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Supporting Material

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SUPPLEMENTAL MATERIAL

MITIGATING UNWANTED PHOTOPHYSICAL PROCESSES FOR IMPROVED SINGLE-MOLECULE FLUORESENCE IMAGING

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	Cy Dyes				Alexa Dyes			
	Direct Illumination		Dual Illumination		Direct Illumination		Dual Illumination	
	t_{on}	t_{off}	t_{on}	$<t_{off}>$	t_{on}	t_{off}	t_{on}	$<t_{off}>$
	(s)				(s)			
No compounds	14.28	0.19 (0.29) 136.00 (0.71)	5.26	38.68	4.79	0.20 (0.03) 175.51 (0.97)	3.33	31.07
2mM COT	54.97	0.19 (0.18) 141.99 (0.82)	10.0	23.53	12.68	0.30 (0.05) 164.74 (0.95)	8.33	10.68
2mM NBA	47.10	0.38 (0.64) 199.72 (0.36)	9.1	12.22*	14.16	0.19 (0.21) 117.16 (0.79)	7.87	6.99*
2mM Trolox	61.62	0.21 (0.18) 125.63 (0.82)	28.9	22.41	37.88	0.30 (0.10) 144.58 (0.90)	23.08	23.1
All compounds	67.11	0.23 (0.44) 116.48 (0.56)	40.84	24.22	64.46	0.28 (0.31) 120.16 (0.69)	29.94	7.14*

Supplemental Table 1: Trolox, COT, and NBA separately and in combination attenuate photophysical processes in both Cy and Alexa fluorophores under direct and dual illumination. t_{on} and t_{off} were calculated for the DNA system by fitting the distributions of dwells in zero and fluorescent states using maximum log likelihood optimization procedures in QuB as a function of increasing concentrations of COT, NBA and Trolox for cyanine and Alexa fluorophores. Both the lifetime and relative populations of short- and long-lived dark state components are reported. For dual illumination experiments a weighted average of dark state lifetimes, $<t_{off}>$, is reported. * indicates that these values are only estimates because few transitions were observed.

A

	Cy Dyes				Alexa Dyes			
	Total t_{on}	Total t_{off}	% Total t_{on}	$n_{transitions}$	Total t_{on}	Total t_{off}	% Total t_{on}	$n_{transitions}$
	(s)		(%)	(s^{-1})	(s)		(%)	(s^{-1})
No compounds	6.5	4.0	62.89	0.3	5.5	19.1	29.8	0.4
0.5 mM COT	7.4	5.	58.4	0.3	6.7	18.7	35.6	0.3
1mM COT	7.1	3.9	65.78	0.2	7.7	19.4	40.0	0.2
2mM COT	10.2	6.1	63.1	0.2	9.2	13.3	46.9	0.2
0.5mM NBA	7.8	0.4	94.0	0.3	7.2	0.6	91.9	0.4
1mM NBA	5.2	0.4	94.9	0.2	6.5	0.3	94.1	0.3
2mM NBA	4.9	0.3	96.6	0.2	5.92	0.12	94.6	0.2
0.5mM Trolox	14.6	4.9	73.2	0.2	10.3	7.5	60.1	0.2
1mM Trolox	17.6	5.0	76.9	0.2	14.4	6.3	68.5	0.2
2mM Trolox	25.8	2.9	85.3	0.1	24.6	4.4	80.9	0.1

B

	Cy Dyes				Alexa Dyes			
	Total t_{on}	Total t_{off}	% Total t_{on}	$n_{transitions}$	Total t_{on}	Total t_{off}	% Total t_{on}	$n_{transitions}$
	(s)		(%)	(s^{-1})	(s)		(%)	(s^{-1})
No compounds	3.7	3.3	55.3	0.8	2.2	4.8	30.7	1.0
0.5 mM COT	4.4	3.8	54.5	0.6	2.4	4.6	33.7	0.9
1mM COT	4.8	4.5	52.6	0.5	3.4	7.0	34.1	0.6
2mM COT	6.8	5.2	58.1	0.4	4.2	6.2	39.7	0.5
0.5mM NBA	2.9	0.3	93.1	0.5	2.3	0.2	90.4	0.9
1mM NBA	2.5	0.3	93.3	0.4	2.3	0.2	94.7	0.5
2mM NBA	2.0	0.2	93.9	0.3	1.8	0.1	93.8	0.4
0.5mM Trolox	7.7	3.4	69.9	0.4	3.7	2.9	57.6	0.4
1mM Trolox	9.6	3.5	73.0	0.3	4.7	1.9	71.2	0.3
2mM Trolox	12.6	3.2	80.45	0.2	8.5	2.3	83.0	0.2

Supplemental Table 2: Trolox, COT, and NBA attenuate photophysical behavior of both Cy and Alexa fluorophore pairs. Total t_{on}, total t_{off}, the percent time in non-zero FRET states, % Total t_{on} (Total t_{on}/Total t_{on} + Total t_{off}), and the average number of transitions per trace ($n_{transitions}$) is calculated for increasing concentrations of COT, NBA and Trolox for cyanine and Alexa dyes at 0.65kW/cm^2 (A) and 1.95kW/cm^2 (B). Total t_{on} increases the most in the presence of 2mM Trolox whereas the total t_{off} decreases the most with 2mM NBA and the trends are consistent for both dye pairs. Exponential fitting was performed in Origin. In each case a minimum of 570 number of events were observed and fitting resulted in R^2 values >0.995.

	DNA System											
	Cy Dyes						Alexa Dyes					
	0.65kW/cm^2			1.95kW/cm^2			0.65kW/cm^2			1.95kW/cm^2		
	t_{on}	$<t_{off}>$	Total t_{on} + Total t_{off}	t_{on}	$<t_{off}>$	Total t_{on} + Total t_{off}	t_{on}	$<t_{off}>$	Total t_{on} + Total t_{off}	t_{on}	$<t_{off}>$	Total t_{on} + Total t_{off}
	(s)						(s)					
No Compounds	2.7	0.4	10.5	1.2	0.5	6.8	1.6	2.3	27.2	0.7*	1.5*	7.0
0.5mM COT	2.9	0.3	13.9	1.5	0.6	8.3	2.2	1.5	27.3	0.8	1.2	7.2
1mM COT	3.2	0.3	11.5	1.6	0.4	9.2	2.8	1.0	29.9	1.1	1.2	10.5
2mM COT	3.6	0.1	17.9	2.2	0.2	12.6	3.9	1.7	28.1	1.4	1.6	10.9
0.5mM NBA	3.9	0.2	8.3	1.6	0.2	3.1	3.0	0.4	7.9	1.0	0.1	2.5
1mM NBA	3.1	0.1	6.2	1.5	0.1	2.6	3.5	0.3	6.9	1.3	0.1	2.4
2mM NBA	2.8	0.1	5.4	1.4	0.1	2.1	3.9	0.3	6.2	1.1	0.1	1.9
0.5mM Trolox	4.6	0.1	21.7	2.4	0.2	11.1	5.0	2.9	18.6	1.8	1.5	6.5
1mM Trolox	5.9	0.1	26.7	3.3	0.3	13.5	7.1	4.8	26.2	2.6	1.4	6.6
2mM Trolox	7.1	0.1	33.9	4.8	0.2	16.3	11.1	0.3	39.9	4.6	1.3	10.6
All Compounds	6.3	0.1	18.7	3.7	0.2	7.2	9.1	0.1	28.4	4.2	0.7	7.7

Supplemental Table 3A: Trolox, COT, and NBA attenuate photophysical processes in both Cy and Alexa fluorophore pairs. t_{on} and $<t_{off}>$ were calculated using the distributions of dwells in non-zero and zero FRET states via maximum likelihood optimization procedures in QuB as a function of increasing concentrations of COT, NBA and Trolox for cyanine and Alexa dyes at both 0.65kW/cm^2 and 1.95kW/cm^2. Data for individual and all three compounds are shown. * denotes values obtained from the systems fitting to a single-exponential process.

Ribosome System			
Cy Dyes			
	0.65kW/cm^2		
	t_{on}	$<t_{off}>$	Total t_{on} + Total t_{off}
	(s)		
No Compounds	1.0	0.5	2.5
2mM COT	1.2	0.5	2.8
2mM NBA	2.4	0.6	3.6
2mM Trolox	1.1	0.3	1.2
All Compounds	5.3	0.5	6.1

Supplemental Table 3B: Trolox, COT, and NBA affect the fluorescence and dark states lifetimes observed for the cyanine dye pair in the ribosome system in a manner distinct from the DNA system.

	Cy Dyes					
	0.65kW/cm^2			**1.95kW/cm^2**		
	Total t_{on}	Total t_{off}	% Total t_{on}	Total t_{on}	Total t_{off}	% Total t_{on}
	(s)		(%)	(s)		(%)
No Compounds	7.1±0.8	4. 3±1.3	65.6±5.9	3.6±0.3	2.8±0.7	60.6±5.4
0.5 mM COT	8.0±1.2	5.7±1.2	60.6±3.6	4.3±0.4	3.3±0.7	56.3±3.1
1mM COT	8.2±1.4	4.7±1.5	65.2±4.5	5.5±0.2	4.6±0.9	52.8±3.1
2mM COT	10.7±1.3	4.7±0.9	63.2±1.1	6.3±0.5	4.8±0.3	57.4±2.9
0.5mM NBA	7.7±0.7	0.5±0.1	94.1±0.7	3.0±0.5	0.2±0.0	93.2±0.2
1mM NBA	6.0±0.5	0.2±0.0	94.9±0.2	2.3±0.2	0.1±0.0	93.2±0.8
2mM NBA	5.2±0.4	0.1±0.0	96.6±0.4	1.9±0.3	0.1±0.0	94.5±1.3
0.5mM Trolox	17.0±2.3	5.8±1.3	72.5±1.1	8.0±1.2	3.4±0.9	70.7±3.8
1mM Trolox	21.1±2.2	5.0±0.5	76.8±1.2	9.4±0.7	3.4±0.4	73.3±0.9
2mM Trolox	27.4±3.3	3.1±0.4	85.6±0.9	12.0±1.4	2.6±0.5	81.1±1.7

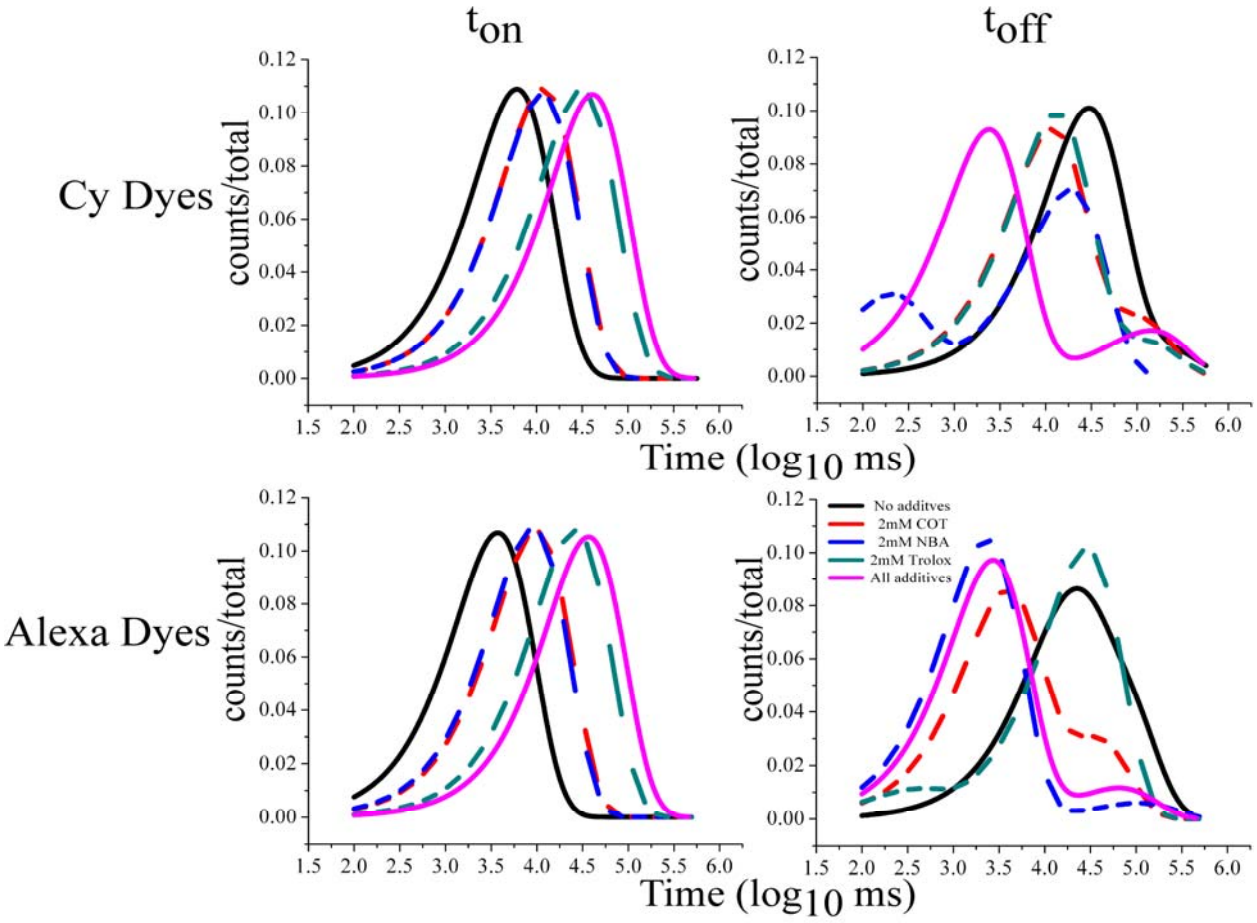
Supplemental Table 4: Dye photophysical processes can be robustly quantified. Total t_{on}, total t_{off}, and the percent time in non-zero FRET states, % Total t_{on} (total t_{on}/total t_{on}+total t_{off}), were obtained in three different datasets for Cy dye pairs linked to a twelve basepair oligonucleotide are provided at both 0.65kW/cm^2 and 1.95kW/cm^2. Exponential fitting of dwell times was performed using Origin software. In each case a minimum of 450 number of events were observed and fitting resulted in R^2 values >0.995. Standard errors provided demonstrate the reproducibility and robustness of data.

	Total t_{on} (s)	Total t_{off}	Total t_{on} (s)	Total t_{off}
	0.65kW/cm^2		1.95kW/cm^2	
No Compounds	10.1	7.6	2.3	2.0
2mM Trolox	31.9	4.6	10.5	2.3
All Compounds	17.68	1.5	5.6	0.5

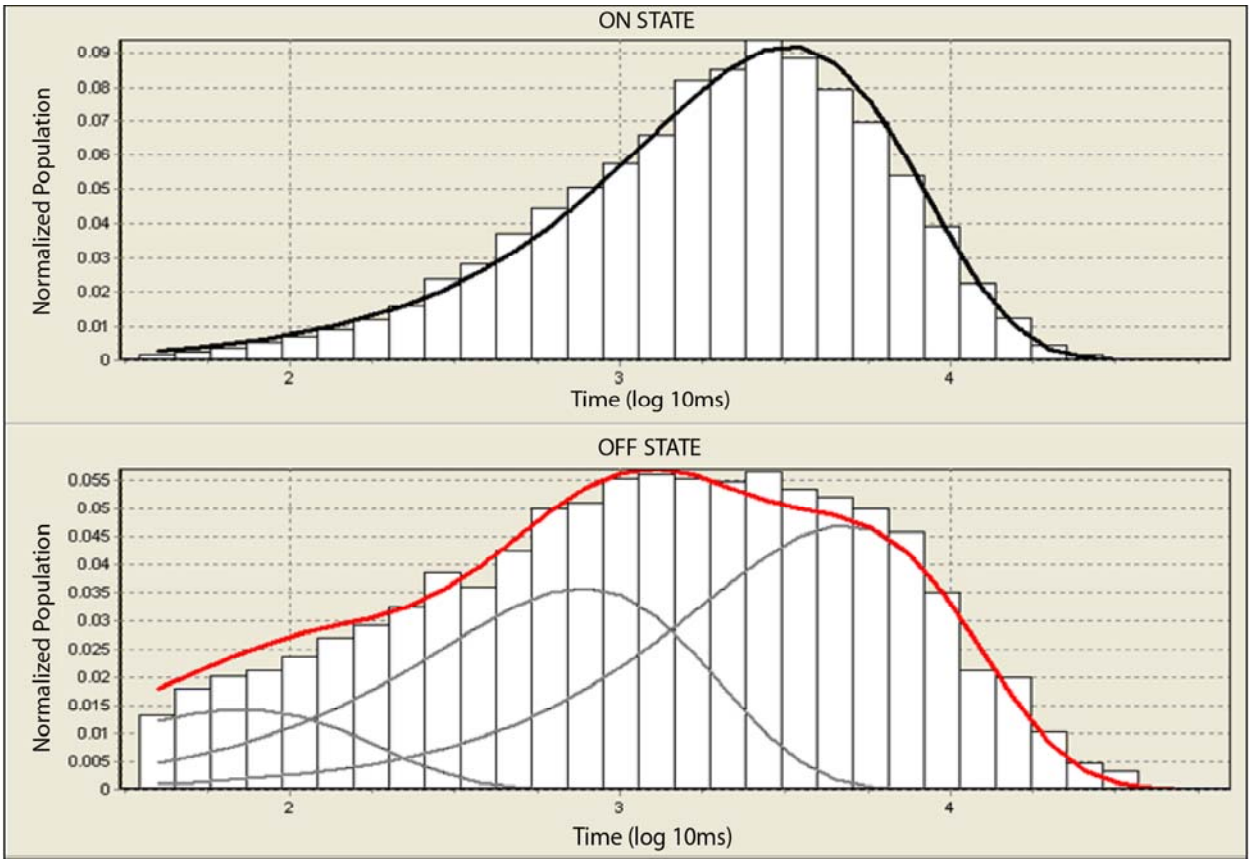
Supplemental Table 5: Protocatechuic acid and Protocatechuate-3,4-dioxygenase (PCD/PCA) oxygen scavenging system can also be used to attenuate dye photophysics in the DNA system. The results obtained indicate that total t_{on} and total t_{off} for the protocatechuic acid and protocatechuate-3,4-dioxygenase (PCD/PCA) (above) and Glucose Oxidase and Catalase (GOD/CAT) oxygen scavenging system **(Tables 1 and 2)** are comparable for the Cy labeled DNA system.

	Total t_{on} (s)	Total t_{off}	Total t_{on} (s)	Total t_{off}
	0.65kW/cm^2		1.95kW/cm^2	
5mM BME	8.7	0.3	4.4	0.1
50mM BME	7.1	0.3	3.8	0.4

Supplemental Table 6: Photophysical processes in the Cy-labeled DNA system are only modestly affected by BME concentration. The dataset was taken in the presence of 1mM COT, 1mM NBA and 1mM Trolox. Modest improvements in total t_{on} and total t_{off} are observed when using lower concentration of BME i.e., 5mM BME.



Supplemental Figure 1: Photophysical processes in Cy5 and Alexa-647N are strongly attenuated under dual 635nm and 532nm illumination in the DNA system by the solution additives Trolox, COT and NBA. The lifetimes of fluorescence, t_{on}, and dark states, t_{off}, are shown as overlaid histograms for both Cy5 and Alexa 647N fluorophores linked DNA oligonucleotides dual illumination in the absence (black), and presence of 2mM COT (dashed red), 2mM NBA (dashed blue), 2mM Trolox (dashed green), and all three additives combined (2mM each) (magenta). Each histogram fit represents a minimum of 724 events where R^2 values were >0.84 for t_{on} and >0.65 for t_{off} with the exception of 2mM NBA and all three additives combined where the R^2 value of t_{off} fit, due to a limited number of observed transitions, was ≤ 0.5.



Supplemental Figure 2: Dwell time analysis reveals the multiexponential nature of photoresurrection rates. Individual dwells for Cy3/5-labeled DNA in the absence of additives were analyzed by maximum log likelihood optimization procedures in QuB. Shown here is a modified screen capture of the QuB interface. The ON STATE (the rate of transition to the dark state) is well defined by single exponential fitting whereas the OFF STATE (the rate of return from the dark state, photoresurrection) is only adequately defined by multiexponential fitting. Here, the OFF STATE is most adequately defined by fitting to a triple exponential process where rates span approximately two orders of magnitude. The histograms shown contain >5000 molecules where similar data was obtained in six independent trials of the experiment. The multiexponential nature of photoresurrection was observed in all experiments, under all conditions tested.

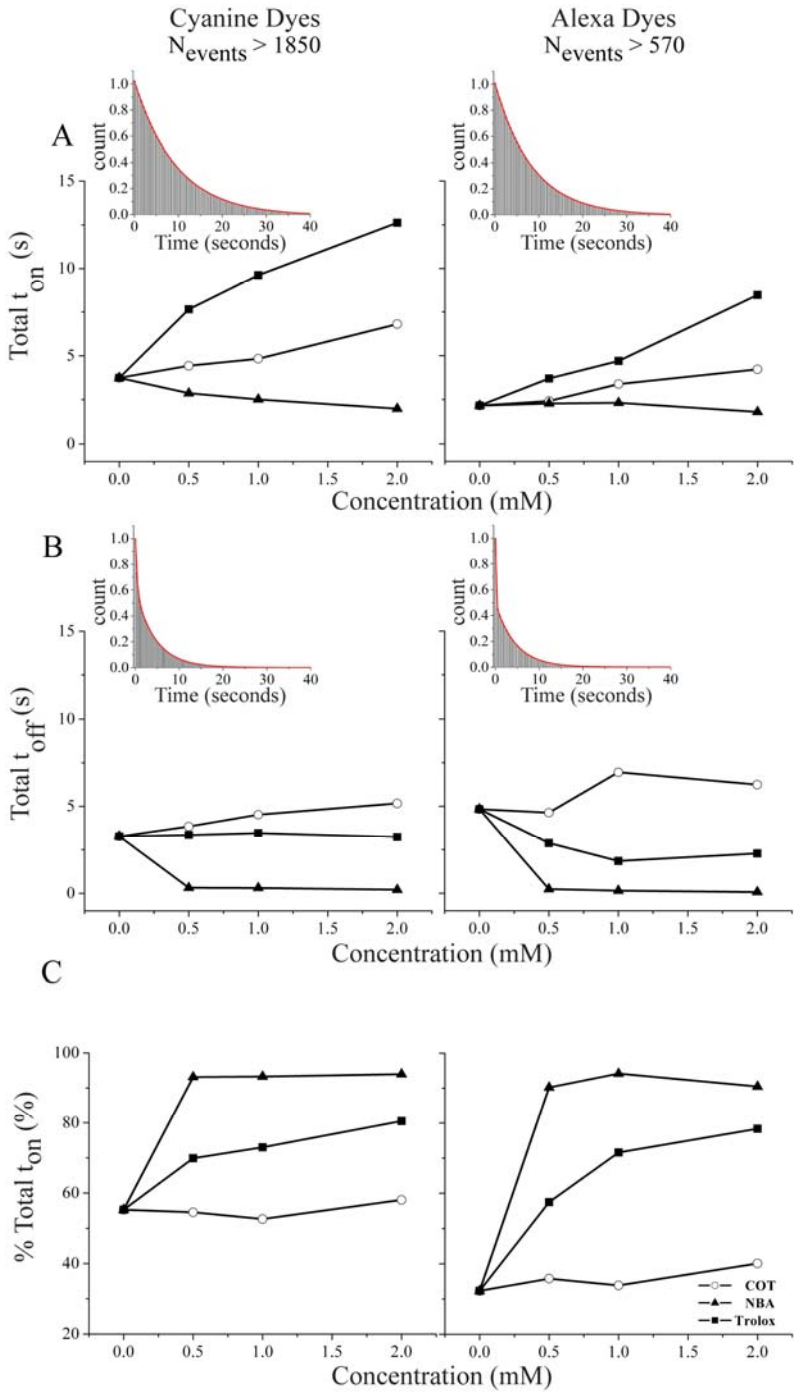
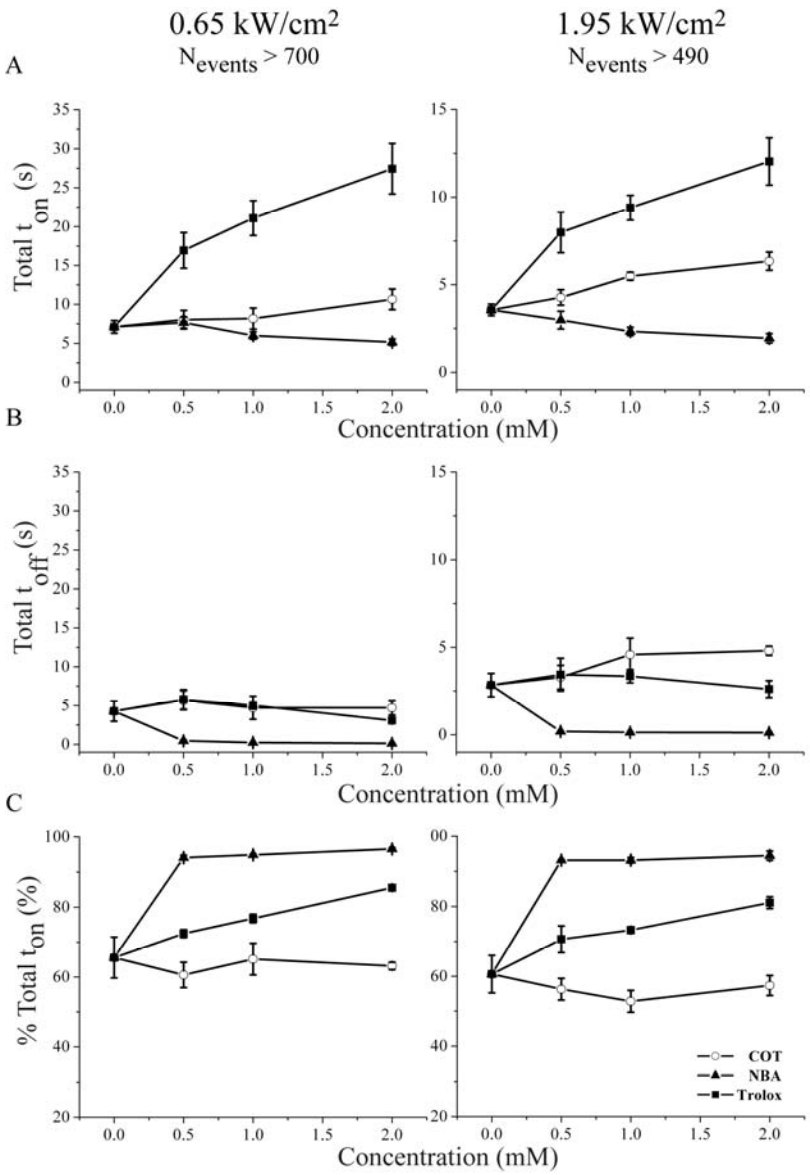


Figure 3: Photophysical processes in Cy5 and Alexa-647N are strongly attenuated in FRET-based experiments performed on the DNA system by the solution additives Trolox, COT and NBA. FRET-based experiments (1.95kW/cm^2 532nm illumination) performed on donor- and acceptor labeled DNA oligonucleotides show that COT (-○- *circles*), NBA (-▲- *triangles*) and Trolox (-■- *squares*) attenuate (*A*) Total t_{on} (*B*) Total t_{off} and (*C*) the percent time in non-zero FRET states % Total t_{on} (total t_{on}/total t_{on} + total t_{off}), in a concentration dependent manner. Inset figures show examples of the single exponential decay fittings of Total t_{on} and double exponential decay fittings of Total t_{off}.



Supplemental Figure 4: Dye photophysical processes can be robustly quantified. The kinetic parameters and error estimates observed for blinking and photoresurrection in Cy-labeled DNA experiments are shown for three separate analyses of independent TSQ titration experiments; COT (-○- *circles*), NBA (-▲- *triangles*) and Trolox (-■- *squares*).