Supporting Information

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SI Materials and Methods

Fluorescent Dye Characterizations. Biotinylated DNA oligonucleotides conjugated to fluorescent dyes (Cy3, Cy3.5, Cy5, and Cy5.5) were chemically synthesized from Trilink with the following sequence: 5′ (biotin) GCT TAT TCA ACT ACA CTT CTC GGC CTC (C6-NH-dye) 3′. Before each experiment, the single-molecule real-time (SMRT) Cell surface was derivatized with 16.6 μM Neutravidin reagent for 5 min at room temperature and rinsed with Tris-based polymix buffer (50 mM Tris·acetate, pH 7.5, 100 mM potassium chloride, 5 mM ammonium acetate, 0.5 mM calcium acetate, 5 mM magnesium acetate, 0.5 mM EDTA, 5 mM putrescine-HCl, 1 mM spermidine) to remove unbound Neutravidin reagent. We then diluted the DNA oligonucleotide in the Tris-based polymix buffer with oxygen scavenging [1 mM Trolox, 2.5 mM protocatechuic acid, 50 nM protocatechuate-3,4-dioxygenase (PCD)] (1) to 1 nM and immobilized on the SMRT Cell by incubating the mixture at room temperature for 3 min. The SMRT Cell was subsequently rinsed with the same Tris-based polymix buffer with oxygen scavenging to remove unbound oligonucleotides. The SMRT Cell was then loaded onto the custom PacBio zero-mode waveguide (ZMW) -based DNA sequencer (*RS*) instrument, and a 5-min movie was acquired at 10 frames per second (fps) without fluidics delivery and with dual laser illumination (532-nm laser at 0.48 $\mu W/\mu m^2$ and 642-nm laser at 0.22 $\mu W/\mu m^2$ measured at the laser source). All experiments were performed at a room temperature of 22 °C.

Single-molecule fluorescence experiments on total internal reflection fluorescence were performed similarly as described previously (2). PEGylated quartz slides were first derivatized with 1 μM Neutravidin reagent for 5 min at room temperature and washed with Tris-based polymix buffer (same as above) to remove unbound Neutravidin reagent. We then diluted the DNA oligonucleotide in the Tris-based polymix buffer with oxygen scavenging (same as above) to 50 pM and immobilized on the slide by incubating the mixture at room temperature for 5 min. Unbound oligonucleotides were washed with the same Tris-based polymix buffer with oxygen scavenging. The experiments were performed using an in house-built, prism-based total internal reflection microscope. Cy3 fluorophores were excited with 532-nm (0.5 kW/cm^2; measured at the prism) lasers. Cy5 fluorophores were excited with 647-nm (0.5 kW/cm^2) lasers. Fluorescence emission was collected by a 1.2 N.A./60× water-immersion objective. A Quad-View device (Photometrics) separated fluorescence emission into distinct color channels corresponding to Cy2, Cy3, and Cy5 fluorescence and projected this emission signal onto a cooled EMCCD camera (Andor Technology). We performed image acquisition using the MetaMorph software package (Molecular Devices) and subsequent analysis using scripts written in MATLAB (MathWorks). Automated MATLAB scripts were in house-designed for spot picking from the original image movies and then converted to time traces for subsequent state assignments. We assigned states using a hidden Markov model approach (3) and confirmed these assignments by visual inspection.

Real-Time Ribosomal Translation Assays. *Escherichia coli* ribosomal subunits (30S and 50S) and translation factors (IF2, EF-Tu, EF-G, and EF-Ts) were prepared and purified as described before (4, 5). The ribosomal subunits have hairpin loop extensions that were introduced into phylogenetically variable, surface-accessible loops of the *E. coli* 16S rRNA in helix 44 and 23S rRNA in helix 101 using previously described site-directed mutagenesis (5); 3′ dye-labeled DNA oligonucleotides complementary to the mutant ribosome hairpins (4, 5) were purchased from Trilink. The 30S labeling oligonucleotide has the sequence 5′ GGG AGA TCA GGA TA (dye) 3′, with the dye being either Cy3B or Cy3.5. The 50S labeling oligonucleotide has the sequence 5′ GAG GCC GAG AAG TG (dye) 3′, with the dye being Cy5, black hole quencher-2, or Cy5.5.

Right before each experiment, purified 30S and 50S ribosomal subunits (final concentration = 1 μM) were mixed in a 1:1 ratio with the 3′ dye-labeled oligonucleotides specific for the hairpin extensions in each subunit at 37 °C for 10 min and then, 30 °C for 20 min in a Tris-based polymix buffer system (50 mM Tris·acetate, pH 7.5, 100 mM potassium chloride, 5 mM ammonium acetate, 0.5 mM calcium acetate, 5 mM magnesium acetate, 0.5 mM EDTA, 5 mM putrescine-HCl, 1 mM spermidine). fMet-$tRNA^{fMet}$, Lys-$tRNA^{Lys}$, and Phe-$tRNA^{Phe}$ (Sigma) are charged and purified according to published protocols (6, 7). Phe-(Cy5)$tRNA^{Phe}$, Phe-(Cy3)$tRNA^{Phe}$, and Lys-(Cy5)$tRNA^{Lys}$ were labeled with Cy3-NHS or Cy5-NHS (GE Lifesciences) at the elbow position (U47), purified, and aminoacylated as previously described (6, 7). The 6(FK) mRNA used consists of a 5′ biotin followed by a 5′ UTR, a Shine–Dalgarno sequence derived from gene 32 of the T4 phage, an AUG start codon, six alternating repeats of Phe and Lys codons, a UAA stop codon, and four spacer Phe codons with the following sequence: 5′ (biotin) CAA CCU AAA ACU UAC ACA CCC GGU AAG GAA AUA AAA AUG UUC AAA UUC AAA UUC AAA UUC AAA UUC AAA UUC AAA UAA UUUUUUUUUUU 3′. The mRNA was chemically synthesized by Dharmacon.

To assemble 30S preinitiation complexes (PICs), we mixed 0.25 μM Cy3B-30S, preincubated with a stoichiometric amount of S1, 1 μM initiation factor-2 (IF2), 1 μM fMet-$tRNA^{fMe}$, 1 μM biotinylated mRNA 6(FK), and 4 mM GTP in a polymix buffer without reducing agents, and incubated the reaction mixture at 37 °C for 5 min. The ZMW surface was derivatized with 16.6 μM Neutravidin reagent for 3 min at room temperature and rinsed with polymix buffer to remove unbound Neutravidin reagent. Before surface immobilization, we diluted assembled PICs to 10 nM in polymix buffer containing 1 μM IF2 and 4 mM GTP. PICs were immobilized by delivering the diluted PIC mixture to the ZMW surface and incubating at room temperature for 3 min. Then, the surface was rinsed with polymix buffer containing 1 μM IF2, 4 mM GTP, and an oxygen scavenging system containing 1 mM Trolox, 2.5 mM protocatechuic acid, and 50 nM PCD. Immobilized PICs were identified by the Cy3B-30S fluorescence and were distributed in ZMW holes according to Poisson statistics. According to Poisson statistics, maximally, ∼30% of the ZMW holes are occupied for single loading. At our loading concentration of 10 nM, ∼15% of the ZMWs holes are occupied. We intentionally decreased the loading percentage to prevent doubly loaded ZMWs.

SMRT Cells were imaged for 5 min. For the ribosome conformational assay (Fig. 4), 200 nM black hole quencher-50S, 1 μM IF2, 80 nM EF-G, 80 nM ternary complex (Lys-$tRNA^{Lys}$-EF-Tu-GTP and Phe-$tRNA^{Phe}$-EF-Tu-GTP), 4 mM GTP, and oxygen scavenging system were delivered by the automated fluidics robotics at the ∼10-s time point. Ternary complex was preformed in bulk with Phe-$tRNA^{Phe}$ or Lys-$tRNA^{Lys}$, EF-Tu, EF-Ts, GTP, and a energy regeneration system (phosphoenolpyruvate and pyruvate kinase). Experiments were conducted at 10 fps with a single 532-nm laser excitation at room temperature.

For the ribosome compositional assay (Fig. 5), 200 nM Cy3.5-50S, 1 μM IF2, 200 nM ternary complex [Phe-(Cy5)$tRNA^{Phe}$-EF-Tu-GTP],

4 mM GTP, and oxygen scavenging system were delivered by the automated fluidics robotics at the ∼10-s time point. Experiments were conducted at 30 fps, with dual laser excitation (532 and 642 nm) at room temperature.

For the four-color experiment (Fig. 6), the SMRT Cell, ribosomes, and protein factors were prepared as described above, except that the 30S subunit was labeled with Cy3.5 and 50S subunits were labeled with Cy5.5; 200 nM Cy5.5-50S, 1 μM IF2, 200 nM ternary complex [Phe-(Cy3)tRNA^{Phe}-EF-Tu-GTP and Lys-(Cy5)tRNA^{Lys}-EF-Tu-GTP], 200 nM EF-G, 4 mM GTP, and oxygen scavenging system were delivered by the automated fluidics robotics at the ∼10-s time point. Experiments were conducted at 10 fps, with dual laser excitation (532 and 642 nm) at room temperature.

Data Analysis. The PacBio *RS* system contains data processing capabilities to support the SMRT sequencing technology. The customized experimental interface of the custom *RS* does not rely on the built-in data processing capabilities of the PacBio *RS*. Instead, the raw fluorescent intensity traces (saved as a .h5 file) are extracted using custom software writing in Matlab (MathWorks). More information is provided in *SI Results and Discussion*. Fluorescence traces corresponding to Cy3, Cy3.5, Cy5, and Cy5.5 are extracted and analyzed to measure lifetimes, arrival times, and intensities as described previously (8). All lifetime and arrival time estimates are the result of maximum likelihood parameter estimation for single-exponential distributions. The signal-to-noise ratio, a parameter that reports on the variance in photon emission rate in a flourophore, is calculated by the mean of the on state intensity divided by the SD of the on intensity (9, 10). Because of the design of the instrument, the background fluorescent intensity value is not at zero. Thus, background is corrected by simply subtracting each trace by the background intensity value (which can be determined by the intensity level when the dyes have been photobleached). All data analyses and plots are done with Matlab.

SI Results and Discussion

1. Single-Molecule Fluorescence Microscopy Workflow. In experimental or single-molecule fluorescence microscopy (SMFM) mode, runs consist of a single SMRT Cell, where initial chip prep steps are performed manually. Subsequent to immobilization, immobilized chip and reagents are loaded onto the instrument, and the run is initiated. The runs are launched through a custom window on a PC computer that controls the custom *RS* instrument.

The SMFM user interface allows the user to input the run info describing the experiment and conditions being tested. The entries are saved into a metadata.xml file as entered. The SMFM interface also allows the user to select the protocol used to run and input the protocol parameters. For standard manual protocol, the user can select the frame rate (frames per second), length of movie (minutes), toggle on/off solution delivery to the chip, delivery volume (typically 20 μL), and location of the delivery mix on a 96-well plate (for example: A1 or B10).

The laser settings can also be set by the user. For each 532- and 642-nm laser setting, a corresponding backscatter back-reflected (BSBR) reference image needs to be selected. The BSBR reference image is used by the instrument software to align and maintain the position of the SMRT Cell and the laser beamlets as well as prevent drift allowing for stable collection of data for several hours. For a new laser setting, a new BSBR reference must be acquired, which can be completed using a step-by-step process through the SMFM user interface and standard green and red fluorescent dyes. Pressing the Load Parameters button loads the user-entered parameters into the instrument.

The delivery mix (if any) and immobilization mix containing the biomolecule to be immobilized initially on the bottom of the ZMWs should be prepared at the bench first, except for the labile reagents that are time-sensitive (for example, PCD should be added only right before the experiment) (*SI Materials and Methods*). The SMRT Cell should also be prepared as follows: (*i*) wet the chip with wash buffer (for example, the polymix buffer discussed in *SI Materials and Methods*) by pipetting ∼40 μL onto the chip; (*ii*) withdraw the buffer by placing the pipette tip against the wall of the SMRT Cell and pipetting out the liquid (be careful not to scratch the bottom of the chip); (*iii*) add 20–40 μL Neutravidin reagent (or equivalent) (*SI Materials and Methods*) onto the chip, incubate at room temperature for 5 min, and then withdraw the liquid; (*iv*) add 40 μL wash buffer onto the chip, pipette the liquid up and down 10 times, and repeat this wash process three times; and (*v*) leave 40 μL wash buffer on the chip. The SMRT Cell chip can now be placed at room temperature until ready for immobilization for 1–2 h.

If the user is performing an experiment with on-instrument delivery, the user should finish preparing the delivery mix (25 μL if doing a delivery of 20 μL to account for instrument dead volume); the oxygen scavenging reaction should be initiated at this time. The user will pipette the delivery mix into the corresponding well of the 96-well plate specified previously in the parameters. The 96-well plate is placed in the front lower right slot of the reagent/sample drawer of the custom *RS* instrument (Fig. S2*B*). The plate is kept at 4 °C throughout the run. The barcode for the particular SMRT Cell to be used can be scanned for tracking purposes. Check the box indicating that the sample plate has been loaded and press Inventory Scan. This step allows the robotic arm with machine vision cameras to locate the inventories (sample plate, pipette tips, etc.) precisely. This step takes ∼3 min.

During the inventory scan process, the SMRT Cell can be immobilized by removing the buffer and adding 20–40 μL immobilization mix with a pipette, and it is incubated at room temperature for 3–5 min. The SMRT Cell is then washed with wash buffer with oxygen scavenging three times by pipetting up and down 10 times each (similar to the previously described method). Leave 20 μL wash buffer on the SMRT Cell for experiments with on-instrument delivery and 40 μL wash buffer on the SMRT Cell for experiments without delivery.

After inventory scan is completed, the user is prompted to press Clear Workspace, which clears the instrument work deck for the upcoming run. This process takes ∼1 min. The SMRT Cell can then be placed onto the chip holder (called SWATJig) on the lower middle of the SMRT Cell/Tips drawer (Fig. S2*C*). Place the SMRT Cell, with the notched corner facing the upper right side, on the first top left position of the SWATJig. Press Run, and the run will start.

During the run, the automated robotic arm will pick up the SMRT Cell from the SWATJig and move it to the imaging area, which is stabilized on a six-axis stage. The SMRT Cell will be aligned to the cameras as well as the individual laser beamlets (Fig. S3). The alignment is completed by comparison with the BSBR reference, and therefore, the entire process does not require the lasers to be turned on, avoiding photobleaching. The process of moving the SMRT Cell to the stage and aligning it takes ∼7–10 min. For a delivery experiment, the robotic arm will pick up a pipette tip and pipette the delivery mix from the specified position of the 96-well plate. After the start of data acquisition, the laser will turn on, which is followed shortly by the addition of the delivery mix (∼10 s into the movie). After the run is completed, the SMRT Cell will be moved back to the SWATJig.

2. Data Analysis Pipeline. After each run, four files are saved: mcd.h5, upd.h5, metadata.xml, and trc.h5. The metadata.xml, mcd.h5, and upd.h5 files save the run parameters, laser on times, spike times, ZMW positions, and user input information. The trc.h5 file saves the trace data. The h5 files can be viewed with HDFView (www.hdfgroup.org/hdf-java-html/hdfview/) or extracted through

Matlab into matrix format for additional processing. Using the extracted data (a 3D matrix with time, the four spectral colors, and molecules) and the corresponding ZMW positions, the mean fluorescent intensity can be plotted as shown in Fig. S5*B*, and the whole-chip view of intensity can be plotted as shown in Fig. S5*A*. The data can then be fed into Hidden Markov Models (3) or Bayesian inference models for state assignment.

3. Stickiness Problems and Possible Ways to Overcome. Nonspecific interactions between labeled proteins and Neutravidin reagent or the aluminum walls of the ZMWs inhibit single-molecule analysis on the ZMWs. Stickiness is usually manifested in the form of aggregation of fluorescent intensity on delivery of the sticky labeled protein, which prevents analysis of arrival and departure of the protein of interest. A simple way to test whether a labeled protein is sticky is to deliver the protein onto an SMRT Cell that only has Neutravidin reagent immobilized (in the absence of the target biomolecule that the protein is supposed to bind). Although reasons for stickiness are diverse and there are currently no effective ways of eliminating the problem, there are a variety of methods to mitigate the problem. Because most stickiness is a nonspecific interaction between the labeled protein and Neutravidin reagent, one way to overcome this problem is to use SMRT Cells without Neutravidin reagent preimmobilized. Instead, the biotinylated molecule to be studied can be directly coupled to Neutravidin before immobilization on the SMRT Cell surface, greatly reducing the amount of excess Neutravidin present on the chip. Another method is to use additives that may block the surface from nonspecifically interacting with the labeled protein or repel through charges on the labeled protein. The addition of 0.1–5 mg/μL BSA or ∼1 μM DNA double-stranded oligonucleotides helps alleviate the stickiness problem. Because the sources of stickiness are varied, careful testing and controls will be required to determine the appropriate mitigation strategy for the system of interest.

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9. Altman RB, et al. (2012) Enhanced photostability of cyanine fluorophores across the visible spectrum. *Nat Methods* 9(5):428–429.
10. Altman RB, et al. (2012) Cyanine fluorophore derivatives with enhanced photostability. *Nat Methods* 9(1):68–71.

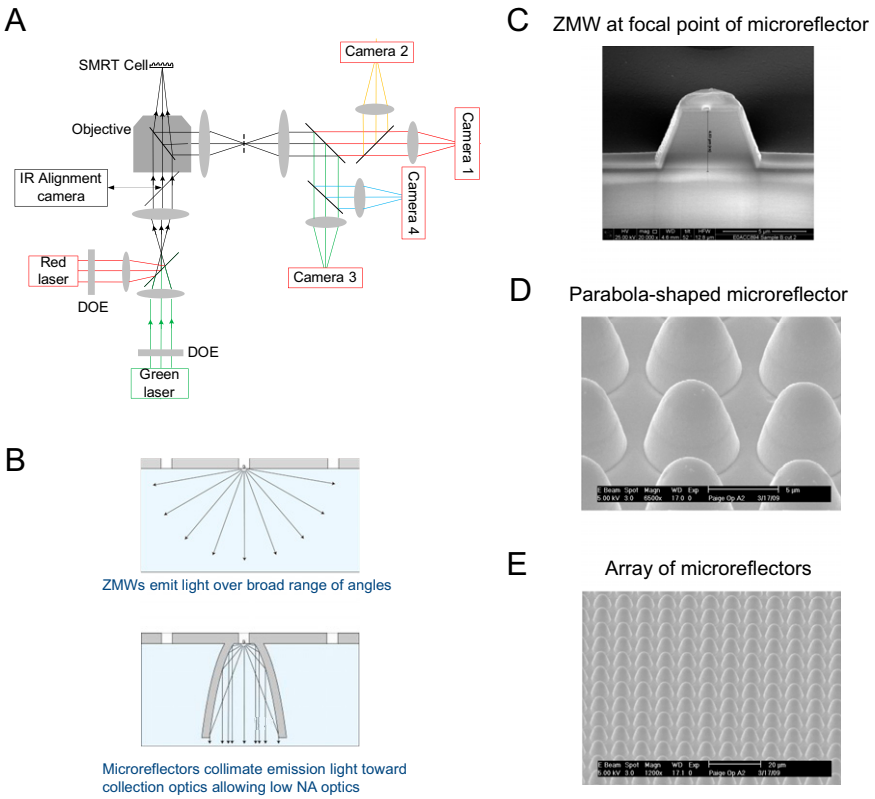

Fig. S1. PacBio *RS* optical path. (*A*) Continuous excitation is provided by a 532-nm laser and a 642-nm laser. A high multiplex is created by passing the red and green laser beams through separate diffractive optical elements (DOEs) before passing through the objective. The ~75,000 beamlets illuminate the SMRT Cell array of ZMWs (metallic apertures of diameter ~ 150 nm patterned on a fused silica substrate); the SMRT Cell sits on a six-axis stage during data acquisition. The emitted light is collected through the same objective in epifluorescence mode; notch filters block transmission of excitation wavelengths. Wavelength separation filters separate dyes into four separate channels, with low cross-talk between ZMW wells (<1%). The collected fluorescence light is imaged onto four high-speed complementary metal-oxide-semiconductor (CMOS) cameras. Alignment and spatial drift control is achieved using an infrared camera that images fiducial structures on the SMRT Cell to stabilize the SMRT Cell and beamlets in real time. (*B*) Microreflectors beneath the ZMWs collimate emission light to collection optics, allowing low N.A. optics. The fluorophore molecule emission is dominated by electric dipole radiation, but the molecule is not stationary; its orientation changes rapidly compared with the time resolution of the detection instrumentation (75 Hx), and thus, the radiation pattern is well-approximated as a Lambertian source. The N.A. of the optical system is 0.50; for highly parallel detection over a large field of view, a higher collection N.A. is impractical. The microreflector fabricated around each ZMW acts to partially collimate the emission, collapsing the radiated photons into a smaller angular range that can be collected and resolved by the optical system. (*C*) A scanning electron microscope (SEM) microphotograph of one such micromirror structure depicts a cross-section of a roughly parabolic shape, with the ZMW located at the focus. An ideal parabolic profile is difficult to achieve in high-volume microfabrication, but in practice, an enhancement of the brightness of ZMWs of over six times (relative to a control ZMW without micromirror) is routinely achieved. (*D*) SEM microphotograph of microreflectors before the patterning of ZMWs. (*E*) SEM microphotograph of a field of microreflectors, where the density is increased to maximize the number of observation sites (ZMW/microreflector combinations) that are in the field of view of the optical system.

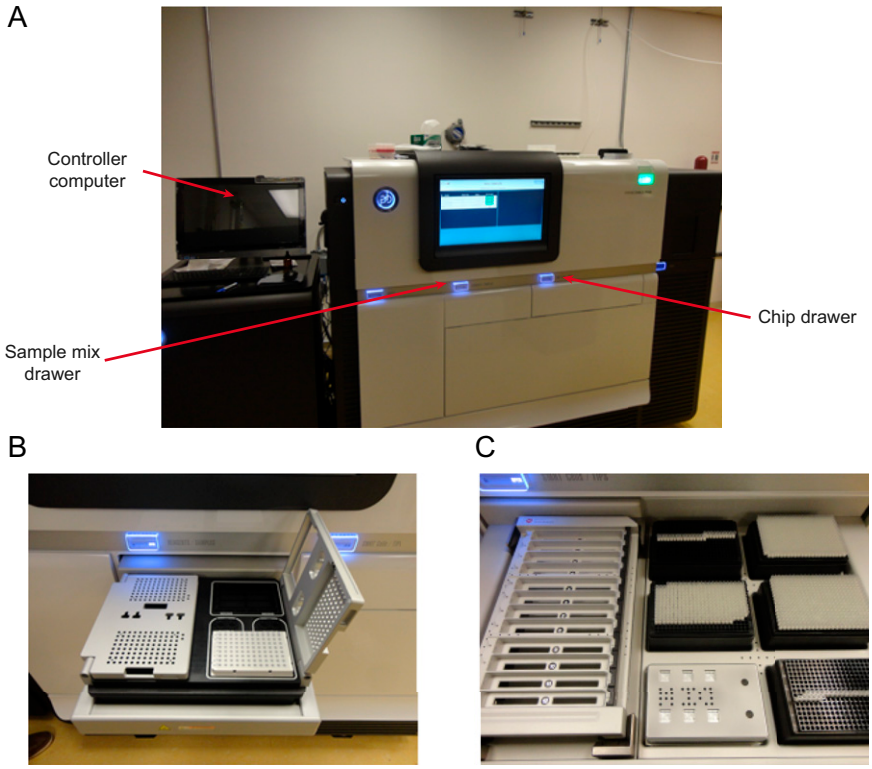

Fig. S2. Custom *RS* Instrumentation. (*A*) Picture of the custom *RS* instrument. The software to control the custom *RS* in the SMFM experimental mode is housed in the PC computer on the left. The computer sits on a podium that houses the temporary data storage and the primary data analysis pipeline for DNA sequencing. The movie data that are collected from the customized experimental runs are also stored temporarily here. The upper portion of the custom *RS* instrument contains the automated fluidics machinery, the lower portion of the instrument houses the optics, and an environmental unit on the right controls the temperature and humidity inside the instrument. (*B*) The sample mix drawer. Delivery mix is pipetted into a 96-well plate, shown on the lower right, which is kept at 4 °C continuously. The automated robot pipettor will aspirate the delivery mix before acquisition and synchronize the spike with the start of the acquisition. (*C*) The chip and pipette tip drawer. The manually prepared SMRT Cell is placed onto the chip holder (middle lower block), and the automated robot arm will move it onto the imaging stage before acquisition. The pipette tips used for real-time delivery are also kept in this drawer.

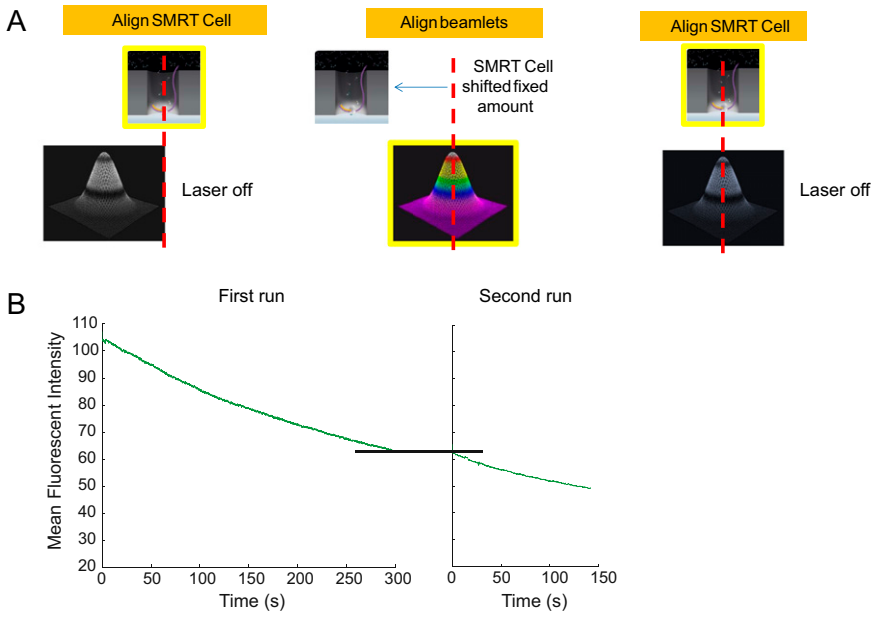

Fig. S3. Unique photobleaching-free alignment. (*A*) The alignment procedure of the PacBio *RS* relies on precisely positioning the beamlets on the ZMWs, which can cause excessive photobleaching of the dyes needed for the custom SMFM assays before the start of the acquisition. The alignment procedure created for the SMFM mode on the custom *RS* relies on the drift control algorithm in a three-step process: (*i*) initial alignment of the SMRT Cell to a reference image (the dashed red line depicts the ideal alignment position from the reference), (*ii*) alignment of lasers while the SMRT Cell is physically shifted away from the lasers, and (*iii*) then a final alignment of the SMRT Cell. This process avoids unnecessary laser exposure to experimental components before the start of data acquisition. (*B*) Example mean Cy3B-labeled DNA oligonucleotide (immobilized on the bottom of the ZMWs) (*SI Materials and Methods*) intensity time traces taken from a 5-min movie followed by an alignment step, which is followed by another movie on the same SMRT Cell (Fig. S5). The amount of signal lost during the alignment step between movies was negligible (less than 2% on average).

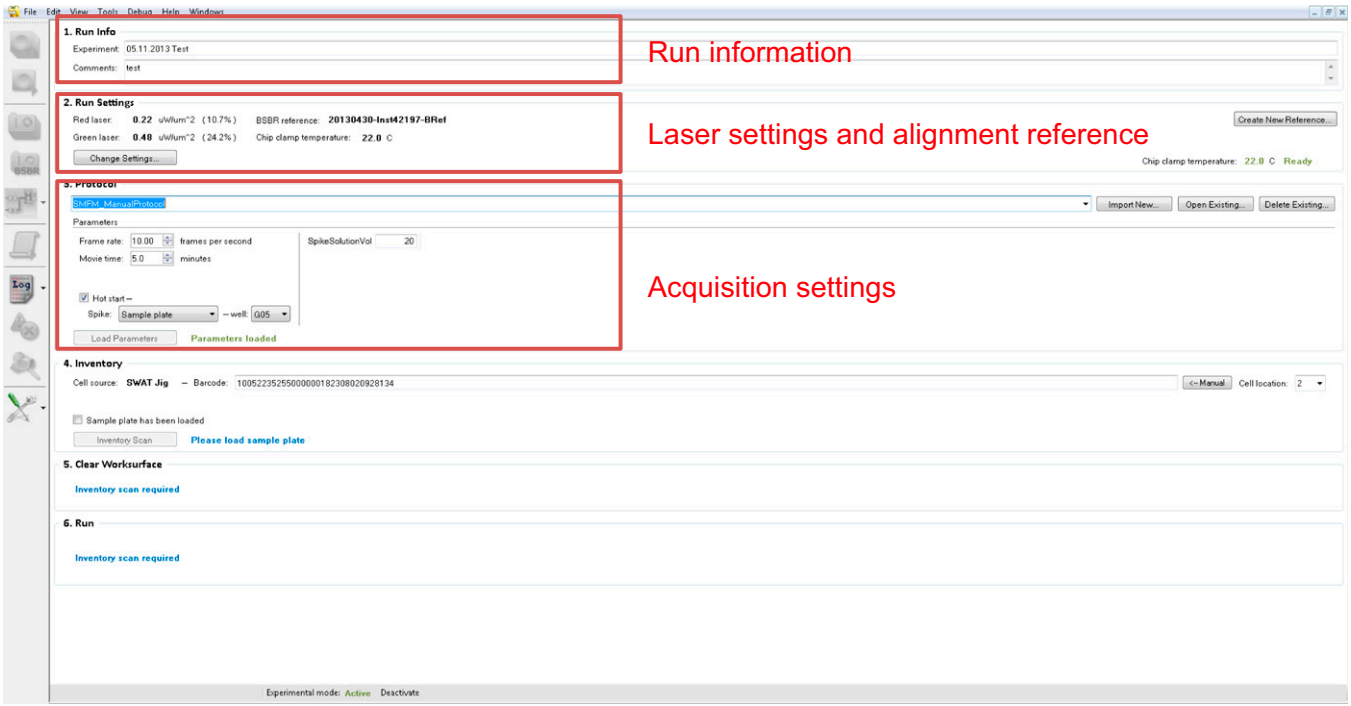

Fig. S4. Controller software. Screenshot of the user interface for controlling the SMFM experimental mode. Run information is entered at the top and will be saved along with the data at the end of the acquisition. Laser settings can be modified, although for each combination of laser intensities, a new alignment reference image (BSBR reference) will need to be acquired (*SI Results and Discussion*). The run parameters can also be modified, including the movie time, frame rate, delivery mix volume, and the particular well of the 96-well plate holding the delivery mix. Before the acquisition, an inventory scan (~3 min) and a clear work surface (~1 min) need to be performed before the run can be started. It is possible to switch from the customized experimental mode to regular DNA sequencing mode simply by deactivating the experimental mode at the bottom of the user interface. More information on the experimental workflow is provided in *SI Results and Discussion*.

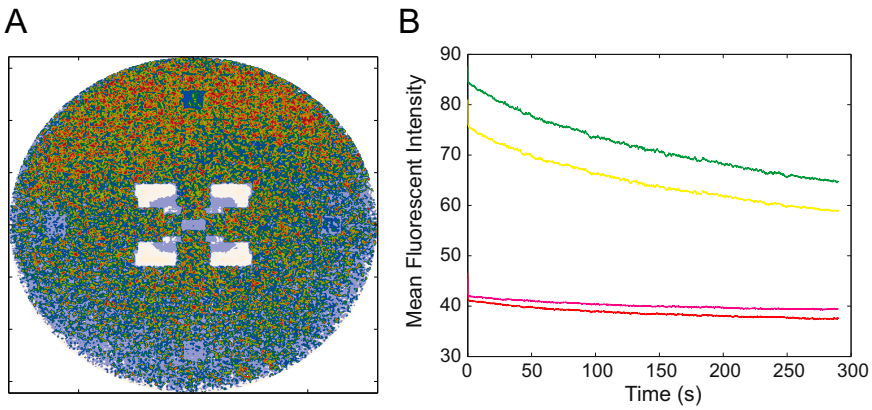

Fig. S5. Preanalysis scripts. (*A*) In-house Matlab scripts to preanalyze the data after acquisitions are completed. The chip view shows a heatmap of fluorescence intensity (in this case, it is Cy3 intensity) at the start of acquisition (in this case, $t = 10$ s), with the red being the highest intensity and blue being the lowest intensity. The chip view allows visualization of immobilization concentration. A loading percentage of ∼30% is the maximum loading concentration based on Poisson statistics (1) to have statistically singly loaded ZMW holes. We usually load at lower concentration (∼15%) to underload and ensure that most of the ZMW holes are singly loaded. (*B*) A mean fluorescence intensity plot for the four spectral channels (green, Cy3; yellow, Cy3.5; red, Cy5; purple, Cy5.5) averaged over all of the ZMWs in an SMRT Cell as a function of time. The mean fluorescence intensity plot allows quick visualization of key experimental parameters to determine quickly whether the run was successful.

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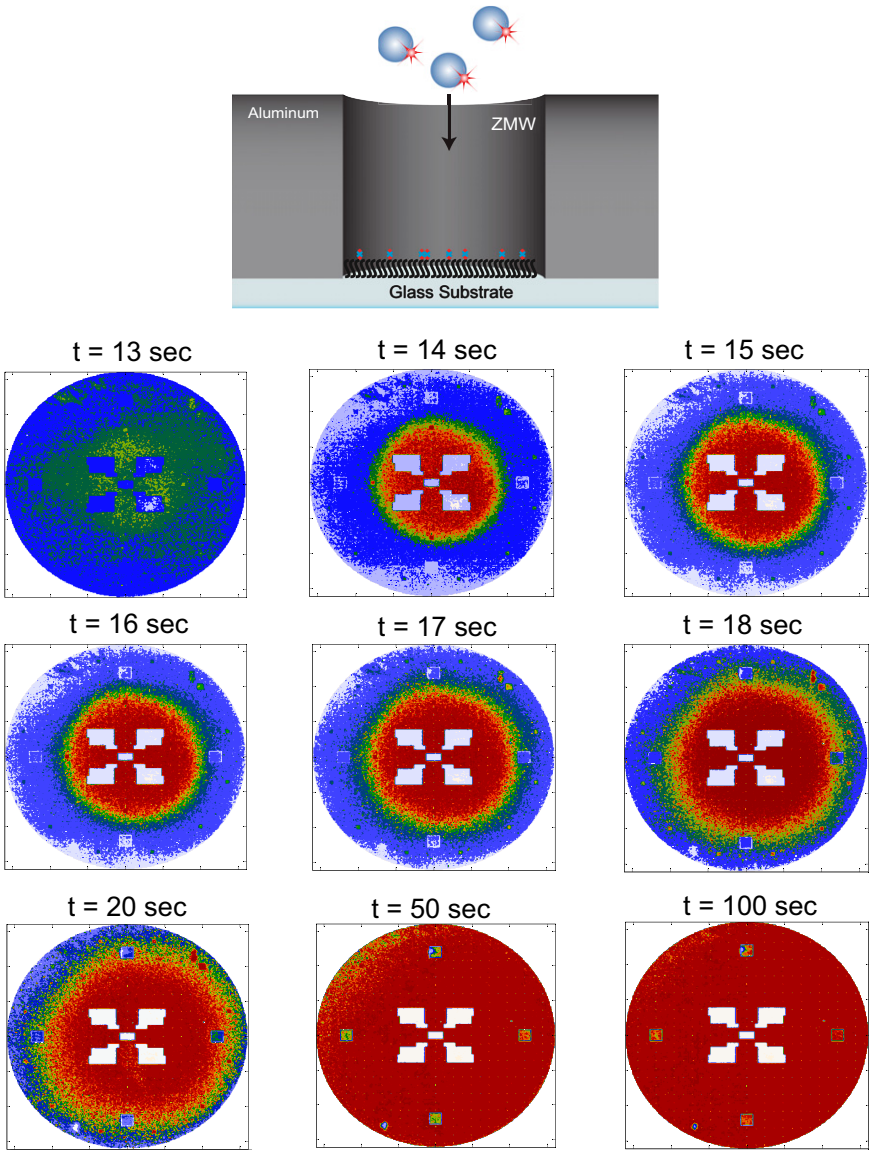

Fig. S6. Delivery characterization with automated fluidics. To characterize the automated robotics for fluidics, we delivered 1 μM Cy5-labeled protein [Cy5-labeled elongation factor G at a single cysteine at the 301 position, which was purified and labeled as described previously (1)] to an SMRT Cell preincubated with Neutravidin reagent. This particular mutant protein is known to nonspecifically interact with Neutravidin reagent, which we took advantage of for a simple assay of the mixing kinetics on delivery. Shown is a heatmap of Cy5 intensity (explained in Fig. S5) as a function of time. On delivery at the ∼13-s time point, the delivery mix containing the Cy5-labeled protein is centralized. The wavefront of the delivery mix expands, and finally, the entire chip is well-mixed at ∼20 s. This short delay before uniform mixing is problematic for experiments where the first arrival time of a ligand is important, although subsequent arrival times will not be a problem after uniform mixing. To overcome this problem, we have developed a script to only pick out the central portion of the chip where mixing is immediate for postanalysis.

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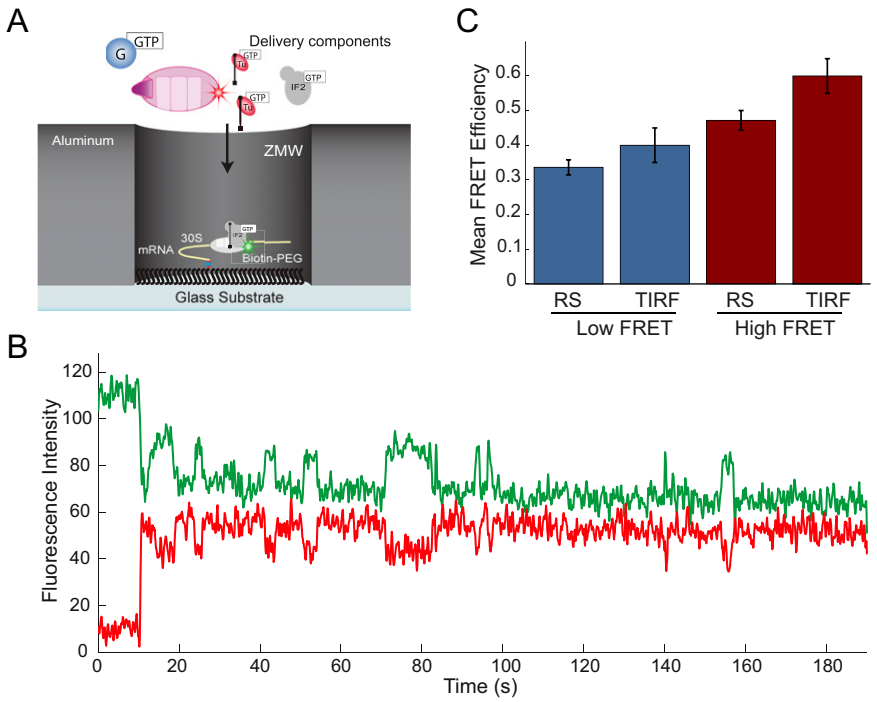

Fig. S7. Analysis of Cy3/Cy5 FRET. (*A*) Schematic of experiment: we immobilized Cy3B-labeled 30S preinitiation complex on the bottom of the ZMW through a biotinylated mRNA; after acquisition had started, we introduced the rest of the elongation factors, including Cy5-labeled 50S, EF-G, IF2, GTP, and tRNA ternary complex (Lys-tRNA^{Lys}-EF-Tu-GTP and Phe-tRNA^{Phe}-EF-Tu-GTP) (*Materials and Methods*). The mRNA translated is 6(FK). (*B*) Example trace of Cy3/Cy5 ribosomes elongating, with the low-FRET state corresponding to the rotated state of the ribosome and the high-FRET state corresponding to the nonrotated state of the ribosome. (*C*) The mean FRET efficiency (corrected for background) for both low and high FRET is ∼0.15 FRET values lower on the *RS* than total internal reflection fluorescence (TIRF). Number of molecules analyzed was $n = 794$.

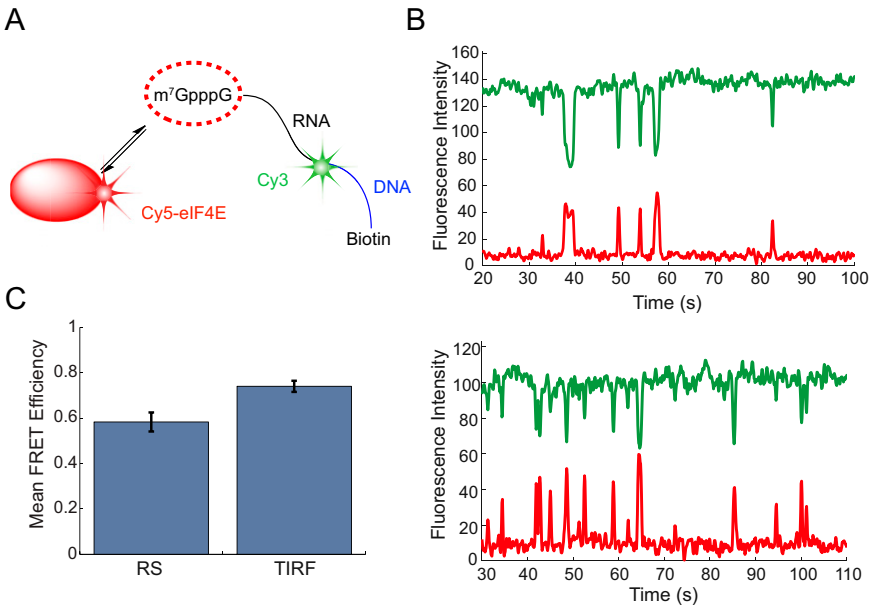

Fig. S8. FRET with eukaryotic translation initiation system. (*A*) Schematic of the eukaryotic translation initiation assay. Binding of the Cy5-labeled *Saccharmoyces cerevisiae* RNA cap binding initiation factor eIF4E to a capped RNA ligated to Cy3-labeled biotinylated DNA results in FRET between the two dyes, which was described by O'Leary et al. (1). The protein was labeled by introduction of a single-cysteine mutation [eIF4E(A124C)], expression of the recombinant N-terminally His_6-tagged protein in *E. coli*, and labeling with Cy5-maleimide. The RNA was immobilized at 1 nM at the bottom of the ZMW (protocol similar to the ribosome translation assay described in *SI Materials and Methods*), and 200 nM Cy5-eIF4E was delivered to the surface. (*B*) Example traces of Cy3/Cy5 FRET between the Cy3-RNA and Cy5-eIF4E, with the FRET events indicating the binding of eIF4E to the RNA. The experiment was performed with a single 532-nm excitation and acquired at 10 fps. (*C*) The mean FRET efficiencies on the custom *RS* compared with TIRF. The FRET value on the custom *RS* is around ~0.15 FRET values lower than on TIRF (0.58 ± 0.02 on the custom *RS* and 0.74 ± 0.04 on TIRF). Number of molecules analyzed was $n = 1193$.

1. O'Leary SE, Petrov A, Chen J, Puglisi JD (2013) Dynamic recognition of the mRNA cap by Saccharomyces cerevisiae eIF4E. *Structure* 21(12):2197–2207.

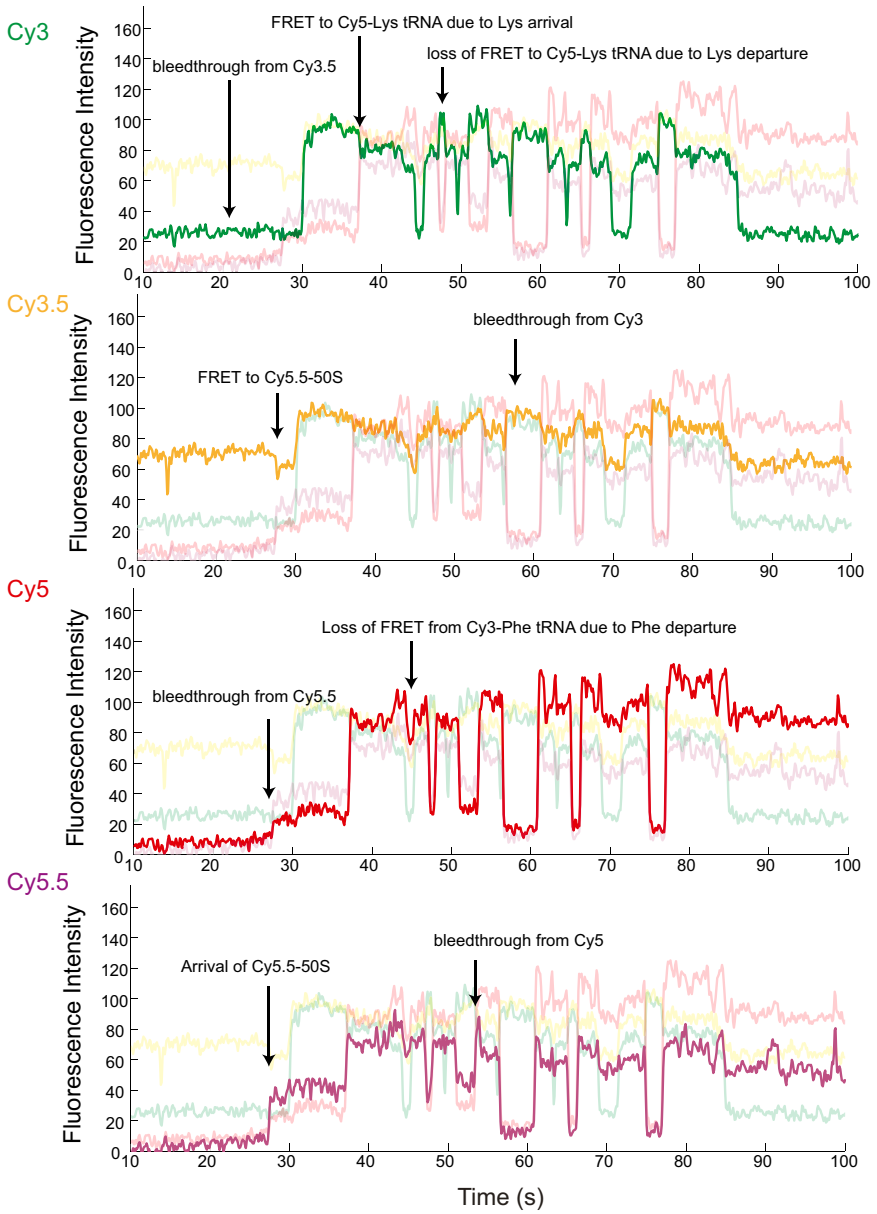

Fig. S9. Explanation of the four-color trace. Explanation of the trace in Fig. 6*A* by walking through each of the individual spectral colors. At the start of the trace, only Cy3.5-30S preinitiation complexes are immobilized, and therefore, only yellow fluorescent signal is present. The Cy3.5 signal bleeds into the Cy3 channel. After delivery, the arrival of Cy5.5-50S is manifested through the appearance of a purple signal. Because the position of the dyes on the 30S and 50S subunits allows FRET and because Cy3.5 and Cy5.5 have sufficient spectral overlap for FRET, we see a corresponding decrease in Cy3.5 signal on 50S arrival because of FRET. The red Cy5 signal increases because of bleedthrough from the Cy5.5 signal. The appearance of the green signal signifies the arrival of Cy3-Phe tRNA to the ribosome A site, with a corresponding increase in the Cy3.5 channel caused by bleedthrough. The arrival of Cy5-Lys tRNA, which was specified by the mRNA sequence, is manifested through the appearance of the red signal. Simultaneously, because of the proximal positioning of the two tRNAs in the ribosome (now in the A and P sites), the green Cy3 signal drops because of FRET to Cy5. The Cy5.5 signal also increases because of bleedthrough from the Cy5 channel. After translocation, the Cy3-Phe tRNA departs, which is signified by the loss of green signal. Correspondingly, we see a small dip in the Cy5 signal as it is lost from FRET from Cy3 to Cy5. The next Phe tRNA then arrives, and this cycle repeats for 12 codons specified by the mRNA. Note that, because of the simultaneous excitation by the 532- and 642-nm lasers, FRET is sometimes not obvious.

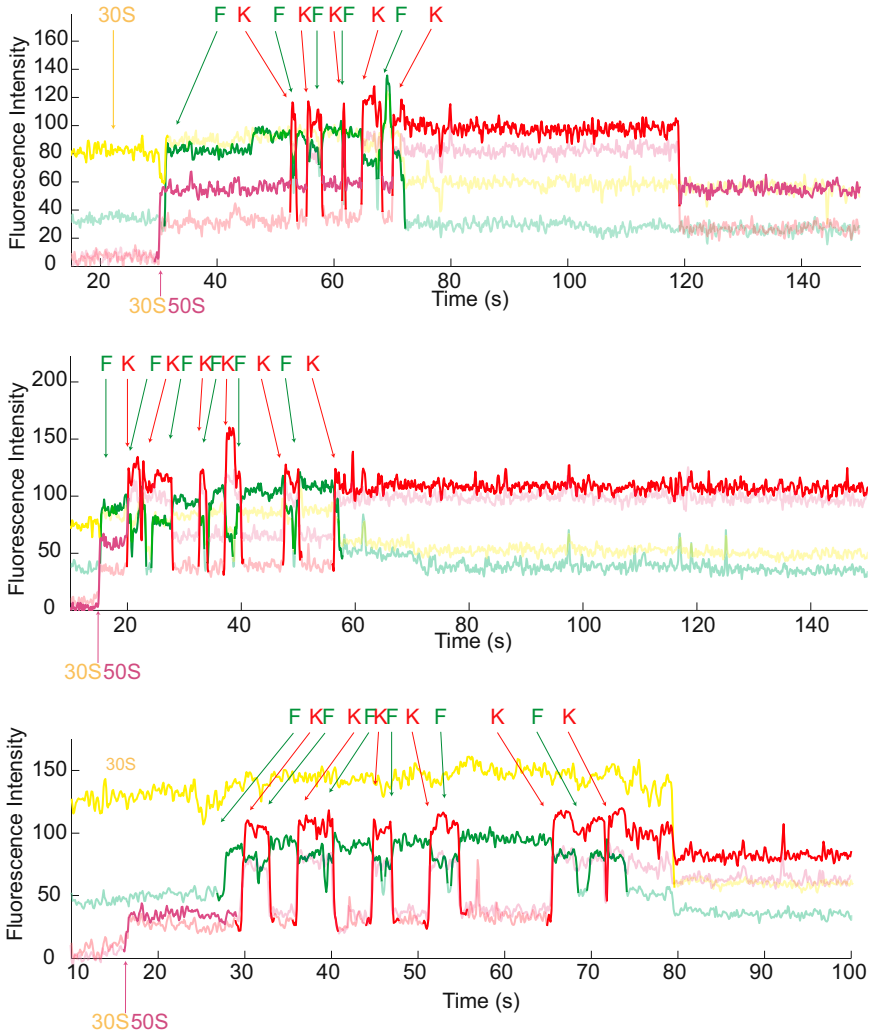

Fig. S10. Gallery of single-molecule four-color traces. More example traces of the experiment described in Fig. 6 and Fig. S9, and they show the ability of the custom *RS* to track complex biology through four spectral channels.

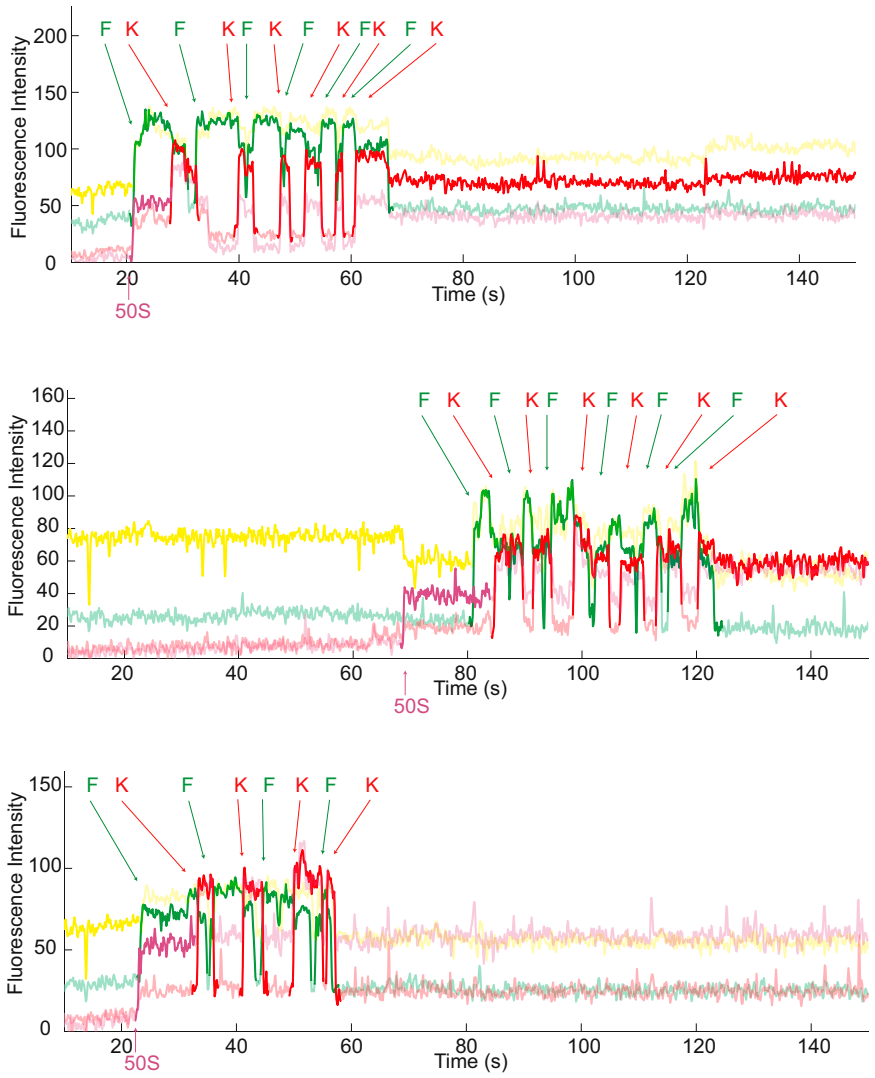

Fig. S11. Gallery of single-molecule four-color traces. More example traces of the experiment described in Fig. 6 and Figs. S9 and S10, and they show the ability of the custom *RS* to track complex biology through four spectral channels.