Single-molecule imaging of non-equilibrium molecular ensembles on the millisecond timescale

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Single-molecule fluorescence microscopy is uniquely suited for detecting transient molecular recognition events, yet achieving the time resolution and statistics needed to realize this potential has proven challenging. Here we present a single-molecule imaging and analysis platform using scientific complementary metal-oxide semiconductor (sCMOS) detectors that enables imaging of 15,000 individual molecules simultaneously at millisecond rates. This system enabled the detection of previously obscured processes relevant to the fidelity mechanism in protein synthesis.

The potential of single-molecule fluorescence resonance energy transfer (smFRET) microscopy to capture transient, non-accumulating events at the level of individual molecules enables biological investigations unobserved by ensemble averaging1,2 and unconstrained by the need for large amounts of homogeneous material. The smFRET approach is based on the detection of biological interactions in terms of motion, with reaction coordinates represented by changes in distance between individual particles or domains to which donor and acceptor fluorophores are site-specifically attached (Fig. 1a). Although a growing number of biological systems are accessible by camera-based smFRET methods2, cellular processes governed by micromolar affinity interactions3,4 and dynamics that occur on millisecond timescales5–7 are typically out of reach as a result of limited readout rates (Fig. 1b). Sub-millisecond time resolution can be achieved with avalanche photodiodes1,6, but only one molecule can be imaged at a time. Non-equilibrium processes can also be studied, but the time course of events must be inferred from snapshots recorded at precise lag times after microfluidic mixing8. In contrast, camera-based methods simultaneously record the entire reaction coordinate in each molecule of the ensemble. Electron-multiplying charge-coupled device (EMCCD) cameras are well suited for single-molecule experiments but are generally restricted to ~1,000 simultaneous observations at 10-ms time resolution. Reducing the EMCCD pixel array size2,9 allows for increased imaging speeds, but at the expense of the number of observations (Fig. 1c), thereby defeating the goal of collecting sufficient statistics to fully characterize the ensemble.

Here we demonstrate a camera-based imaging platform that enables the acquisition of millisecond-timescale steady-state and pre–steady-state smFRET measurements of more than 10,000 individual molecules simultaneously (Fig. 1c). To achieve this goal, we used sCMOS detectors, rapid solution exchange (Fig. 1d), photostabilized fluorophores and nearly real-time data analysis.

Commercially available sCMOS cameras have great potential for single-molecule imaging10, but their performance in smFRET applications has not yet been characterized. Our investigations demonstrate two key advantages of sCMOS cameras for smFRET. First, sCMOS cameras have a higher effective quantum efficiency (QE) than EMCCDs because signal multiplication by EMCCDs introduces excess noise that effectively halves their QE10. Consequently, sCMOS cameras yield superior signal-to-noise ratios at signal levels of 200 or more detected photons per frame (Fig. 1e and Online Methods) and narrow FRET distributions by up to 25% (Fig. 1f). The advantage of EMCCD cameras extends only to photon counts below the typical prerequisite for accurately distinguishing FRET states1,2. Second, sCMOS cameras have readout rates exceeding 400 megapixels per second, compared to just 30 for the fastest EMCCDs. This advantage provides marked increases in imaging throughput that exceed one order of magnitude (Fig. 1c). Using one camera per spectral channel further increases throughput relative to that of commonly used split-camera optics1 (Fig. 1d) to more than 10,000 molecules per movie at full frame.

At this scale of data generation, conventional analysis methods that include manual trace inspection and parameter tuning become prohibitively time consuming11. To address this challenge, we developed the smFRET data analysis software package SPARTAN (Single-molecule Platform for Automated Analysis), optimized for sCMOS data sets (Fig. 1g, Online Methods and Supplementary Software 1; also available at http://www.scotcblanchardlab.com/software). This platform provides automated tools for the analysis procedure, including (1) identification of molecules in each spectral channel, (2) integration of pixel intensities into appropriately scaled and corrected time-dependent fluorescence and FRET trajectories, (3) selection of desired fluorescence and FRET features according to user-defined criteria (Supplementary Table 1), (4) calculation of FRET observables (FRET value and state lifetimes) using hidden Markov modeling12,13 and (5) visualization of key experimental statistics for data interpretation. Minimizing the time and effort for such procedures facilitates real-time evaluations during data collection.
Figure 1 | Imaging platform for enhanced-throughput smFRET. (a) Schematic of a molecular interaction monitored by FRET ($K_0$, dissociation constant; $\tau$, interaction lifetime). (b) FRET signal for a, modeled at infinite time resolution and downsampled to 1-ms and 10-ms resolution, highlighting how insufficient sampling hinders the detection of events. (c) Comparison of fields of view and numbers of molecules observed ($N$) with typical EMCCD and sCMOS cameras at 1-ms and 10-ms time resolution. (d) Experimental setup with prism-type total internal reflection excitation, fluorescence detection via a 60× water-immersion objective, one sCMOS camera per spectral channel, and a microfluidic reagent delivery system. (e,f) Comparison of (e) average signal-to-noise ratio (SNR) and (f) width of the observed FRET distribution of data obtained from a dye-labeled DNA duplex standard at various signal-to-noise ratios ($\gamma$). (g) Schematic of automated data-analysis pipeline. From left to right: molecules are detected and aligned in each spectral channel, where $(x_D,y_D) \rightarrow (x_A,y_A)$ indicates the mapping function, and their signals are summed to create fluorescence-time traces; corrections are made for baseline, spectral bleed-through ($\alpha$) and unequal apparent brightness ($\beta$), and FRET-time traces are calculated; descriptive statistics are calculated for each trace, and traces are selected that meet user-defined fitness criteria; and Hidden Markov modeling with a kinetic (left) and emission (middle) model is used to interpret the dynamic behavior in FRET traces (right) and assign the underlying physical state of the system at each point in time (idealization; red line).

As demonstrated by other high-throughput single-molecule platforms10, large observation numbers make it possible to apply stringent criteria to select long-lived FRET trajectories exhibiting functional heterogeneities or rare events that inform on the biological system. Imaging throughput is particularly critical for pre–steady-state, non-equilibrium smFRET measurements, such as measurements of single-turnover enzymatic reactions, in which the biological system evolves rapidly over time7,19–22. In contrast to steady-state observations, with which data can be accumulated from multiple fields of view, the goal of pre–steady-state measurements is to acquire sufficient information on transient, non-accumulating events from the molecular ensemble in a single acquisition.

To evaluate the effectiveness of our imaging platform for pre–steady-state measurements, we investigated the process of tRNA selection on the bacterial ribosome, a fidelity-determining step in protein synthesis that occurs at a bulk rate of approximately $10^{-3}$ s$^{-1}$ by tracking the evolution of smFRET as an acceptor-labeled aminoacyl tRNA (aa-tRNA)—bound in a ternary complex (TC) with elongation factor Tu and GTP—enters an mRNA-bound in a ternary complex (TC) with elongation factor Tu and GTP—enters an mRNA-bound in a ternary complex (TC) with elongation factor Tu and GTP—enters an mRNA-bound in a ternary complex (TC) with elongation factor Tu and GTP—enters an mRNA-bound in a ternary complex (TC) with elongation factor Tu and GTP—enters an mRNA-bound in a ternary complex (TC) with elongation factor Tu and GTP—enters an mRNA-bound in a ternary complex (TC) with elongation factor Tu and GTP—enters an mRNA-bound in a ternary complex (TC) with elongation factor Tu and GTP. As expected on the basis of theoretical considerations24, the initial phase of tRNA selection requires aa-tRNAs to be rapidly inspected by the ribosome, as cognate aa-tRNAs represent only a small fraction of the cellular pool. This molecular recognition process takes place within a short-lived codon-recognition (CR) state that is poorly resolved at 10-ms time resolution7, where more than 60% of traces seem to skip the CR state. To better elucidate this initial selection step, we devised a distinct structural perspective wherein the CR state exhibits a high FRET efficiency that is more readily distinguished from background noise than the low-FRET CR state observed in the tRNA-tRNA system. Here energy is transferred from a donor-labeled ribosomal protein (S12) to acceptor-labeled aa-tRNA entering the ribosome (Fig. 2b and Online Methods). We added the translation inhibitor tetracycline to promote rejection of aa-tRNA from the CR state, which enabled the detection of repeated, short-lived tRNA binding events at 1-ms time resolution (Fig. 2c), more than 80% of which would
Figure 2 | Robust detection of millisecond-scale transient events by smFRET. (a) Schematic of the tRNA-tRNA FRET experiment. TC is delivered to surface-immobilized ribosomes (no FRET; left) containing a cognate mRNA codon in the aminoacyl site. Accommodation (high FRET; right) occurs via short-lived intermediates (intermediate FRET; center). EF, elongation factor. (b) Equivalent schematic of the S12-tRNA FRET experiment. Here intermediates correspond to high FRET and accommodation corresponds to intermediate FRET. Cy3B is used instead of Cy3; all other color-coding corresponds to the key in a. (c) Fluorescence (top) and FRET (bottom) time traces of a single ribosome displaying short-lived TC binding events in the presence of the tRNA-selection inhibitor tetracycline (20 μM). The idealized FRET trace is shown in red in the lower graph. (d) Fraction of binding events that are detected as a function of time resolution, obtained by downsampling 1-ms data and normalizing to the number of events observed at 1 ms. Data shown are the mean ± s.d. of three technical replicates. (e) Exponential fits to the survival time in the high-FRET state reveal lifetimes of 4.6 ms for the cognate (τ_{c}) and 2.2 ms for a near-cognate codon-anticodon interaction (τ_{nc}). Data shown are the mean ± s.d. of three technical replicates. (f–h) Pre–steady-state tRNA-selection experiments (2-ms time resolution) comparing the tRNA-tRNA (f,g) and S12-tRNA (h) signals. (f,h) Example FRET traces (left) and post-synchronized ensemble plots (right). (g) Occupancy of each FRET state as a function of time (blue line, no FRET/unbound; red line, low FRET/codon recognition; orange line, intermediate FRET/GTPase activation; green line, high FRET/accommodated). Color-coding in g corresponds to shading in a, c, f and h. Error bars denote ±s.e. from 1,000 bootstrap samples.
of molecular recognition processes with microsecond lifetimes and millimolar affinities.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

M.F.J. and D.S.T. designed and implemented the sCMOS TIRF (total internal reflection fluorescence) microscope and analyzed the data. M.F.J. and M.R.W. performed smFRET experiments. D.S.T. designed and implemented the analysis software, with help from S.C.B., M.F.J. and M.R.W. M.F.J. designed and implemented data-acquisition software. M.R.W. developed the S12 ribosome-labeling strategy. M.R.W., R.B.A. and M.F.J. prepared ribosome and smFRET reagents. Z.Z. and H.Z. synthesized fluorophores. All authors contributed to experimental design and writing of the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details are available in the online version of the paper.

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ONLINE METHODS

Ribosome and reagent preparation. Escherichia coli ribosomes, translation factors, fluorescently labeled tRNAs, microfluidic chambers, buffers, and other reagents were prepared as previously described. For tRNA-tRNA FRET experiments, we used ribosomes with ribosomal protein L1 deleted to reduce the appearance of hybrid states.

Preparation of S12-labeled ribosomes. Ribosomal protein S12 was PCR cloned from E. coli strain K12 genomic DNA into the pVB4 vector (Vectornt Biosolutions) with an eight-residue peptide encoding the A4 epitope for the Acps phosphopantetheinyl transferase reaction (amino acid sequence: DSLDMLEW) fused at the C terminus. After plasmid shuffling into an E. coli AS12 knockout strain, cells were cultured and ribosomes were harvested, cleaved and labeled with Cy3B in situ as done previously for enzymatic labeling of S13-tagged subunits. For the formation of intact 70S ribosome complexes, Cy3B-S12 30S and unlabelled BL21 (DE3) 50S subunits were heat activated at 42 °C for 10 min in Tris-polymix buffer containing 50 mM Tris-acetate, pH 7.5, 5 mM Mg(CH3COO)2, 100 mM KCl, 5 mM NH4(CH3COO), 0.5 mM CaCl2, 0.1 mM EDTA, 5 mM putrescine, 1 mM spermidine, 1.5 mM β-mercaptoethanol, and 1 mM GTP. Ribosomes were then initiated with fMet-tRNAMet as previously described. The assignment of FRET states for the S12 system (Fig. 2b) was validated using well-characterized translation inhibitors and similarly to the approach used for previous assignments for the tRNA-tRNA signal.

Single-molecule fluorescence microscopy. Single-molecule FRET imaging of tRNA selection was performed using a custom prism-based total internal reflection fluorescence microscope as previously described. Ribosomes programmed with a biotinylated mRNA were surface immobilized to streptavidin, which was adhered to surface-linked biotin-polyethylene glycol. The mRNA codon in the ribosomal aminoacyl (A) site was either cognate (UUC) or near-cognate (UCU) to the tRNAPhe anticodon. All experiments were performed in Tris-polymix buffer (defined above) in the presence of an oxygen scavenging system (2 mM protocatechueic acid, 50 mM protocatechuate 3,4-dioxygenase and photostabilizing agents (1 mM Trolox, 2 mM cyclooctatetraene, 1 mM nitrobenzyl-alcohol). Cy3 fluorophores (Cy3 or Cy3B) on P-site tRNA or S12 were illuminated with a 532-nm diode-pumped solid-state laser (Opus, LaserQuantum) at ~0.45 to 2 kW/cm² (at the total internal reflectance (TIR) interface) for time resolutions between 10 ms and 1 ms. An area larger than the imaging field of view was illuminated to optimize uniformity by appropriate placement of the f = 150 mm focusing lens relative to the TIR prism. Additionally, a telescopic arrangement of cylindrical lenses (f1 = -50 mm and f2 = 100 mm) was placed in the excitation beam path to correct for the nonuniform projected beam aspect ratio at the TIR interface. It is also worth noting that any poorly illuminated or aberrant molecules in the corners of the field of view can be removed using the automated analysis procedure and that the large data sets obtainable with sCMOS ensure that the resulting subsets are large enough to be used to determine the parameters of interest. An ET555lp long-pass filter (Chroma) was used to remove scattered laser light. Fluorescence emission from Cy3 (or Cy3B) and LD650 (Lumidyne Technologies) fluorophores was collected using a 60×/1.27-NA (numerical aperture) PlanApo water-immersion objective (Nikon), spectrally separated using a MultiCam-LS device (Cairn) using a T635pxr-U2 dichroic mirror (Chroma) and imaged onto two Hamamatsu ORCA-Flash 4.0 v2 sCMOS cameras (2,084 × 2,048 pixels, 6.5-μm pixel size) connected to a PC with Camera Link acquisition boards. The instrument was also equipped with additional laser lines (Ciel 473 nm, LaserQuantum; Genesis MX 639 nm, Coherent; 730-nm collimated diode, Leading-Tech Laser), two additional cameras (connected to the same MultiCam-LS device) and dichroic mirrors (ZT532rdc-U2, T740pxr-U2) for additional spectral channels in the Cy2 and Cy7 regions.

Data were acquired using custom software implemented in LabView (National Instruments). We chose 2 × 2 pixel binning (217-nm effective pixel size in the sample plane, accounting for magnification) to minimize the data size without sacrificing data quality. A pixel size satisfying the Nyquist sampling criterion is not required for measurement of the total intensity of each diffraction-limited molecule image, but excessive binning decreases the resolvable molecule density. In contrast to EMCCD cameras, binning on an sCMOS sensor does not increase the achievable frame rate. The total field of view was 222 μm × 222 μm in the sample plane at 10-ms time resolution. Synchronization of the cameras was achieved with an external pulse to trigger the acquisition of each frame. The pulse sequence was generated in real time by a USB device (USB-6501, National Instruments) controlled by the acquisition software. We verified the timing and reproducibility of this approach both by examining oscilloscope outputs and by testing the synchronization of camera frames with an external light source. Movies were streamed to a solid-state drive during acquisition using the built-in HD recorder of the Hamamatsu camera driver. The generated raw data files were tiled side by side and automatically converted to the BigTIFF format, a proposed standard for image files exceeding 4 GB in size, using a custom library implemented in C (Supplementary Software 2). New movie files were automatically detected and processed as described under “Particle Detection and Generation of Fluorescence Traces” (below), and were ready for immediate inspection by the user.

For EMCCD measurements of tRNA selection, a DualCam device (Photometrics) using a 640DCLP dichroic mirror (Chroma) was used to spectrally separate Cy3 and LD650 fluorescence and image the molecules on two Evolve 512 cameras (Photometrics) with 512 × 512 pixels and a 16-μm physical pixel size. Data were acquired in Meta Morph (Molecular Devices) with the 10-MHZ chipset and 2 × 2 pixel binning (533-nm effective pixel size), chosen to balance throughput and data quality. We achieved synchronization by triggering each frame with a custom timing circuit. A rectangular region of interest (137 × 68 μm in the sample) covering the bottom half of the field of view was used to achieve 10-ms imaging (Fig. 1c). For direct quantitative comparisons of sCMOS and EMCCD measurements, the two pairs of cameras were attached to the same two output ports of the MultiCam device and integration regions (described below) chosen to account for the different effective pixel sizes of the two camera models. Comparative measurements were performed using DNA standards labeled with LD550 and LD650.
fluorophores (Lumidyne Technologies) according to previously established protocols. SNR and FRET distribution statistics were computed as described below.

**Fast (1–2 ms) tRNA-selection experiments.** A rectangular region of interest consisting of 200 (400) lines at the center of the field of view was used to achieve 1-ms (2-ms) imaging (Fig. 1c), corresponding to a 222 µm × 22 µm (222 µm × 43 µm) field of view in the sample. Multiple movies were combined for each condition. Measurements at 1-ms time resolution were performed in the presence of 20 µM tetracycline (Fig. 2c) or with TC containing the nonhydrolysable GTP analog GDPNP (Fig. 2d) to terminate selection events before completion of the initial selection phase. To verify that the resulting short FRET events represented specific interactions, we carried out control experiments with Cy5-labeled DNA oligonucleotides instead of TC, which showed low numbers of nonspecific or misidentified events (41.5 and 4.8 events on average per movie for TC and control experiments, respectively). We determined the lifetimes of CR interactions (Fig. 2e) by fitting a sum of two exponentials, \( a e^{-k_1 t} + (1 - a) e^{-k_2 t} \), to the survival plot and calculating the inverse \( \tau \) as the average of the amplitude-weighted average of the rates \( k_1 \) and \( k_2 \). The results with 95% confidence intervals were as follows: cognate, \( a = 0.687 \pm 0.025, k_1 = 0.293 \pm 0.012 \text{ ms}^{-1}, k_2 = 0.058 \pm 0.004 \text{ ms}^{-1} \); near-cognate, \( a = 0.967 \pm 0.035, k_1 = 0.459 \pm 0.035 \text{ ms}^{-1}, k_2 = 0.043 \pm 0.057 \text{ ms}^{-1} \).

To achieve reproducible timing for pre–steady-state experiments, we prepared TC at the final concentration in oxygen-scavenging buffer and loaded it into a pressure-resistant steel syringe (Harvard Apparatus) that was then attached to a syringe pump (PHD Ultra, Harvard Apparatus). Reagent delivery from the pump to the microfluidic devices was triggered by the custom acquisition software to ensure reproducibility to within 2 ms (Supplementary Fig. 2). The synchronization of the ensemble process is limited by the reproducibility and rise time of the reagent delivery (inherent properties of the instrument) and the stochastic arrival time distribution determined by the bimolecular rate constant of the interaction. It should be noted, however, that one of the strengths of single-molecule experiments is that one can eliminate this asynchrony by synchronizing individual traces to the point of the first registered FRET transition as described below. The purpose of hardware synchronization is mainly to ensure that mixing is complete before photobleaching so that the full process can be observed.

**Particle detection and generation of fluorescence traces.** smFRET data analysis was performed using SPARTAN, a custom software package written in MATLAB (Supplementary Software 1). The process begins with the detection of fluorophore-labeled particles, which appear as local intensity maxima (point-spread functions (PSFs)) above baseline levels in fixed positions within the field of view (Fig. 1g). Because they are fixed, particles are detected from an average image from the first ten frames of the movie. Prior to detection, a background image is subtracted to flatten baseline levels and ease molecule detection, particularly when the illumination is nonuniform. For generation of the background image, the field is divided into a grid and an average of the 5% lowest intensity pixels in each grid area is taken, and those values are interpolated to generate a full-field image, similar to other methods. Intensity maxima associated with fluorophore-labeled particles are distinguished from background using a threshold of eight s.d. above background noise. Any molecules that are stochastically closer than three average PSF s.d. apart are rejected to avoid signal contamination between neighboring particles.

Next, intensity maxima in each spectral channel corresponding to the same particle are associated using the iterative closest point (ICP) algorithm, resulting in a transformation that accounts for translation, rotation and magnification. The algorithm is robust to large alignment deviations, particles without a signal in all spectral channels and background fluorescence. The procedure is automated and does not require the user to select control points. In difficult cases where one channel has low signal, the alignment transformation can instead be derived using fluorescence beads before the experiment. Robust software alignment is critical to the success of sCMOS imaging because its larger field of view and higher pixel density result in larger deviations in pixel units than found with EMCCDs.

After image registration using the transformation derived above, particles are re-detected from a summed image of all spectral channels to minimize any potential selection bias. For example, particles with a low FRET efficiency might not have sufficient intensity on the acceptor channel to be detected, causing the remaining population to be biased toward particles with higher FRET efficiency. When the intensities from all spectral channels are summed, particles have roughly the same overall intensity regardless of FRET efficiency.

Finally, fluorescence-time traces for each particle are extracted from the movie via summation of the intensity of the highest-intensity pixels within a small neighborhood around the maximum. The neighborhood size is chosen to be five times the average PSF s.d. The optimal number of pixels balances the trade-off between collecting more fluorescence intensity and collecting more background noise, but it needs to be determined only once for a particular instrument. This is similar to aperture photometry in that it is computationally simple and fast, whereas PSF fitting is relatively slow even with extensive optimization. Although PSF fitting has potential advantages for handling nonuniform PSFs, uneven focus, stage or focal drift, and noise characteristics of the detector, in our experience it does not significantly improve the quality of data obtained with a stable, well-corrected instrument.

**Baseline subtraction, spectral corrections and FRET calculation.** In order for FRET efficiency to be interpreted as a measure of the distance between donor and acceptor fluorophores, several experimental considerations must be taken into account: fluorescence baseline, spectral cross-talk, and the apparent sensitivity for each fluorophore (Fig. 1g). First, the baseline intensity levels after donor photobleaching are subtracted from fluorescence traces to set the baseline level close to zero, refining the earlier estimate obtained by subtracting an approximate background image. The donor photobleaching point is detected as the last large drop in the gradient of median–filtered total fluorescence intensity (six s.d. beyond the noise). Although aperture photometry has the advantage of adjusting for changing baseline levels, it also requires a large, clear area around each PSF, limiting the density of particles that can be imaged simultaneously.
Next, the fraction of donor fluorescence appearing on the acceptor channel (spectral cross-talk, denoted by $\alpha$), estimated from the acceptor channel intensity after acceptor photobleaching, is subtracted from acceptor fluorescence traces so that the apparent cross-talk is zero. To ensure that the relative brightness of the fluorophores is equal, the change in donor and acceptor fluorescence after acceptor photobleaching is used to estimate the relative brightness ($\gamma$), and the average value is used to scale the acceptor intensity. In both cases, a single, average correction value is used for all traces in each movie because the imperfect estimates in each trace can add variability, rather than reducing it.

Finally, FRET-time traces are calculated as $E_{\text{FRET}} = I_A/(I_A + I_D)$, where $I_A$ and $I_D$ are the acceptor and donor fluorescence intensity at each point in time, respectively. FRET is set to zero after donor photobleaching and during donor blinking events, which are detected whenever the total intensity drops below six s.d. of the background noise. The high noise levels and nonideal photophysical behavior apparent in millisecond smFRET data necessitate an alternative strategy that is more robust to noise: the total fluorescence intensity trace ($I_A + I_D$) is idealized with a two-state model for fluorescent and dark states using the segmental $k$-means algorithm (SKM) implemented in MATLAB.

### Calculation of trace statistics and trace selection

For automated trace selection, the user calculates a set of statistics for each trace and chooses a useful subset according to defined criteria (Fig. 1g). Commonly used statistics, selection criteria, and the purpose of each are listed in Supplementary Table 1.

The signal-to-noise ratio (SNR) is a measure of trace quality, which ultimately determines the capability to distinguish distinct FRET states. SNRBG is defined as the mean total fluorescence intensity divided by the s.d. of the background total intensity immediately after donor photobleaching (100 frames). A distinct ‘background noise’ statistic defines the s.d. of the entire trace after photobleaching, which is useful for detecting unstable background levels. SNRSignal is defined as the mean total fluorescence intensity divided by the s.d. of the total intensity before donor photobleaching, ignoring regions where the donor is dark. This metric more closely corresponds to the noise in the measurement because it takes into account photon statistics and photophysical noise.

For detecting traces with an acceptor signal above baseline, a threshold of 0.125 FRET is used, corresponding to two s.d. above typical background noise levels. 'FRET lifetime' is a statistic that quantifies the observation time for FRET, where the acceptor signal is above background, primarily for experiments where the acceptor fluorophore is stably bound. To reduce false positives from traces that happen to have high background noise, only runs of five frames or more above the threshold are considered.

Single-molecule traces are generally useful only if they contain exactly one donor and one acceptor signal. One can determine the number of donor fluorophores by counting the number of photobleaching steps, which appear as instantaneous, irreversible drops in the total fluorescence intensity (donor + acceptor). Detection is accomplished by median filtering of the signal with a window size of nine frames to remove high-frequency noise while preserving sharp changes and taking the gradient to detect the large, sudden drops in fluorescence intensity associated with photobleaching steps. A threshold of four s.d. of the gradient signal is used for detecting these events. A photobleaching event is counted if the intensity never returns to the level observed before the event.

One can estimate bias in these parameters by comparing the population behavior of a selected subset of data to that of the full data set. This procedure should be repeated for any new biological sample or type of experiment to ensure that selection is unbiased.

#### Hidden Markov modeling

FRET events, corresponding to RNA binding to the ribosome, are isolated and postsynchronized as previously described. State assignment (Fig. 1g) is performed using SKM. Although several other Markov modeling utilities are available and could be used for this purpose (e.g., HaMMy, vbFRET, SMART, iSMS, and TwoTone), SKM is much faster than the alternatives, and this facilitates analysis of the large data sets associated with sCMOS.

#### Code availability

Source code, updates and documentation for the SPARTAN software are freely available at [http://www.scottblanchardlab.com/software](http://www.scottblanchardlab.com/software).

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