Supporting Information

Reversible fluorescence photoswitching in DNA

*Darren A. Smith,[1] Philipp Holliger,[2] Cristina Flors[1,3]**

[1]EaStChem School of Chemistry, University of Edinburgh, Joseph Black Building, The King's Buildings, West Mains Rd, EH9 3JJ Edinburgh, United Kingdom

[2] MRC Laboratory of Molecular Biology, Hills Road, Cambridge CB2 0QH, United Kingdom

[3] IMDEA Nanociencia, Calle Faraday 9, Ciudad Universitaria de Cantoblanco, 28049 Madrid, Spain

*Corresponding author e-mail: cristina.flors@imdea.org

MATERIALS AND METHODS

Materials

CyDNA was synthesized by polymerase chain reaction (PCR) using a mutant *Pyrococcus furiousus* (Pfu) polymerase, E10, as previously described.[1] Samples were prepared in 1x ThermoPol buffer (New England Biolabs) and 1% (v/v) formamide. Plasmid (1 μg) from *Thermococcus gorgonarius*, pASKTgoT, was used as template. Primers (0.4 μg) were 5'-ACC ACC GAA CTG CGG GTG ACG CCA AG-3' and 5'-GCT GAG GAA GGC CTA CGA GAG-3'. Increasing labeling density in Cy5DNA was achieved by increasing the concentration of Cy5-dCTP in the PCR mix as follows (Table S1):

Sample name	[Cy5-dCTP] / μM	[dCTP] / μM	[d(A,T,G)TPs] / μM
Sample 1	2	8	10
Sample 2	5	5	10
Sample 3	25	25	50

It is worth noting that "Sample 2" and "Sample 3" result from PCR mixtures with the same ratio of labeled vs unlabeled dCTP. However the incorporation of Cy5-dCTP into CyDNA (labeling density) is higher in "Sample 3", as seen in Figure 1A. The exact reason for this is unknown, but this observation reflects that the relation between incorporation efficiency of the enzyme vs concentration in the PCR mixture is not the same for dCTP and Cy5-dCTP.

Cy3DNA was prepared using the most concentrated Cy3-dCTP conditions as in the above table ("Sample 3", 25 μM). Labeled nucleotides were purchased from GE Healthcare, and unlabeled ones from New England Biolabs. HPLC water was used to give a total reaction volume of 100 μl in all cases. PCRs were performed in a thermal cycler (Mastercycler, Eppendorf). Reaction conditions were as follows: 94°C for 2 minutes, then 50 cycles of (94°C for 10 s, 55 °C for 1 minute and 72 °C for 20 minutes). PCR products were purified by ethanol precipitation (with 2.0x volume ethanol and 0.1x volume sodium acetate pH 5.2). Samples were re-dissolved in Tris-EDTA buffer and further purified with an S-400 column (GE Healthcare). PCR product size was confirmed by agarose gel electrophoresis. The amplified fragment

(1.3 kb) had 50% GC content, and therefore the maximum ratio of labeled base pairs that could be achieved using the above conditions was 25%.

Cy3/5DNA heteroduplex was assembled by mixing 1:1 solutions of Cy3DNA and Cy5DNA (conditions as in "Sample 3", Table S1, corresponding to a maximum achievable labeling density of 25% of total base pairs), heating to 94 ºC and cooling to room temperature at 1 ºC/min. This procedure yields a mixture with 2:1:1 of heteroduplex : pure Cy5DNA : pure Cy3DNA (which reflects the four possible permutations of dsDNA created from two duplex Cy5DNA and two duplex Cy3DNA of the same sequence, and assuming that hybridization efficiency is independent of dye type). Although the 532 nm laser (pulsed at 0.2 Hz) can excite Cy3DNA, the filter combination we use precludes the detection of its fluorescence by the camera (we are detecting in the Cy5 spectral region, see below), and in practice it can be considered as a 2:1 mixture of heteroduplex : pure Cy5DNA. It is worth noting that the presence of pure Cy5DNA will result as an offset in Figure 2A. The same mixture above is used in the OLID experiments in Figure 4. Indeed, we can roughly observe that there are 2/3 of photoswitchable molecules and 1/3 of non-photoswitchable molecules, as expected from the mixture composition.
Cy*DNA was prepared using 10 μM d(A,T,G)TP, 5 μM dCTP, 2.5 μM of Cy3-dCTP and 2.5 μM of Cy5-dCTP, equivalent to an estimated labeling density of 25% of the total base pairs.

The "switching buffer" contained 10 mM phosphate buffered saline (PBS, pH 7.4, Sigma P3813) with an oxygen scavenger (0.5 mg/ml glucose oxidase (Sigma), 40 μg/ml catalase (Sigma) and 10% w/v glucose (Fischer Scientific)) and 50 mM β-mercaptoethylamine (MEA, Fluka).

Steady-state spectroscopy

Absorption spectra were carried out in a Cary 50 Bio UV-Vis spectrophotometer (Varian). Corrected excitation and emission spectra were recorded on a FluoroMax spectrophotometer (Horiba Jobin Yvon), with 0.2 s integration time and 1 nm step size.

Single molecule and OLID microscopy

For microscopy experiments, an aliquot of a CyDNA or Cy5-dCTP (~0.4 ng/µl) solution (20 µl) was added to the centre of a polylysine-coated coverglass. After binding for 20-30 s, the sample was rinsed with water and the coverglass was dried with compressed air. The coverglass was then attached to a CoverWell imaging chamber (Grace Bio Labs), which contained switching buffer.

Fluorescence imaging was performed on a Nikon Eclipse TE2000 inverted microscope, equipped with a total internal reflection fluorescence (TIRF) oil-immersion objective (Apochromat, 60x, NA 1.49, Nikon). Excitation was provided by a 633 nm He/Ne CW laser (Coherent model #31-2140-000, 17 mW at the sample) passing through appropriate bandpass filters (Chroma Technology). Fluorescence photoactivation was achieved with a 532 nm DPPS CW laser (Cobolt Samba), passing through an electronic shutter (Newport) controlled by a function generator (USB-6218, National Instruments), resulting in pulses of 0.2 Hz, 50 ms and 0.4 mW. Wide-field illumination was achieved by focusing the expanded and collimated laser beam onto the back-focal plane of the objective. The resulting illuminated area was roughly 60 µm in diameter. Emission was collected by the same objective and imaged by an Andor Luca(S) EMCCD camera after passing through a dichroic mirror (z633rdc, Chroma Technology) and additional spectral filters (HQ645LP and HQ700/75, Chroma Technology). Additional lenses resulted in a final pixel size of 74 nm. Integration time per frame was 100 ms, and the total number of frames collected was typically 200 for OLID and 1000 for PALM imaging.

Data in Figure 2 were generated by localizing and counting the total number of fluorescent molecules in each frame with the localization microscopy toolkit *Localizer* (http://www.igorexchange.com/project/Localizer). *Localizer* was also used to analyze the PALM super-resolution images shown in Figure S2.

OLID analysis was based extensively on the methodology outlined by Marriott *et al.*[2] For every pixel in the image, a correlation coefficient was calculated via cross-

correlation analysis of the pixel trace and a reference waveform. The reference waveform used characterized the photoswitching behavior which was to be amplified in the image. In this case, the waveform followed intensity changes which are associated with photoswitching an ensemble of dyes, specifically, a rapid increase in intensity followed by decay back to equilibrium – see Figure S3. The reference waveform could be created in a number of different ways. Marriott *et al.* utilized the intensity trace of a small region in the image which was known to have a high concentration of photoswitchable molecules in it. In this study, the reference waveform was constructed by using the average intensity of the whole image in each frame. This gave a statistically reliable trace and was possible because the sample contained a relatively high proportion of photoswitchable molecules (meaning that photoactivating pulses caused a discernible change in average intensity). It was also possible to use a trace of the number of fluorescent molecules in each frame as the reference waveform.

The correlation coefficient, ρ, for a pixel at position (x,y) in the image, was calculated via

$$\rho(x,y) = \sum_{t=1}^{N} \frac{[I(x,y,t) - \mu_{I(x,y)}][R(t) - \mu_R]}{\sigma_{I(x,y)} \cdot \sigma_R}$$

N is the total number of frames, t is time (in frames), I(x,y,t) is the pixel intensity at time t, R(t) is the reference waveform intensity at time t, $\mu_{I(x,y)}$ and μ_R are mean intensity values and $\sigma_{I(x,y)}$ and σ_R are standard deviation values for the pixel and reference waveform, respectively.

Mean values were calculated via

$$\mu_\alpha = \frac{1}{N} \sum_{t=1}^{N} \alpha(t)$$

Standard deviation values were calculated via

$$\sigma_\alpha = \sqrt{\frac{1}{N-1} \cdot \sum_{t=1}^{N} [\alpha(t) - \mu_\alpha]^2}$$

Negative correlation coefficients (which represented anti-correlation) were set to zero and positive values were normalized by N-1 so that a trace identical (within a scaling

factor) to the reference waveform had a correlation coefficient of 1. It is important to realize that correlation coefficients are intensity-independent. This is evident in Figure 4C (main text) which shows great enhancement of relatively weak Cy3/5DNA molecules (S1, S2) and diminishment of the more intense, but uncorrelated, Cy5DNA molecules (N1, N2) with OLID analysis.

OLID correlation images were created with Java (version jdk1.6.0_23) and then visualized using ImageJ (version 1.44p).

SUPPLEMENTARY FIGURES

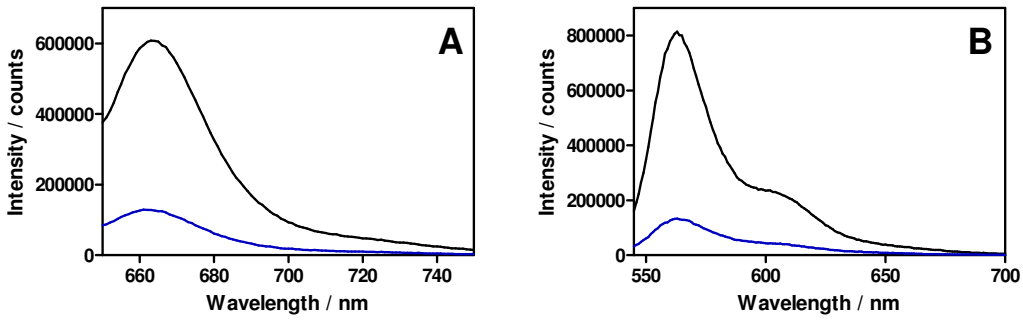

Figure S1. Fluorescence quenching in CyDNA. (A) Emission spectra at λ_{exc} = 644 nm of free Cy5-dCTP (black) and Cy5DNA (blue) optically matched solutions at 644 nm. (B) Emission spectra at λ_{exc} = 532 nm of optically matched solutions of free Cy3-dCTP (black) and Cy3DNA (blue).

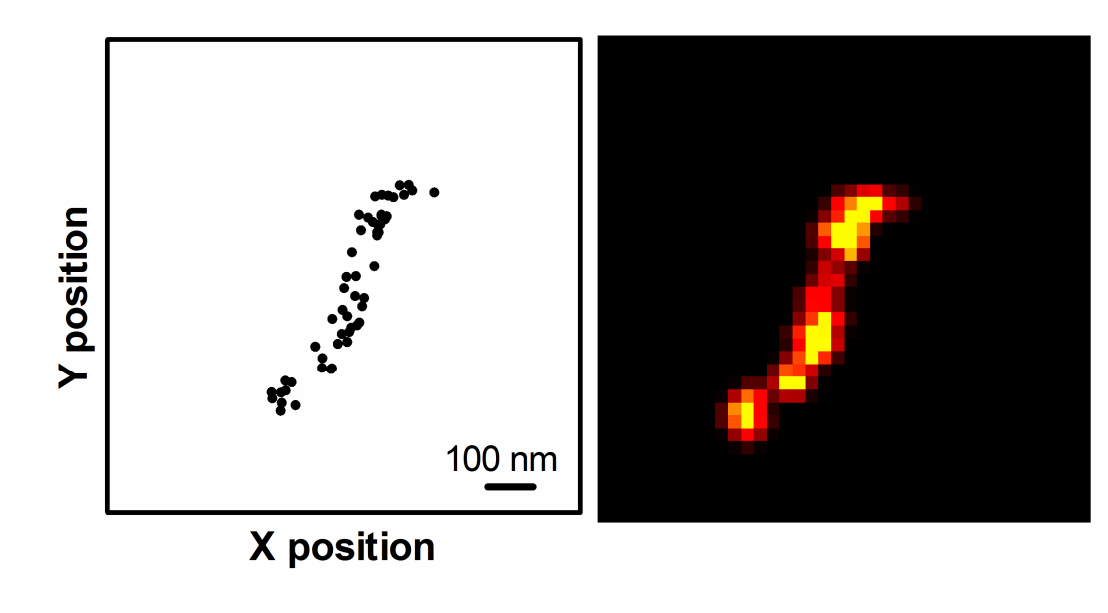

Figure S2. The structure of a 1.3 kb fragment of Cy3/5DNA can be resolved by single-molecule photoswitching and localization. Raw localization data (left) and PALM reconstructed image (right).

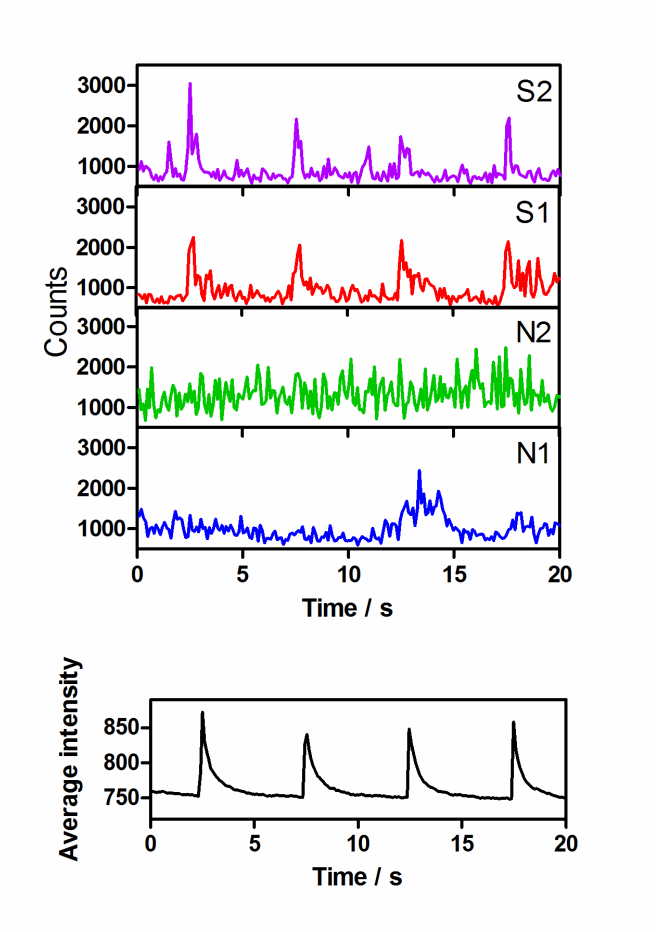

Figure S3. Above: pixel values for molecules in Figure 4 (main text). Below: reference waveform for OLID imaging. Each pixel time trace was cross-correlated with the reference waveform to obtain a correlation coefficient. A correlation image was then built up using these calculated coefficients (Figure 4B and C in the main text).

(1) Ramsay, N.; Jemth, A. S.; Brown, A.; Crampton, N.; Dear, P.; Holliger, P. *J Am Chem Soc* **2010**, *132*, 5096-104.

(2) Marriott, G.; Mao, S.; Sakata, T.; Ran, J.; Jackson, D. K.; Petchprayoon, C.; Gomez, T. J.; Warp, E.; Tulyathan, O.; Aaron, H. L.; Isacoff, E. Y.; Yan, Y. L., Proc Natl Acad Sci U S A 2008, 105 (46), 17789-17794.