Supporting information for the manuscript:

Sensing DNA opening in transcription
using quenchable FRET

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ADDITIONAL EXPERIMENTAL RESULTS AND SUPPLEMENTAL FIGURES

18-bp standard:

\[
\text{Cy3B-5'}^1\text{-TTATCTAAGTTACAACAACAGTAACTTACGTCCATTCGCG-3'}^1
\]

\[
\text{ATTO647N}
\]

Figure S1. Oligonucleotide sequence of dsDNA with 18-bp separation for dye pair Cy3B/ATTO647N.

Figure S2. Functional RNAP assay using radioactivity: Comparison between *in vitro* transcription product profiles when using an unlabeled (lanes 1 and 2) and labeled (lanes 3 and 4) dsDNA template (lacrCy3B,-5/ATTO647N,-3) in the presence of...
0.3μCi/μl α-32P GTP: lanes 1&3 – full transcript, lanes 2&4 – 7 nt-long transcript corresponding to the RP<sub>inc,7</sub> reaction. In designating the mRNA products, the letter corresponds to the 3'-most base of the transcript (G, A, U), while the number indicates its length (5-8, 11, 13, 14-18). RO stands for run-off.

Figure S3. Photophysical control experiments for Cy3B/ATTO647N on an 18bp standard DNA: ALEX-based $E^*$ histograms of single diffusing molecules (Cy3B/ATTO647N fluorophore pair on dsDNA with 18-bp separation between the two dyes) with an apparent FRET of $E^* \approx 0.5$. The DNA sequence is found in Figure S1. Measurement time was 20 min for each panel.

Figure S4. PCH analyzing quFRET: Photon counting histograms for different states of dsDNA (lac<sup>Cy3B,-5/ATTO647N,-3</sup>) for (a) donor-excitation donor-detection $D_{ex}D_{em}$, (b) acceptor-excitation acceptor-detection $A_{ex}A_{em}$, and (c) donor-excitation acceptor-detection $D_{ex}A_{em}$. Please note that the histograms only incorporate bursts with an acceptor present and have an additional per-bin threshold of $A_{ex}A_{em} > 30$. 


Figure S5. Analysis of the quFRET principle including all photons: The figure shows the same experimental results from ALEX-based smFRET as in Figure 1 (main text). One-dimensional FRET histograms were obtained by selecting all molecules in the lower panels with intermediate S (0.45 < S < 0.8). Both experiments were conducted for 20 minutes at 37°C at a concentration of 50-100 pM. Bursts corresponding to a single-molecule were identified using parameters M = 12, T = 500 µs and L = 30 and an additional per-bin threshold for all photons > 100.

Figure S6. quFRET with lac\textsuperscript{Cy3B,-3/ATTO647N,-3}: ALEX-based $E^*$ histograms of single diffusing molecules of transcription complexes formed using lac\textsuperscript{Cy3B,-3/ATTO647N,-3} DNA at 37°C and at a concentration of ≈50-100 pM of DNA. The figure
shows the number of events of the apparent FRET $E^*$ for different buffer conditions (100 bins). Presence of different nucleotides in the buffer is indicated in the different panels. Measurement times were ≈60 min for each panel.

Figure S7. Absorption spectra of Cy3B and lac$_{Cy3B,-3}$ATTO647N,-3: a) Absorption spectra of free Cy3B: data points in grey, smoothed data as a black line. b) Absorption spectra of Cy3B in lac$_{Cy3B,-3}$ATTO647N,-3: data points in grey, smoothed data as a black line. Note that the absorption of ATTO647N (maximum ≈650) in lac$_{Cy3B,-3}$ATTO647N,-3 is not easily observable due to the low concentrations of the sample and due to possible changes in the absorbance spectrum of ATTO647N when participating in quFRET.