides (Nunc/VWR, The Netherlands) with a volume of 750 µL as described in ref.¹¹. After cleaning with 0.1 % 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/800 mL BSA and 1 mg/800 mL BSA/biotin (Sigma Aldrich, The Netherlands) at 4 °C in PBS buffer overnight. After rinsing with PBS buffer, a 0.2 mg/mL solution of streptavidin was incubated for 10 minutes 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 µL 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, Germany). The respective mixtures of two oligomers were heated to 98 °C for 4 minutes and cooled down to 4 °C with a rate of 1 °C/min 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 minutes leading to a typical surface coverage of fluorophore-labeled dsDNA as shown in Figures 2.5 and Supplementary Figures S2.1-S2.7.
All single-molecule experiments were carried out at room temperature (22 ± 1 °C). Oxygen was removed from the buffer system utilizing an oxygen-scavenging system (PBS, pH = 7.4, containing 10% (wt/vol) glucose and 10% (vol/vol) glycerine, 50 µg/ml glucose-oxidase, 100-200 µg/ml 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 photophysics of organic fluorophores and hence do not convolute with effects from intramolecular stabilization. Glucose-oxidase catalase (GOC) was used instead of a combination of protocatechuic acid and protocatechuate-3,4-dioxygenase (PCA/PCD) to avoid convolution of inter- and intramolecular photostabilization with PCA.

2.4.4 Confocal scanning microscopy and data analysis

A custom-built confocal microscope, described in ref.18, 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 centered at 640 nm or 532 nm. The spatially filtered beam was coupled into an oil immersion objective (60×, NA 1.35, UPLSAPO 60XO mounted on an IX71 microscope body, both Olympus, Germany) by a dichroic beam splitter (zt532/642rpc, AHF Analysentechnik, Germany). Surface-scanning was performed using a XYZ-piezo stage with 100×100×20 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/s, Picoquant, Germany) with appropriate spectral filtering (green: HC582/75, red: ET700/75 AHF, both Analysentechnik, Germany). The detector signal was registered using a Hydra Harp 400 picosecond event timer and a module for time-correlated single photon counting (both Picoquant, Germany). The data was 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.

2.4.5 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 total-internal-reflection fluorescence (TIRF) configuration as described
Images were collected with a back-illuminated emCCD camera (512×512 pixel, C9100-13, Hamamatsu, Japan in combination with either ET585/50 or ET700/75, AHF Analysentechnik, Germany). 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 \( \approx 50 \text{ W/cm}^2 \) at the sample location. To quantitatively characterize photostability, we imaged areas with the size of \( \approx 25 \times 35 \mu\text{m} \) 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 standard deviations above background noise (Standard Deviation of all pixels over all frames of the movie) and summing the intensity in a 3×3 pixel area. Neighboring peaks closer than 5 pixels were not taken into account (see typical examples of fluorophore density and fluorescent time traces in Supplementary Figures S2.1-S2.7). 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 \( y(t) = C + A \cdot e^{-c t} \) (with \( c = 1/\tau_{\text{bleach}} \) and \( \tau_{\text{bleach}} \) being the characteristic bleaching time constant). For some samples with a more complicated behavior, 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 standard deviations were derived from multiple repeats of the same experiment on different days, where each compound was tested in \( \geq 5 \) movies. The signal-to-noise ratio (SNR) was determined using fluorescent time traces, by dividing the standard deviation 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.

**Author Contribution**

Thorben Cordes conceived the study. Jasper H. M. van der Velde, Jens Oelerich, Andreas Herrmann, Gerard Roelfes and Thorben Cordes designed experiments. Jingyi Huang, Jens Oelerich, Jochem H. Smit provided new reagents including chemical characterization. Jingyi Huang performed the synthesis, purification and characterization of NPA-Alexa 555, NPA-ATTO647N, TX–PG–Cy5 and all the DNA-photostabilizer-dye conjugates. Jingyi Huang performed TIRF microscope imaging and data analysis, Jasper H. M. van der Velde and Atieh Aminian Jazi performed confocal microscope imaging and ALEX experiments.
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Appendix

Supplementary Figure 2.1. Further details of photophysical characterization of Alexa555. (a) Schematic view of the photostabilizer-dye conjugate on DNA and the experimental strategy for surface immobilization. (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 τf. (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 were autocorrelated, 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.
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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.

**Supplementary Figure 2.2.** 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 \(\tau_0\). (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.
Supplementary Figure 2.3. 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 \( \tau_f \). (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.
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(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.

Supplementary Figure 2.4. 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 \( \tau_f \). (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.
Supplementary Figure 2.5. Details of 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 $\tau_{fl}$. (d) Representative fluorescence time traces of the selected single emitters, indicating blinking characteristics. (e) Histogram of off-state lifetimes (longer (main panel: triplet) and shorter (inset: cis-trans isomerization) lifetimes), with Gaussian fit (red line) and mean plus standard deviation value. Here all individual fluctuating fluorescence time traces were autocorrelated, 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 measurement 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.

Supplementary Figure 2.6. 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 τfl. (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.
**Supplementary Figure 2.7.** 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 $\tau_f$. (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, respecti