Supporting information

On the Mechanism of Trolox as Antiblinking and Antibleaching Reagent

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1. Material and Methods

Sample preparation

LabTek 8-well chambered cover slides (Nunc) with a volume of ~750 µl were treated with 0.1 % HF for 30 seconds and were washed three times with PBS-buffer. They were subsequently incubated with a solution of 5 mg/ml BSA and 1 mg/ml BSA/biotin (as received from Sigma Aldrich) in PBS for at least ten hours at 4 °C. After washing three times with PBS, the surface was incubated with a ~0.1 mg/ml solution of streptavidin for five minutes and washed again for three times with PBS. Then a biotinylated 40mer oligonucleotide (biotin-CGT ATA GCT ATA CAA TAT AAG TGT AAG GAA TCG AAT CGT A (strand I); as received from IBA, Göttingen) was incubated on the surface for one minute. Subsequently, a counter-strand terminally labelled with ATTO655 (GCA TAT CGA TAC ATT ATA TTC ACA TTC CTT AGC TTA GCA T-ATTO655 (strand I); as received from IBA, Göttingen) was hybridized to strand I giving dsDNA sample I. The same procedure was performed using two different ssDNA-strands with the same DNA sequence but ATTO647N as marker dye. This resulted in dsDNA sample II.

Single-molecule experiments of the different samples were carried out at room temperature (22 ± 1 °C) under different buffer-conditions: (i) Standard phosphate buffered saline (PBS) with a pH of 7.4 was used for all experiments. (ii) Additionally, oxygen could be removed using an oxygen-scavenging system (PBS, pH = 7.4, containing 10% (wt/vol) glucose and 12.5% (vol/vol) glycerine, 50 µg/ml glucose-oxidase, 100-200 µg/ml catalase, and 0.1 mM Tris(2-carboxyethyl)phosphine hydrochloride (TCEP)).[1] (iii) Different concentrations of Trolox (TX) were added to the PBS-buffer as described in the main text.

Single molecule fluorescence spectroscopy

To study fluorescence on the level of single molecules, a custom built confocal microscope was used as described in refs 2,6,7 found in the main text. The excitation wavelength was selected by filtering a laser beam of a pulsed supercontinuum-source (SuperK Extreme, Koheras, Denmark) with an acousto-optical tunable filter (AOTFnc-VIS, AA optoelectronic) that was subsequently coupled into a single-mode fiber. The excitation wavelength was fixed to 640 nm (spectral width of 2 nm, excitation ATTO647N/ATTO655). The spatially filtered beam entered an inverse microscope and was coupled into an oil immersion objective (60×, NA 1.35, UPLSAPO 60XO, Olympus) by a dual-band dichroic beam splitter for recording fluorescent transients (Dualband z532/633, AHF Analysentechnik, Germany). The average light intensities for these measurements were 1.5 kW/cm^2 at 640 nm. The resulting fluorescence was collected by the objective, focused onto a 50 µm pinhole, filtered (ET-Bandpass 700/75M, AHF Analysentechnik, Germany), and detected by an avalanche photodiode (SPCM-AQR-14, PerkinElmer). The detector signal was registered and evaluated using custom made LabVIEW software. For characterization of the fluorescent properties of ATTO655 the off- and on-times were determined according to established procedures (ref 7 in the main text): i) an autocorrelation of the fluorescence transients was generated. ii) the autocorrelation-curve was fitted using an exponential function; iii) the off-times τ_{off} and on-counts N_{on} were derived from the amplitudes and the characteristic time-constant of the autocorrelation after background correction. ~30 molecules were measured and evaluated for each data point as described for each experimental condition as seen in Figure 2 (main text). The concept of the single-molecule redox-sensor is explained in greater detail in ref 7 (main text).

2. Further Results and Discussion

The photostabilizing effect of Trolox and ROXS

A qualitative picture of the photostabilizing effect of Trolox (ref 1, main text) and ROXS (ref 2, main text) is given in Figure S1: a) shows a typical fluorescence transient of ATTO647N in pure PBS-buffer where blinking and fast bleaching occurs. The right inset of (a) shows the autocorrelation function G(τ) together with an exponential fit revealing off-times τ_{off} = 125 ± 5 ms. Strong effects on the fluorescence of ATTO647N can be observed by addition of oxidant/reductant with oxygen removal. The transient shown in Figure S1b was recorded applying ROXS-conditions with addition of 1 mM MV/AA and oxygen-removal. Here, no blinking is observed (no amplitude is observable in the autocorrelation function) and the photostability is

increased as shown in ref 2 (main text). The same effect can be achieved by the addition of 1 mM TX together with oxygen removal which also results in stable fluorescence and increased stability (almost no amplitude is observable in the autocorrelation function) (Figure S1c).

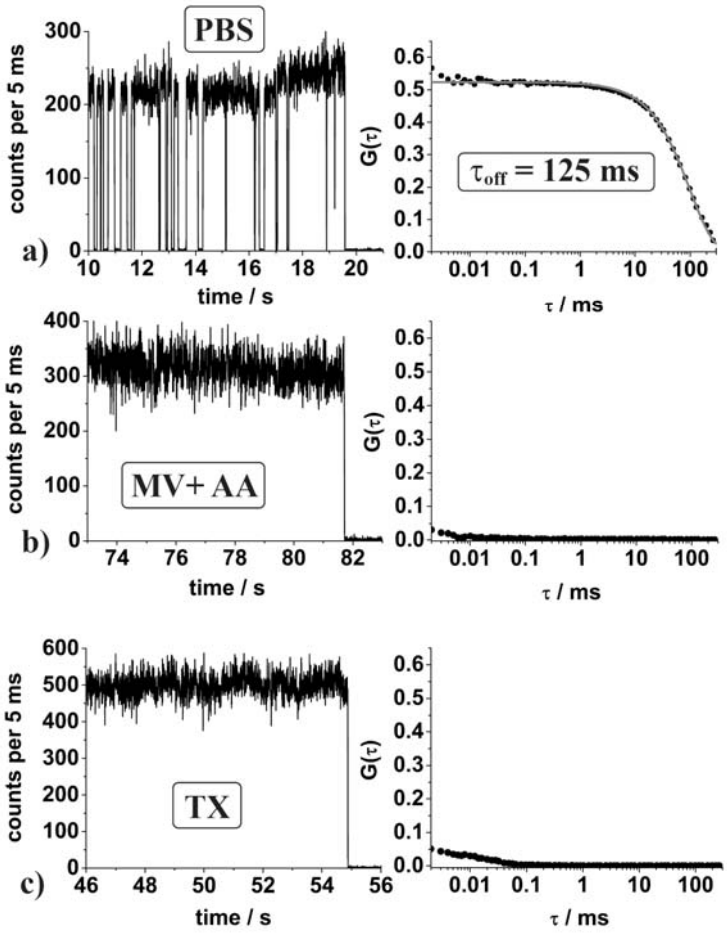

Figure S1. (a–c) Fluorescence transients of ATTO647N-labeled dsDNA (sample II) immobilized in aqueous environment under different buffer conditions. The transients are binned in 5 ms. All transients that are shown in the left panels were recorded by exciting the sample at 640 nm with an average excitation intensity of ~1.5 kW/cm². The results of an autocorrelation analysis together with a mono-exponential fit, if plausible, are shown in the right part of the figure.

Observation of slow oxidation of Trolox in PBS

Our experiments detailed in Figure 2 (main text) show that a fraction of TX is already oxidized during the slow 18 h dissolving process in PBS. During that process TX is randomly exposed to room light and oxygen. The formation of an oxidizing species could be observed when fresh TX (1 mM solution) was dissolved in PBS and the SMRS-values were recorded over time (Figure 2b, main text and Figure S2). We observe that the reducing properties are not changed within experimental error (Figure S2a) while the oxidizing properties are increased. This means that the TX-concentration appears to be nearly constant while the concentration of the oxidant increases on the timescale of ~18 h. A method to determine the TX and oxidant concentration is presented below. This method yields a concentration of TX of ~975 µM after 18 h, and the oxidant is present at a concentration of ~25 µM.

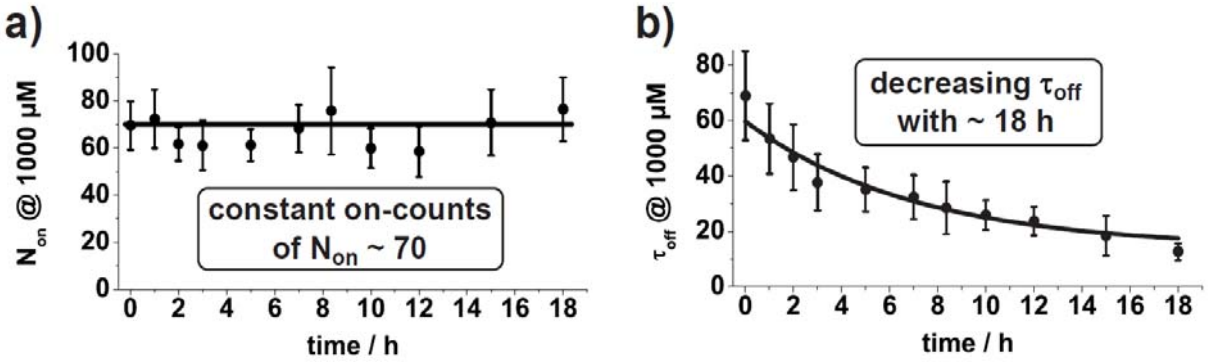

Figure S2. Temporal evolution of the SMRS-parameters when dissolving 1 mM TX in PBS. It is clearly evident that the on-counts remain constant within experimental error while τ_{off} decreases significantly on the timescale of several hours. The TX-sample and the SMRS were stored in a Labtek-Chamber and were randomly exposed to room light.

The difference between freshly prepared TX and TX slowly dissolved in aerated PBS is also apparent for the fluorescent dye ATTO647N, for which the close to complete removal of blinking by TX at millimolar concentrations has already been demonstrated.[2] At 10 µM concentration, off-times of 4 ± 1 ms are observed for TX sample I (see main text & material and methods), 19 ± 4 ms for TX sample III and 28 ± 8 ms in the presence of 1 mM ascorbic acid, i.e. in the absence of oxidizing agents (Figure S3 and ref 2). These data indicate some oxidizing effect already for the fresh TX sample III, which is in excellent agreement with the data shown in Figure 2. This is further evidence that the results obtained with the SMRS can be applied to other fluorescent dyes such as those studied in refs 1,2.

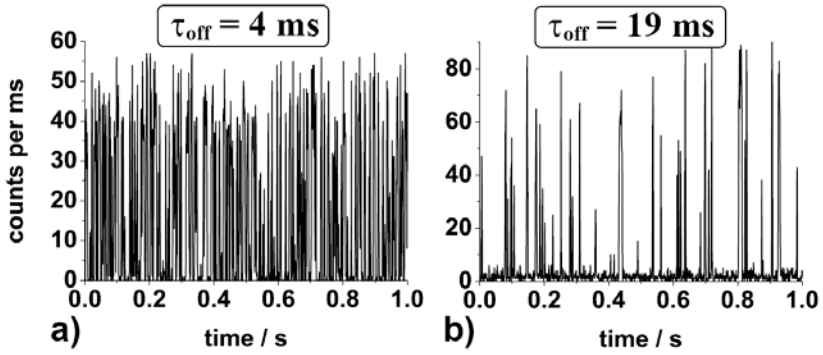

Figure S3. Fluorescence transients of ATTO647N-labeled DNA (sample II) immobilized in aqueous environment. The transients are binned in 1 ms. (a) shows ATTO647N in PBS-buffer with additional 10 µM TX (TX was dissolved in PBS for at least 24 h), this represents TX and its oxidizing form – the duration of the off-times is drastically reduced. Off-times of the order of 4 ± 1 ms are observed by autocorrelation analysis as shown in ref 2 (main text). (b) Longer off-times of τ_{off} = 19 ± 4 ms are observed for ATTO647N in the case of 10 µM freshly dissolved TX. Here, less oxidant is present resulting in strong blinking. All transients were recorded by exciting the sample at 640 nm with an average excitation intensity of ~1.5 kW/cm².

UV-vis detection of the TX-Quinone derivative and correlation with SMRS transients

(i) Detection of the TX-quinone and quantitative determination of its concentration

It is known that TX can form stable quinone-derivatives after enzymatic oxidation or photo-induced oxidation (ref 9, main text). During these processes, the absorption properties of the

sample change considerably: the TX-absorption band of lowest energy is found at ~290 nm in aqueous buffer or water/ethanol mixtures (ref 9, main text). When oxidized to the quinoid form (TX-quinone, TQ) - either by enzymatic reactions or light - a new band appears at ~270 nm. This absorption is clearly associated to the TX-quinone as shown in refs 9 (main text) and references cited therein. We hence use these spectroscopic marker bands to probe the composition of our samples and to verify the presence and influence of TQ in our experiments.

We detected the photoinduced formation of TQ (Figure S4) using a standard absorption spectrometer. Therefore the UV/VIS spectra of TX and TQ were recorded in the region from 225 – 500 nm with a Specord S100 from Analytik Jena. In order to induce the photochemical oxidation of TX, a Hg/Xe lamp (λ_{exc} < 300 nm, ~450 mW, Hamamatsu) was used in combination with suitable filtering (BG20, 1 mm, Schott) to minimize secondary photochemistry.

As seen in Figure S4a the same absorbance changes, as reported in refs 9 (main text), were found when freshly dissolved TX (Figure S4, black, λ_{max} ~290 nm) is illuminated with UV-light. Already after few minutes a distinct absorption band of TQ was clearly observed at ~270 nm (Figure S4, blue). The isosbestic point (λ_{iso} ~280 nm) found for illumination times < 10 minutes indicates a one-step transformation from TX into its quinone derivative. Side reactions, most likely due to photoreactions of the quinone, cause absorbance changes in the range > 300 nm for long illumination periods. The formation of TQ was observed in a less pronounced way for solutions that underwent aging without specific UV-illumination (data not shown). Here, the exact same absorbance changes as observed for UV-illumination (Figure S4a) could be detected and account for the temporal evolution of the SMRS-values (see Figure 2 (main text) and Figure S2).

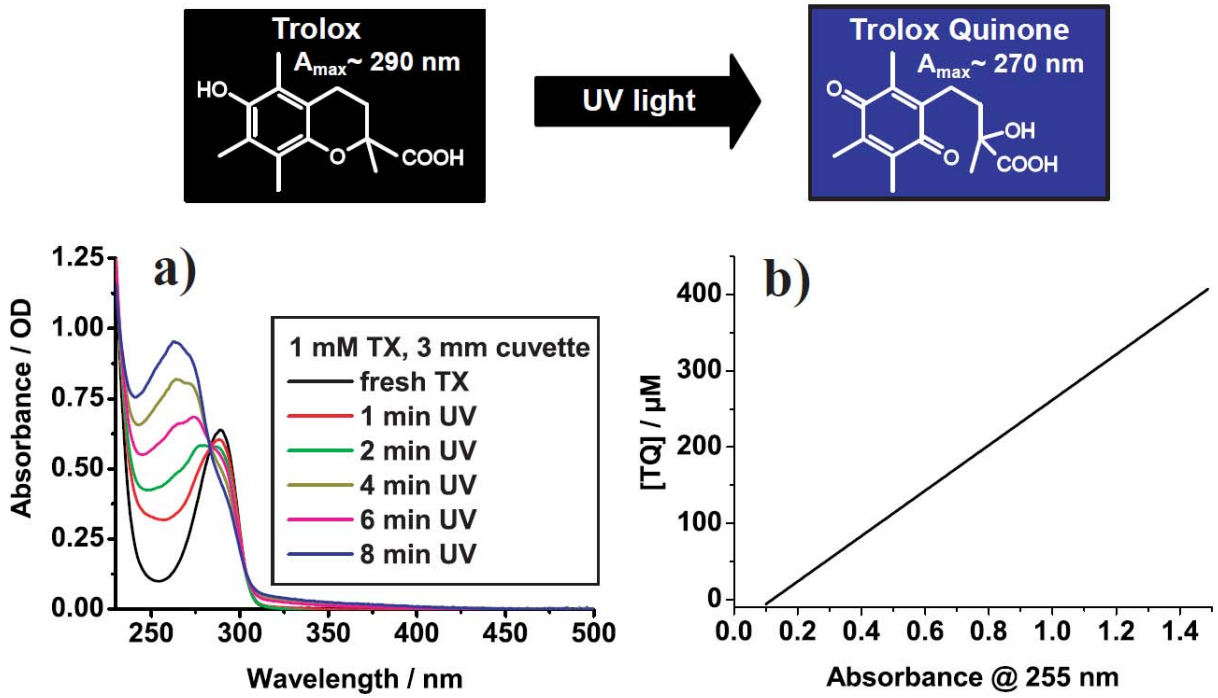

Figure S4. Scheme of TX undergoing light-induced oxidation to its quinone derivative. a) Spectroscopic investigation of TX under UV-exposure: 100 µl of a 1 mM TX-solution were illuminated with UV-light around 300 nm for periods described in the figure. Thereby the absorption peak of TX at ~290 nm decreased with increasing illumination time, while a new peak corresponding to the TX-quinone appeared at ~270 nm. An isosbestic point was

observed around ~280 nm, which is partially lost for long illumination times > 8 min. b) Illustration of the dependence of the TQ concentration [TQ] on the absorbance of the sample at a detection wavelength of 255 nm. This graph was calculated using eqn. 3 and is valid for a starting concentration of Trolox, $[TX]_0$ = 1 mM and a cuvette with an optical path length of 3 mm.

The concentration of the TX-quinone [TQ] in a sample can be determined using absorption spectroscopy. The observed absorption A of the TX-sample at the specific detection wavelength λ is equal to the sum of the absorption of TX and TQ:

$$A_\lambda = \left[\varepsilon_\lambda(TX)\cdot[TX] + \varepsilon_\lambda(TQ)\cdot[TQ]\right]\cdot d \qquad (1)$$

Here, d is the optical path length of the cuvette and ε_λ(TX) and ε_λ(TQ) are the respective extinction coefficients. When TX is dissolved in buffer, the starting concentration $[TX]_0$ is equal to [TX] while [TQ] = 0. Our approach assumes that each TX transforms into one TX-quinone, so the sum of both concentrations [TX] and [TQ] is equal to $[TX]_0$.

$$[TX]_0 = [TX] + [TQ] \qquad (2)$$

Integrating eqn (2) into eqn (1) allows to obtain the concentration of TQ as a function of the absorbance at a specific detection wavelength:

$$[TQ] = \frac{\frac{A_\lambda}{d} - \varepsilon_\lambda(TX)\cdot[TX]_0}{\varepsilon_\lambda(TQ) - \varepsilon_\lambda(TX)} \qquad (3)$$

This linear function is plotted in Figure S4b for a starting concentration $[TX]_0$ = 1 mM. This representative sample shows an absorbance of A = 0.1 at 255 nm corresponding to pure TX (Figure S4a, d = 3 mm, black curve). The concentration of TQ in samples that contain both TX and TQ can easily be estimated: as the extinction coefficient of Trolox at 255 nm is only ε_{255}(TX) = 400 $l\cdot mol^{-1}\cdot cm^{-1}$ while that of TQ is considerably larger with ε_{255}(TX-Quinone) ~ 11600 $l\cdot mol^{-1}\cdot cm^{-1}$, even small amounts of TQ are detectable at this particular wavelength (see ref 9c, main text). So, the concentration of TQ formed by UV-radiation or aging of the sample, which results in absorbance changes as shown in Figure S4a, is quantitatively determined using the absorbance of a specific sample at 255 nm.

(ii) Correlation of TX and TQ concentration with blinking kinetics

To correlate the TX and TQ concentration with the on-counts and the off-times of the SMRS we recorded single-molecule transients at different degrees of photo-oxidation, that is with changing ratio of [TX]/[TQ]. Absorption spectra were recorded after certain illumination periods to determine the sample composition using eqn. 3 (see also Figure S4). Single-molecule transients with indicated concentrations of TX and TQ are shown in Figure S5.

The following trend is found: freshly dissolved TX (1 mM solution) with a spectrum similar to the one in Figure S4a (black) shows off-times of 65 ± 12 ms and on-counts of 64 ± 14(Figure S5a or Figure 2, main text). UV-radiation forms detectable amounts of TQ after only several minutes of exposure (Figure S4a, red). A sample with 25 µM TQ and 975 µM TX shows off-times of 14 ± 5 ms and on-counts of 87 ± 9 as seen in Figure S5b. Further on, this trend is confirmed in Figure S5, panels c,d, where increasing on-counts (Figure S5, c: τ_{off} = 6 ± 2 ms, N_{on} = 104 ± 15; d: τ_{off} = 3 ± 2 ms, N_{on} = 150 ± 20) represent the consumption of TX while decreasing off-times show that more oxidant (= TQ) is formed. In contrast to the data shown in Figure 2 (main text) and Figure S2, the TX concentration is significantly changing in this experiment, which is manifested by the increase of on-counts.

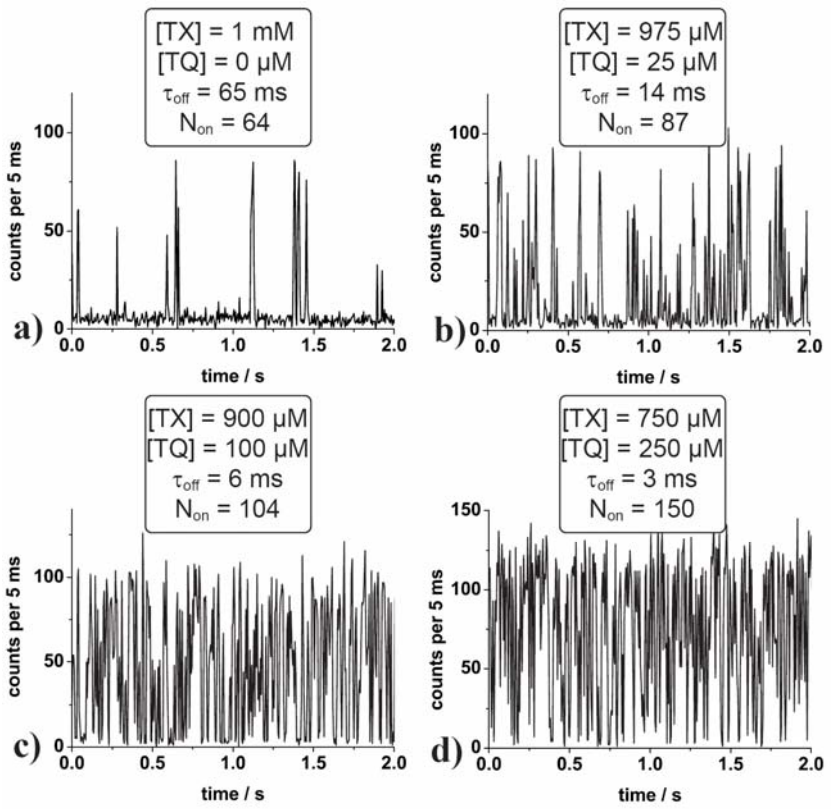

Figure S5. Correlation of TX and TQ concentration with blinking kinetics of the SMRS (concentration of TX before the illumination process was $[TX]_0$ = 1 mM). Representative transients and the corresponding on-counts and off-times are displayed. All transients were recorded by exciting ATTO655 terminally attached to dsDNA at 640 nm with an average excitation intensity of ~1.5 kW/cm².

It is noteworthy that these results have implications on the interpretation of the oxidizing properties of TQ. The observed off-times of only 14 ms for ~25 µM of oxidant show clearly that TQ is a stronger oxidant than N,N-methylviologen (MV), because MV shows much longer off-times (τ_{off} ~ 250 ms) under similar conditions (ref 7, main text). The reduction potential of MV is known to be -0.69 V (vs. SCE, ref 2, main text) which allows estimating the one of the TQ to be higher, i.e. in a range suggested in the main text. Hence, TQ is a very effective oxidant and can be used at lower concentrations than MV. Since Trolox is a vitamin E analogue, i.e. related to natural compounds, TQ should also be less toxic and therefore well suitable for dye stabilization based on ROXS.

A practical guide how to use Trolox as reliable antifading and antiblinking agent

Users of Trolox for dye stabilization commonly encounter problems with the reproducibility of the obtained results. This is likely due to the different extent of TQ formed in their samples. Obviously, partly degraded TX will, in many experiments, yield better results in terms of blinking suppression and photostability than pure and freshly prepared TX. In our lab, TX in buffer is prepared freshly the day before use. Two possibilities of oxidizing fresh samples to obtain a system of reductant and oxidant (ROXS) are easily applicable:

(i) As TX dissolves slowly in water or buffers, the suspension of solid TX and solvent should be stirred or shaken for at least 18 hours at room temperature. The aging process (slow oxidation into the quinone) starts already without UV-illumination immediately after dissolving at room light. This commonly yields a sufficiently high concentration of TQ without significantly degrading the reducing capability.

(ii) Dissolve TX, i.e. as a 100 mM stock solution in methanol and use small amounts of this solution to create a 1-2.5 mM solution in buffer. In less polar solvents such as methanol the oxidation proceeds significantly slower compared to water allowing better storage (ref 9c, main text). Buffers prepared from methanol solution, however, have a less defined composition of TX and TQ, which should be checked by UV-Vis spectroscopy (see below). In some cases additional aging or UV-illumination is necessary.

The composition of the TX/TQ ROXS solution can be monitored at ~255 nm. The concentration of TQ is calculated according to eqn. 3/Figure S5b. We suggest to use 1-2.5 mM solutions of TX with at least 25 µM TQ. On the other hand, the fraction of TQ should not be too high as better photostability is observed for an excess of reductant for most dyes. Moreover, colored secondary photoproducts accumulate during the oxidation. From our experience the TQ fraction should be in the range of 2-30 %. The following form of equation 3 can be simply used for [TQ] calculation from the absorption at 255 nm A_{255} using a 1 cm cuvette.

$$[TQ] = \frac{\frac{A_{255}}{d} - \varepsilon_{255}(TX)\cdot[TX]_0}{\varepsilon_{255}(TQ) - \varepsilon_{255}(TX)} = \frac{\frac{A_{255}}{1cm} - 400\cdot[TX]_0 lmol^{-1}cm^{-1}}{(11600-400)lmol^{-1}cm^{-1}} = \frac{A_{255}(mol\cdot l^{-1}) - 400\cdot[TX]_0}{11200} \quad (4)$$

We finally note that the amount of oxidant, that is necessary to stabilize fluorophores, of course, depends on the specific dye. Dyes with low lying reduced states as oxazines will need considerably more oxidant for efficient recovery of the radical anion than fluorophores such as ATTO647N where the re-oxidation is energetically more favorable. The qualitative amount (ratio from oxidant to reductant) may be estimated by consideration of the free enthalpy of the re-oxidation of the radical-anion state using the Rehm-Weller Equation[2] as described in refs 2,7 (main text).

3. Additional references

1. Ha, T.; Rasnik, I.; Cheng, W.; Babcock, H. P.; Gauss, G. H.; Lohman, T. M.; Chu, S., "Initiation and re-initiation of DNA unwinding by the Escherichia coli Rep helicase." *Nature* **2002,** *419*, 638-641.
2. Rehm, D., and Weller, A. "Kinetics of fluorescence quenching by electron and H-atom transfer." *Israelien Journal of Chemistry* **1970**, *8*, 259-271.