The Transcription Bubble of the RNA Polymerase–Promoter Open Complex Exhibits Conformational Heterogeneity and Millisecond-Scale Dynamics: Implications for Transcription Start-Site Selection

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Abstract

Bacterial transcription is initiated after RNA polymerase (RNAP) binds to promoter DNA, melts ~ 14 bp around the transcription start site and forms a single-stranded “transcription bubble” within a catalytically active RNAP–DNA open complex (RPo). There is significant flexibility in the transcription start site, which causes variable spacing between the promoter elements and the start site; this in turn causes differences in the length and sequence at the 5′ end of RNA transcripts and can be important for gene regulation. The start-site variability also implies the presence of some flexibility in the positioning of the DNA relative to the RNAP active site in RPo. The flexibility may occur in the positioning of the transcription bubble prior to RNA synthesis and may reflect bubble expansion (“scrunching”) or bubble contraction (“unscrunching”). Here, we assess the presence of dynamic flexibility in RPo with single-molecule FRET (Förster resonance energy transfer). We obtain experimental evidence for dynamic flexibility in RPo using different FRET rulers and labeling positions. An analysis of FRET distributions of RPo using burst variance analysis reveals conformational fluctuations in RPo in the millisecond timescale. Further experiments using subsets of nucleotides and DNA mutations allowed us to reprogram the transcription start sites, in a way that can be described by repositioning of the single-stranded transcription bubble relative to the RNAP active site within RPo. Our study marks the first experimental observation of conformational dynamics in the transcription bubble of RPo and indicates that DNA dynamics within the bubble affect the search for transcription start sites.

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Introduction

Transcription, the synthesis of RNA from a DNA template, is the first step in gene expression and is a highly regulated process. In Escherichia coli and other bacteria, RNA polymerase (RNAP) initiates transcription after binding to specific sequences within promoter DNA, where binding is controlled by transcription initiation factors known as sigma (σ) factors. In typical bacterial promoters controlled by the main sigma factor σ70, the RNAP-σ70 holoenzyme initially binds to the −10 and −35 elements of the promoter (reviewed in Ref. 1), melts ~14 bp in the DNA surrounding the transcription start site to form a single-stranded “transcription bubble” and yields the catalytically active RNAP–DNA open complex (RPo).
RNAP can initiate transcription from multiple positions within the same promoter, in both prokaryotes and eukaryotes. In bacteria, transcription is mainly initiated by purines located within a region 4–12 bp downstream of the −10 element, which extends from position −7 to −12 relative to the +1 site. Such preferences for start sites can be used for regulation of gene expression, since variation at the 5′ ends of transcripts can affect transcript stability or control the formation of secondary structures that in turn can affect translational initiation. For example, expression of the pyrC gene in E. coli is regulated by a translational control mechanism dependent on the presence or absence of a 5′ hairpin loop structure whose synthesis depends on start-site selection. In addition, different start sites can affect the extent of abortive initiation or transcriptional slippage, both of which can influence the frequency of initiation at a particular promoter.

Flexibility in transcription start sites has also been observed in eukaryotes. An early study identified heterogeneity in the 5′ termini of adenovirus mRNAs, which are transcribed by cellular RNAP II. In yeast, RNAP II initiates transcription at multiple start sites located 40–120 bp downstream of the TATA box, presumably by actively scanning (through an ATP-driven process) for start sites downstream of the transcription bubble, as in the case of the U4 small nuclear RNA gene SNR14. The observed flexibility in transcription start sites implies that there must be static or dynamic flexibility in the positioning of the single-stranded transcription bubble relative to the RNAP active center in RPo (Fig. 1a). The discoveries that initial transcription by RNAP involves transcription-bubble expansion (“scrunching”) and that promoter escape by RNAP involves transcription-bubble contraction (“unscrunching”) provide precedents for the functional importance of transcription-bubble flexibility. The discovery of scrunching and unscrunching also supported a mechanistic model for the role of transcription-bubble flexibility in start-site selection: namely, transcription-bubble expansion (similar to scrunching) in RPo would place further downstream DNA in contact with the RNAP active center, resulting in a more downstream start site, and transcription-bubble contraction (similar to unscrunching) in RPo would place more upstream DNA in contact with the RNAP active center, resulting in a more upstream start site.

In this work, we studied the mechanisms for start-site heterogeneity by examining the E. coli lac promoter. Specifically, we have performed single-molecule FRET ( Förster resonance energy transfer) measurements on individual, freely
Results

Start-site selection at lacCONS+2 and lacCONS+2 derivatives

The lacCONS+2 promoter used in this work is a derivative of the E. coli lacUV5 promoter; lacCONS+2 differs from the lacUV5 promoter by having a single-base-pair substitution in the −35 element (to form a consensus −35 element), a single-base-pair deletion in the spacer region between the −35 and −10 elements (to form a consensus −10/−35 spacer region) and a 2-bp insertion at position +9 in the initial transcribed region. We examined the distribution of transcription start sites at lacCONS+2 and at three lacCONS+2 derivatives containing substitutions in the start-site region (Fig. 2). As anticipated from previous work on the start sites at the lac promoter and substituted lac promoter derivatives, lacCONS+2 exhibited a major start site at position +1, and substituted lacCONS+2 derivatives exhibited different distributions of start sites (Fig. 2). Specifically, lacCONS+2(T−3A) exhibited start sites at −2, +1, +2 and +3, while lacCONS+2(A+2C) exhibited a start site at +1 and lacCONS+2(G−2T;A+1C) exhibited start sites at −1 and at +2.

Transcription-bubble flexibility in RP0: smFRET between DNA segments upstream and downstream of the bubble

Having confirmed that RNAP can initiate transcription from multiple sites on the lacCONS+2 derivatives, we used smFRET to investigate the dynamics of RP0. Experiments were conducted using lacCONS+2(T−3A) (Fig. 2b) labeled with the donor fluorophore Cy3B and the acceptor fluorophore ATTO647N in a variety of positions (for DNA sequences and labeling positions, see Supplementary Fig. 1). In a first set of experiments, the FRET ruler (“downstream DNA ruler”) monitored the distance between DNA segments upstream and downstream of the transcription bubble, by monitoring FRET between an acceptor fluorophore at position +15 and a donor fluorophore at position −15 (Fig. 3a, top panel; see Ref. 24). This FRET ruler detects RP0 formation through an increase in FRET due to opening of the transcription bubble (Fig. 3a). smFRET spectroscopy with ALEX (alternating-laser excitation)30,31 on diffusing molecules of double-stranded DNA (dsDNA) and RP0 resulted in FRET histograms for molecules containing both donor and acceptor fluorophores (Fig. 3a; see Materials and Methods for details).

The FRET distribution of free dsDNA showed a single species with low apparent FRET (mean value of $E^* \sim 0.23$; Fig. 3a). As expected,24 addition of RNAP to DNA to form RP0 resulted in a bimodal distribution that represents two species: dsDNA (due to dissociation of nonspecific RNAP–DNA complexes during heparin challenge; see Materials and Methods) and RP0 (Fig. 3a, third panel). The DNA distribution was centered at $E^* \sim 0.23$, whereas the RP0 distribution was centered at $E^* \sim 0.39$. The FRET distribution of RP0 was also unusually wide, both relative to dsDNA (which serves as a “static” standard; Fig. 3a, third panel) and to the expected width for a static species with a known mean FRET efficiency and photon count distribution (“shot-noise-limited” width); the latter is due to the low photon counts inherent to single-molecule fluorescence measurements.32 The calculated shot-noise-limited width for the dsDNA peak ($\sigma = 0.038$; Fig. 3a, red Gaussian, first panel) was comparable with the actual width of the FRET distribution ($\sigma = 0.041$); in contrast, the width for the RP0 peak ($\sigma = 0.085$; Fig. 3a, black Gaussian fit, third panel) substantially exceeded its shot-noise width ($\sigma = 0.044$; Fig. 3a, red Gaussian, third panel), pointing to the presence of heterogeneity in the FRET distribution.

A possible explanation for the wide distribution of the RP0 FRET peak is the presence of conformational heterogeneity in RP0.26,33,34 which may exist in multiple static conformational states that do not interconvert within the millisecond-timescale transit of single RP0 complexes through the detection volume. Alternatively, RP0 may be dynamic and interconvert between different conformational states within the millisecond timescale.26 To deconvolve static from dynamic heterogeneity, we analyzed the FRET data using burst variance analysis (BVA).27 which detects dynamics by examining how FRET efficiency fluctuates over time within single transits of individual molecules. Essentially, molecules with
dynamic fluctuations in FRET are characterized by an increased FRET standard deviation compared to that expected from shot noise; BVA compares the experimentally observed standard deviations with those expected for static limit (i.e., the expected standard deviation at a certain FRET value), thus providing information on the source and timescale of any dynamics.

As expected, BVA showed that the experimental standard deviations for free dsDNA (black triangles in Fig. 3a, second panel) are close to the static limit (black continuous arc in Fig. 3a, second panel). In contrast, BVA for RPo suggests dynamic behavior as the values deviate significantly from the shot-noise expectation curve (Fig. 3a, bottom panel). In this sample, the free dsDNA population acts as an internal control, remaining close to the static limit. These results are consistent with the hypothesis that the DNA within RPo has a dynamic component; the results further suggest that RPo can interconvert between multiple conformational states within the 0.1- to 5-ms timescale, wherein BVA is sensitive to FRET fluctuations.27

Fig. 2. Start-site selection at lacCONS+2 and lacCONS+2 derivatives. (a) In vitro transcription reactions using lacCONS+2 promoters with base-pair substitutions; RNAP, dsDNA, ATP, UTP, CTP and [α32P]GTP were incubated in transcription buffer at 37 °C for 5 min followed by heparin challenge and separation of the products on a polyacrylamide gel. Labeled RNA standards were used to determine the size of the products. (b) Sequences of the lacCONS+2 promoter and its derivatives. Boxes are drawn around the −10 and −35 elements and the +1 position is marked. The primary RNA products observed for each sequence are labeled in green.

Transcription-bubble flexibility in RPo: smFRET between nontemplate and template strands of the bubble

In a second set of experiments, the FRET ruler ("bubble DNA ruler") monitored the distance between the nontemplate strand and template strand of the transcription bubble, by monitoring FRET between a
fluorophore at position −5 of the nontemplate strand and a fluorophore at position −3 of the template strand (Fig. 3b, top panel). This labeling scheme has been used previously and is based on contact-mediated quenching of the two fluorophores in dsDNA followed by removal of the quenching and appearance of FRET in RPo due to the separation of the two DNA strands in the transcription bubble. This labeling scheme allows the detection of RPo formation with no background from free dsDNA, as the latter is not visible due to the contact-mediated quenching. As a result, free dsDNA appears as a broad, unstructured and sparsely populated FRET distribution (Fig. 3b, top panel). Upon formation of the transcription bubble in RPo, the fluorophores were separated to remove the contact-induced quenching and produced a high FRET distribution with mean $E^* \approx 0.72$ (Fig. 3b, third panel) and a width ($\sigma = 0.091$; Fig. 3b, black Gaussian fit, third panel) that substantially exceeds the expected shot-noise width ($\sigma = 0.057$; Fig. 3b, red Gaussian, third panel), confirming the presence of heterogeneity within RPo. BVA analysis showed that the mean FRET standard deviation values of RPo deviated from the static limit curve (Fig. 3b, fourth panel), consistent with the downstream ruler results that identified a dynamic component in the FRET heterogeneity.

Fig. 3. Transcription-bubble flexibility in RPo. (a) Top panel: dsDNA, lacCONS+2(T−3A), labeled with donor and acceptor fluorophores at positions −15 and +15, respectively (with respect to the +1 position), was analyzed using smFRET. A FRET histogram was derived from the mean values of (at least) triplicate experiments. Sizable FRET distributions were fitted with a Gaussian function (black curve) to determine the center and width of the distribution. The calculated shot-noise-limited width is shown as a red Gaussian fit. Second panel: BVA of the FRET distribution of the dsDNA. Black arc represents static limit, colored contour plots represent frequency distributions (red contour, most abundant region; blue, less abundant) and triangles represent the standard deviation of a particular part of the two-dimensional histogram of the experimental data. Third panel: samples containing dsDNA and RPo analyzed using smFRET. Fourth panel: samples containing dsDNA and RPo analyzed using BVA. (b) Top and second panels: dsDNA labeled with donor and acceptor fluorophores at positions −5 and −3, respectively, was analyzed by smFRET and BVA. Third and fourth panels: samples containing dsDNA and RPo analyzed by smFRET and BVA, respectively. (c) Top and second panels: dsDNA labeled with donor and acceptor fluorophores at positions −25 and −15, respectively, was analyzed by smFRET and BVA. Third and fourth panels: samples containing dsDNA and RPo analyzed by smFRET and BVA, respectively.
Transcription-bubble flexibility in RPo: smFRET between positions within upstream dsDNA

In a third set of control experiments, the FRET ruler (“control ruler”) monitored the distance between positions within upstream dsDNA, by monitoring FRET between a fluorophore at position −15 of the non-template strand and a fluorophore at position −25 of the template strand. Since there are no known or suggested dynamics associated with this region of DNA in RPo, we reasoned that the control FRET ruler would exhibit a static behavior in RPo.

The apparent FRET distribution for free dsDNA was centered at mean $E^* \sim 0.85$ (Fig. 3c, top panel), whereas RPo formation led to a small decrease in the mean FRET value ($E^* \sim 0.83$; Fig. 3c, third panel). To verify that the FRET histogram for RPo actually represented a substantial amount of complex formation (typically defined as having 40–60% of the DNA being involved in RPo formation), we used fluorescence correlation spectroscopy to show that the diffusion times increased as expected for formation of RPo (Supplementary Fig. 2). The widths of the FRET distributions of free dsDNA and of RPo were similar ($\sigma = 0.047$ for dsDNA and $\sigma = 0.055$ for RPo; Fig. 3c, black Gaussian fits in the first and third panels), and both distributions were close to their expected shot-noise widths ($\sigma = 0.041$ for dsDNA and $\sigma = 0.042$ for RPo; Fig. 3c, red Gaussians in the first and third panels). We note that the decrease in the mean FRET efficiency between free dsDNA and RPo (from 0.85 to 0.83) suggests that part of the small increase in the FRET width (from 0.042 to 0.055) arises from the inability to resolve the free DNA and RPo distributions and, thus, is static in nature. This assessment is supported by the BVA analysis, which shows that the FRET standard deviation values for both free dsDNA and for RPo remain close to the static limit (Fig. 3c, second and fourth panels). These results suggest that the dynamic behavior observed for RPo using the downstream and bubble FRET rulers is specific to the DNA within the transcription bubble.

Reprogramming start-site selection changes the distance between DNA segments upstream and downstream of the bubble

We subsequently examined the relationship between reprogramming of start-site selection and repositioning of transcription-bubble DNA within RPo. To assess the ability of initiating nucleotides to reposition transcription-bubble DNA within RPo, we used a “downstream ruler” based on the lacCONS+2(G−2T; A+1C) promoter, which initiates transcription at positions −1 and +2 (confirmed by in vitro transcription assays; Fig. 2). We used this ruler to study the effect of the addition of initiating

![Fig. 4](attachment:image.png)

**Fig. 4.** Start-site reprogramming changes the distance between DNA segments upstream and downstream of the transcription bubble. (a) Reprogramming by addition of initiating nucleotides. A lacCONS+2(G−2T; A+1C) promoter DNA fragment that can initiate transcription at positions −1 and +2 was mixed with RNAP to form open complexes (RPo). The dsDNA promoter was labeled with fluorophores at positions −15 and +15 on either side of the transcription bubble. FRET histograms of RPo alone (gray histogram), RPo with GTP (blue histogram) and RPo with ATP (green histogram) were overlaid (top panel). The difference between the FRET histograms of (RPo +GTP) and (RPo +ATP) was calculated (bottom panel). Both histograms were normalized to the area of the Gaussian fit function of the RPo distribution. (b) Reprogramming by base-pair substitutions in the start-site region. A lacCONS+2(T−3A) promoter DNA fragment that initiates transcription at a range of positions from −2 to +3 was incubated with RNAP to form open complexes; these complexes were compared to open complexes formed using a lacCONS+2 promoter DNA fragment that initiates transcription from the +1 position. Both promoter fragments were labeled with fluorophores at positions −15 and +15 on either side of the transcription bubble. FRET histograms of RPo of the lacCONS+2 promoter (top panel) and lacCONS+2(T−3A) promoter (lower panel) were compared.
nucleotides that are complementary to, and therefore that are expected to favor, different start sites. The FRET distribution of RPo in the absence of nucleotides was centered at $E^* = -0.43$ (Fig. 4a, top panel, gray histogram). Similar to the RPo FRET curve for the lacCONS+2(T–3A) promoter (Fig. 3), the RPo FRET curve for lacCONS+2(G–2T;A+1C) was significantly wider than the static DNA curve, suggesting heterogeneity in RPo. Upon addition of the initiating nucleotide GTP, which is complementary to position −1, the FRET distribution shifted to slightly lower FRET values (Fig. 4a, top panel, blue histogram). In contrast, upon addition of the initiating nucleotide ATP, which is complementary to position +2 and that therefore is expected to favor a start site at position −1, the FRET distribution shifts to slightly higher FRET values upon ATP addition (Fig. 4a, top panel, green histogram). The nucleotide-dependent FRET differences are small but reproducible; a difference histogram relating the FRET distributions for RPo +GTP and RPo +ATP shows the FRET differences more clearly (Fig. 4a, lower panel). Analysis of a structural model of RPo along with estimates of the positions of the donor and acceptor fluorophores (Supplementary Fig. 3) suggests that the magnitude of the observed FRET changes (from a mean $E^* = -0.44$ for RPo +ATP to 0.42 for RPo +GTP) are consistent with a small change in dye positions due to a 2-bp difference in start-site selection (from a mean donor–acceptor distance of ~72 Å for RPo +ATP to ~76 Å for RPo +GTP) and the corresponding changes in translocational register of transcription-bubble DNA relative to the RNAP active site.

According to our model, it may be expected that NTP binding favors a single state of RPo, thereby making the histograms for RPo +ATP/GTP more static and thus narrower. While the dsDNA peak in Fig. 4a always has a mean $\sigma$ value of 0.03, the widths of the RPo histograms upon nucleotide addition change slightly (for RPo, $\sigma = 0.08$; RPo +ATP, $\sigma = 0.085$; RPo +GTP, $\sigma = 0.09$). Therefore, although slight changes are observed, we do not see a decrease in the width of the FRET distributions. This may reflect the fact that the NTP concentration is not fully saturating for binding to RPo; however, even at saturating NTP concentrations, it is entirely possible that the NTP-bound state is still dynamic, albeit biased for the NTP-based −1 or +2 translocation register, and hence, the observed widths of the histograms in our experiments may indeed represent true heterogeneity.

We also investigated the effects of reprogramming of start-site selection by base-pair substitutions in the start-site region. For this, we compared downstream rulers based on lacCONS+2 (major start site at +1; Fig. 2) and lacCONS+2(T–3A) (start sites at −2, +1, +2 and +3; Fig. 2). We found that, whereas the FRET distribution for lacCONS+2 RPo was centered at $E^* = -0.45$ (Fig. 4b, top panel), the FRET distribution for lacCONS+2(T–3A) RPo was shifted to a lower FRET efficiency (mean of $E^* = -0.42$; Fig. 4b, lower panel). We note that this change is, in fact, more significant than the mean change in $E^*$ suggests due to the asymmetry of the lacCONS+2 RPo FRET peak. We interpret this shift as reflecting a change in start-site utilization between the two promoters due to a corresponding shift in translocational register of transcription-bubble DNA relative to the RNAP active site.

Discussion

Using smFRET techniques, we have obtained evidence for the presence of DNA conformational heterogeneity and millisecond-timescale DNA conformational dynamics within the single-stranded transcription bubble of RPo. We have observed DNA conformational heterogeneity and dynamics both in experiments assessing the apparent “length” of the transcription bubble (distances between DNA segments upstream and downstream of the transcription bubble) and in experiments assessing the apparent “width” of the transcription bubble (distances between the nontemplate and template strands of the transcription bubble). Our experiments assessing changes in the apparent length of transcription bubble upon reprogramming of start-site selection also suggest that transcription-bubble DNA conformational heterogeneity accounts for flexibility in start-site selection.

Our data support a model wherein RNAP harnesses thermally driven DNA fluctuations to access a distribution of transcription-bubble translational registers relative to the RNAP active site, with each different translational register corresponding to a different start site. In particular, our data support a model where transcription-bubble expansion (scrunching) places downstream DNA in contact with the RNAP active center, facilitating the usage of downstream start sites, and transcription-bubble contraction (unscrunching) places upstream DNA in contact with the RNAP active center, facilitating the usage of upstream start sites. Numerous factors, such as the DNA sequence, the availability of nucleotides and possibly the presence of transcriptional regulators, can alter the energy landscape describing the ensemble of translocation registers and therefore select one or more transcription start sites.

We have also considered whether part of the fluctuations observed may be a result of photophysical changes in the fluorophores used. We note that the proximity of a certain region of the RNAP to a fluorophore may alter the optical properties of that particular fluorophore, therefore making comparison
between dyes at different positions on the DNA difficult to interpret. However, although we cannot completely exclude the possibility that photophysics play a role in the fluctuations, the comparisons over our entire set of data and the use of control samples (such as free DNA fragments and the control ruler) indicate that the role of photophysics is likely to be minor.

In our experiments analyzing RNAP open complexes, we have assigned the first peak of the bimodal distribution to dsDNA and the second peak to RPo. It is important to note, however, that we cannot exclude the possibility that the DNA-only peak consists of not only unbound DNA but also closed or partially closed states. Indeed, the poor fit of some of the distributions in our experiments does indicate further complexity. Multiple intermediate states in transcription initiation by σ70-RNAP have been described previously (reviewed in Ref. 39). It is therefore possible that we are detecting intermediate complexes in our analysis; additional experiments using immobilized RPo complexes should allow these intermediate states to be studied further.

Our data suggest that at least some of the DNA dynamics occur on the timescale of milliseconds. Considering that each nucleotide addition during transcription elongation occurs on the ~30-ms timescale,40–43 it seems likely that partial or full equilibration among transcription-bubble translocational registers may occur before the formation of the initiating dinucleotide in transcription initiation. Conclusive arguments, however, must await real-time studies of transcription-bubble DNA dynamics using immobilized complexes. In addition, methods used in this study should enable the analysis of transcription-bubble conformational heterogeneity during transcription elongation, pausing and termination.

Materials and Methods

DNA and reagents

Amino-modified oligonucleotides (IBA, Germany) were internally labeled with fluorophores Cy3B (Invitrogen, USA) and ATTO647N (ATTO-TEC, Germany), as previously described,35 and purified using gel electrophoresis. Single-stranded DNAs were annealed in hybridization buffer [50 mM Tris–HCl (pH 8.0), 1 mM ethylenediaminetetraacetic acid and 500 mM NaCl]. Sequences of DNAs and the labeling schemes used are shown in Supplementary Fig. 1.

Formation of RNAP open complexes and initial transcribing complexes

According to published procedures,24,35,44,45 open complexes (RPo) were formed by mixing dsDNA (10 nM) and E. coli RNAP holoenzyme (50 nM; Epicentre, USA) in a total volume of 20 μl KG7 buffer [40 mM Hepes–NaOH (pH 7), 100 mM potassium glutamate, 10 mM MgCl₂, 1 mM DTT, 100 μg/ml bovine serum albumin, 5% glycerol and 1 mM mercaptoethanol] and subsequent incubation at 37 °C for 15 min. After incubation, heparin Sepharose-coated beads (1 mg/ml; GE Healthcare) were added to disrupt nonspecific RNA–DNA complexes and to remove free RNAP. After 30 s at 37 °C, samples were centrifuged, and 13 μl of supernatant was transferred to a pre-warmed tube. Wherever indicated, ribonucleotides ATP and GTP were added to the KG7 buffer at a concentration of 1 mM after RPo formation.

In vitro transcription assays

The in vitro transcription reaction mixtures were set up by adding 0.24 U RNAP (Epicentre, USA), 10 nM dsDNA promoter, 12 U RNasin (Promega, USA), 50 μM UTP, 50 μM CTP, 50 μM ATP (Fermentas, UK) and 0.3 μCi/μl [α³²]GTP (10 μCi/μl (PerkinElmer)) to 1× KG7 buffer [40 mM Hepes–NaOH (pH 7), 100 mM potassium glutamate, 10 mM MgCl₂, 100 μg/ml bovine serum albumin, 1 mM DTT, 1 mM mercaptoethanol and 5% glycerol] and incubated for 5 min at 37 °C. Heparin Sepharose (1 mg/ml; GE Healthcare) was added, and the reaction was allowed to continue at 37 °C for a further 55 min. Reactions were stopped by addition of 5 μl of loading dye (90% formamide, 10 mM ethylenediaminetetraacetic acid, bromophenol blue and xylene cyanol), and mixtures were incubated for 5 min at 95 °C before being loaded on a 6-M urea, 20% polyacrylamide sequencing gel and visualized by autoradiography.

Single-molecule fluorescence spectroscopy

A custom-built confocal microscope was used for smFRET experiments as previously described.28,46 The setup was modified allowing ALEX of donor and acceptor fluorophores.30,31 For this purpose, the fiber-coupled outputs of a green (532 nm, Samba; Cobolt, Sweden) and a red (638 nm; Cube Coherent, USA) laser were alternated with a modulation frequency of 10 kHz. Both beams were spatially filtered and coupled into an inverted confocal microscope IX71 (Olympus, Germany) equipped with an oil-immersion objective (60×, 1.35 NA, UPLSAPO 60XO; Olympus, Germany). In a typical experiment, the average excitation intensities were 250 μW at 532 nm and 60 μW at 635 nm. The same objective was used to collect the resulting fluorescence; the emission was separated from excitation light by a dichroic mirror, focused onto a 200-μm pinhole and subsequently split spectrally on two avalanche photodiodes (SPCM-AQR-14; PerkinElmer, UK) detecting the donor and acceptor fluorescence with two distinct spectral filters (green, 585DF70; red, 650LP). Custom-made LabVIEW software was used to register and evaluate the detect signal. For all experiments, the temperature of the sample was set to 37±1 °C using a custom-made heated collar attached to the objective, which was connected to a heating bath.

Data analysis

Fluorescence photons were assigned to either donor-based (Dexc) or acceptor-based (Aexc) excitation with
respect to their photon arrival time (donor detection channel, $D_{\text{em}}$; acceptor detection channel, $A_{\text{em}}$).

Two characteristic ratios, fluorophore stoichiometries $S$ and apparent FRET efficiencies $E^*$, were calculated for each fluorescent burst above a certain threshold yielding a two-dimensional histogram. Stoichiometry $S$ is the ratio between the overall green fluorescence intensity over the total green and red fluorescence intensity and describes the ratio of donor-to-acceptor fluorophores within a diffusing molecule. The uncorrected FRET $E^*$ efficiency [defined as $D_{\text{exc}}A_{\text{em}}/(D_{\text{exc}}A_{\text{em}} + D_{\text{exc}}D_{\text{em}})$] monitors the proximity between the two fluorophores. We selected bursts characterized by three parameters ($M$, $T$, and $L$) from the data. In this analysis, a fluorescent signal is considered a burst if a total of $L$ photons having $M$ neighboring photons arrive at the detector within a time interval of $T$ microseconds. Acceptor-containing molecules were identified by applying a burst search on $A_{\text{exc}}A_{\text{em}}$ with parameters $M=7$, $T=500$ μs and $L=12$. We additionally applied per-bin thresholds to remove spurious changes in fluorescence intensity and to select for bright donor–acceptor molecules ($A_{\text{exc}}A_{\text{em}}>30–100$ photons). One-dimensional $E^*$ distributions for donor–acceptor species were obtained by using a 0.45<$S$<0.8 threshold. These $E^*$ distributions could be fitted using a Gaussian function, yielding the mean $E^*$ value for a certain distribution and an associated standard deviation $\sigma$. BVA analysis was performed as described previously.  

**Fluorescence correlation spectroscopy**

The same microscope and experimental configuration as described above was used for fluorescence correlation spectroscopy measurements. Excitation was at 532 nm in continuous-wave fashion (150 μW). Photon-by-photon arrival times in the donor and acceptor channels were correlated using a hardware correlator. Data in the manuscript were derived from autocorrelation in the red detection channel after green excitation to detect doubly labeled species.

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**Supplementary Data**

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**Abbreviations used:**

RNAP, RNA polymerase; smFRET, single-molecule FRET; dsDNA, double-stranded DNA; BVA, burst variance analysis.

**References**


