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Supporting Material

A single-molecule study of ribosomes' hierarchic dynamics at the peptidyl transferase center

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Supporting information for

A single-molecule study of ribosomes' hierarchic dynamics at the peptidyl transferase center

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1. Materials and Sample preparations

Buffers

All single molecule experiments were performed in TAM_{10} buffer: 20 mM Tris, pH 7.5; 30 mM NH_4Cl; 70 mM KCl; 10 mM $MgCl_2$ and 1 mM DTT. The ribosomes were purified in B1 buffer: 20 mM Tris, pH 7.5; 100 mM NH_4Cl; 10 mM $MgCl_2$; 3 mM BME (2-mercaptoethanol); 0.5 mM EDTA. The L27, EF-G, EF-Tu, EF-Ts and *E. coli* tRNA synthetase (Phenylalanine specific) are stored in storage buffer: 20 mM Tris, pH 7.5; 400 mM KCl; 10 mM $MgCl_2$; 4 mM BME (2-mercaptoethanol); 0.5 mM EDTA. L27 labeling buffer was composed of: 20 mM Tris, pH 7.5; 100 mM NaCl.

Material preparation and surface immobilization of ribosomes

The His-tagged Proteins EF-G, EF-Tu, EF-Ts and *E. coli* tRNA synthetase (Phenylalanine specific) were expressed and purified using standard methods (Qiagen purification kit or Histrap FPLC column from GE healthcare). N-acetyl phenylalanine-tRNA was prepared essentially as described (*1*). 5'-biotinylated poly(U) was purchased from Dharmacon RNAi Technologies, Thermo Scientific. All the reagents were from Sigma unless otherwise stated.

Ribosomes, Cy5-L27 and Cy3-tRNA^{Phe}. Ribosomes lacking L27 were isolated from *E. coli* strain IW312 (a kind gift from Prof. Zimmermann of U Mass, Amherst MA) as described (*2*), except that in the 10-40% sucrose gradient purification step the 70S is not dissociated. The rpmA gene was subcloned into the PET-20b plasmid from the PET-3b plasmid (a kind gift from Prof. Wower of University Auburn, Auburn AL), The C-terminal His-tagged L27 was expressed, purified and labeled with a Cy5-maleimide mono-reactive dye (Amersham Biosciences) dissolved in DMSO. Yeast $tRNA^{Phe}$ (Sigma) was labeled at the 16/17 position with Cy3-hydrazide (GE healthcare) as described (*3*). The labeled L27 was incorporated into the ribosome 70S subunit as described (*4*). The incorporation efficiency was ~ 100%, whereas incorporation into the MRE600 ribosome (with native L27) was less than 10%. The correct position of L27 incorporation into the ribosome was confirmed by the complete recovery of IW312 ribosome activity (Figure S1) (*4*).

Complex Preparation. The pre-translocation complex was made as described (*4*) and purified by sucrose cushion ultracentrifuge. The post-translocation complex was prepared by incubating the purified pre-complex (0.2 μM) with EF-G (1.0 μM), GTP (1 mM), phosphoenolpyruvate/ATP (1 mM) and Pyruvate kinase (0.02 mg/ml) in the tube. Alternatively, the post-translocation

complex was prepared by directly delivering EF-G·GTP (0.2-1 μM EF-G and 4 mM GTP) solution onto surface immobilized ribosomes.

Surface Tethering of Ribosomes. The glass cover-slips were cleaned and passivated with Biotin-PEG (5) as described. The streptavidin (0.5 mg/ml) was delivered to the surface before the single molecule acquisition. The ribosome complexes were then tethered to the surface via the streptavidin-biotinylated mRNA interaction.

2. Data classification method.

As shown in Figure S2, the ribosomes are categorized according to the following criteria (the bleaching/blinking events have been eliminated forehand):

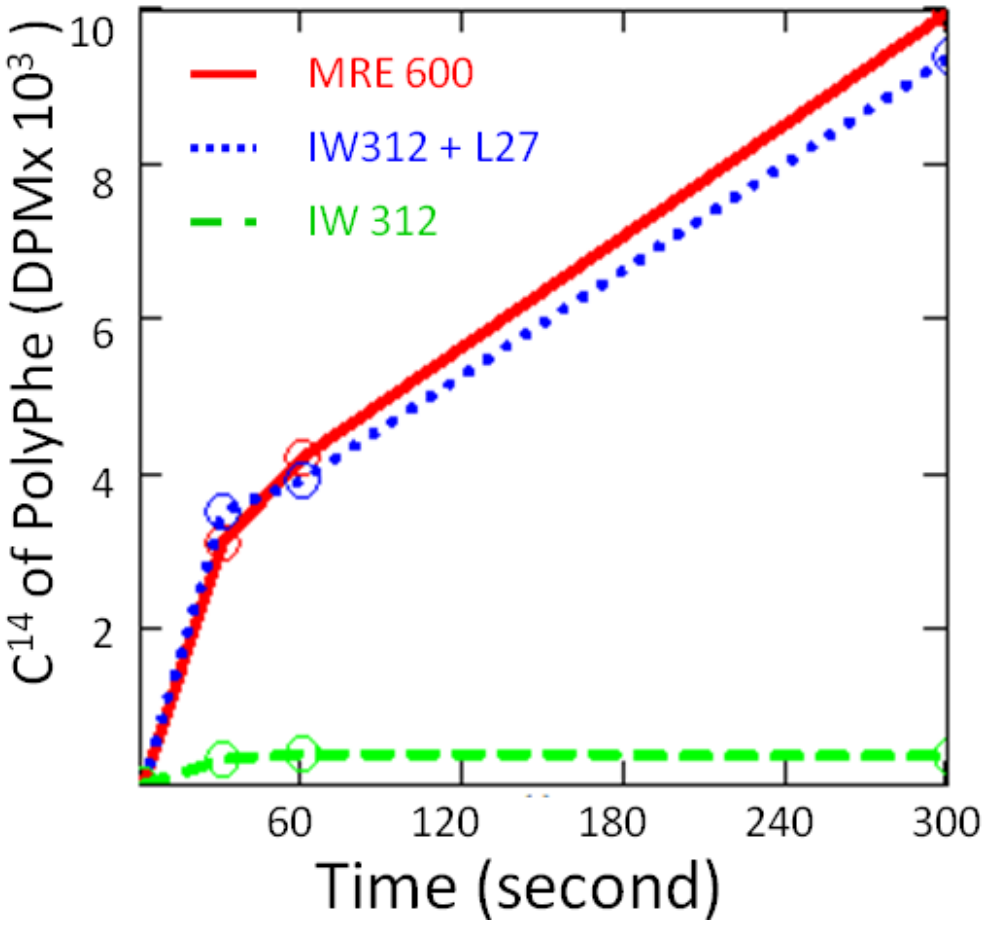
(i) Non-fluctuating (NF) ribosomes generate traces with correlation coefficients within ± 0.15 between donor and acceptor intensities (Fig. S2a). In addition, the ratio of standard deviation vs. average has to be less than 20% in both the donor and acceptor traces, corresponding to a minimum signal-to-noise ratio of 4:1.

(ii) Fluctuating ribosomes (F ribosomes) are defined by the anti-correlation between donor and acceptor fluorescence being less than -0.6. Within this subgroup, Figure. S2b showed the F-low ribosomes sample FRET ≥ 0.6 less than 5 frames (total, not required to be consecutive) in an average imaging window of 110 frames; and Figure S2c showed the F-high ribosomes sample FRET ≥ 0.6 of at least 5 frames. The threshold of 0.6 is chosen according to the FRET distributions detected (as discussed in the text). However, "5 frames" is a somewhat arbitrary threshold to tolerate random noise and was used consistently for all data. Setting this number as "1 frame" or "10 frames" generates similar results (Data not shown). Three facts support this categorization: 1. In the PRE-Complex, the average occupancy of FRET ≥ 0.6 for the F-Low ribosomes is only 0.7%, whereas the occupancy for the F-High ribosomes is 33%; 2. The lack of FRET ≥ 0.6 in the F-Low ribosomes was not due to faster bleaching because the average imaging window before bleaching for both the F-low and F-high ribosomes was ~ 110 frames, which corresponds to the dye bleaching time under our experimental conditions (11 seconds); 3. The FRET ≥ 0.6 states in the F-high ribosomes appeared at early times and were distributed evenly throughout the entire time axis, suggesting that the classification of the F-Low and F-High ribosomes is valid. Therefore, at least two types of fluctuating ribosomes existed in our experiments.

(iii) The NF-ribosomes (Fig. S2a) were sorted using the same threshold to give the fractions of ribosomes that are stable at their respective FRET states. This sorting agrees with a multiple-Gaussian fitting. The biological sensibility of our data analysis strategy is further supported by the fact that the subpopulation change correlates with the biological complexes and the conditions in our study.

2. Supporting figures.

Supporting material Figure. S1.

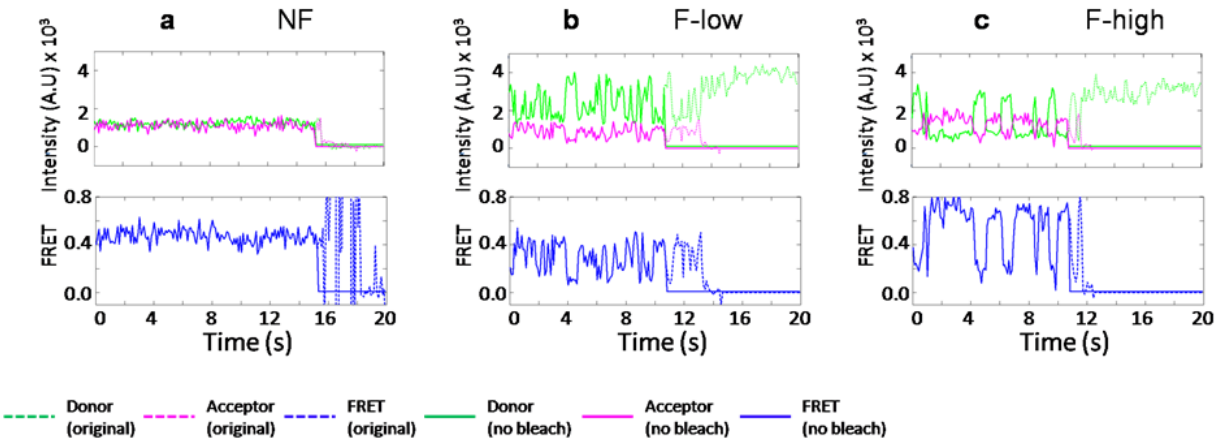


The Poly(Phe) assay of ribosome activity by using Poly(U) mRNA. The ribosome activity was assayed using ^{14}C labeled Poly-Phenylalanine generated at several time points. This figure shows that without L27, the IW312 ribosome has very little activity, whereas incorporation of L27 into IW312 restores the activity comparable to that of the MRE 600 ribosome (the wild type ribosome with intact L27 and normal activity). This assay clearly demonstrates that the L27 incorporated at the right position.

In a typical poly(Phe) assay, three mixtures were made. (1) The ribosome mixture contained 1 μM ribosomes, 1 μM poly(U) and 1 μM N-acetyl phenylalanine-tRNA in TAM_{10} buffer; (2) the factor mixture contained 4 μM EF-Tu, 6 μM EF-Ts, 2 μM EF-G, 0.5 mM GTP, 0.5 mM PEP and 0.006 mg/ml pyruvate kinase in TAM_{10} buffer; (3) the aminoacylation mixture contained 100 mM Tris (pH 7.5), 20 mM $MgAc_2$, 1 mM EDTA, 4 mM ATP, 7 mM BME, 33 μg/ml purified $tRNA^{phe}$ aminoacyl synthetase, 50 mM ^{14}C labeled phenylalanine and 5 mM $tRNA^{phe}$. All three mixtures were incubated separately at 37 ºC for 5 minutes. Then 7 μl of the ribosome mixture, 14 μl of factor mixture and 14

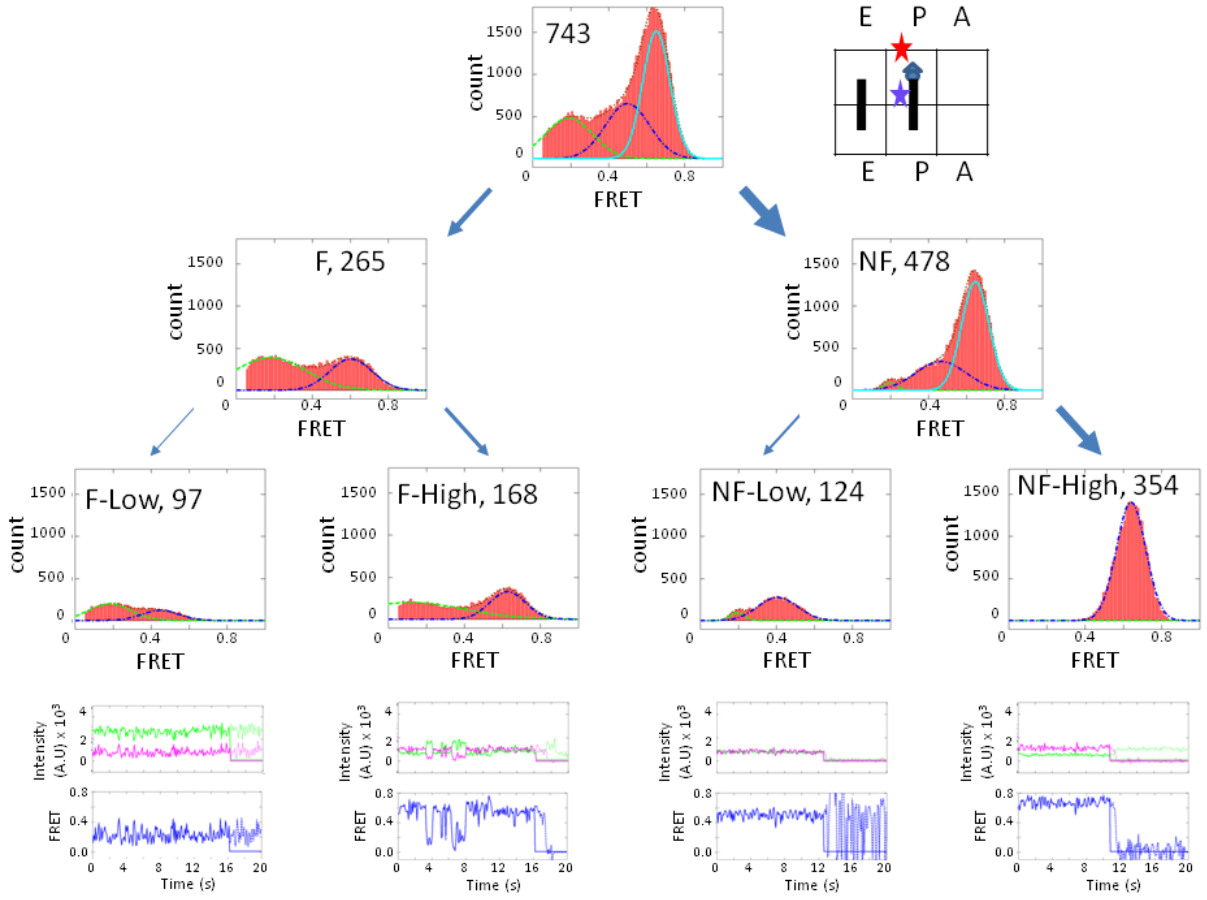
μl of aminoacylation mixture were gently mixed together to form the poly(Phe) assay solution. The poly(Phe) assay solution (10 μl) was sampled at 30 seconds, 1 minute and 5 minutes and added to 1500 μl cold TCA with 0.1 mg/ml phenylalanine on ice. TCA solutions were then heated to 90ºC for 10 minutes and then cooled on ice for at least 30 minutes before being passed through cellulose filters. Each filter was washed with 6 ml cold TCA solution and air dried. The radioactivity of the synthesized poly-phenylalanine on each filter was counted with a scintillation counter.

Supporting material Figure. S2.



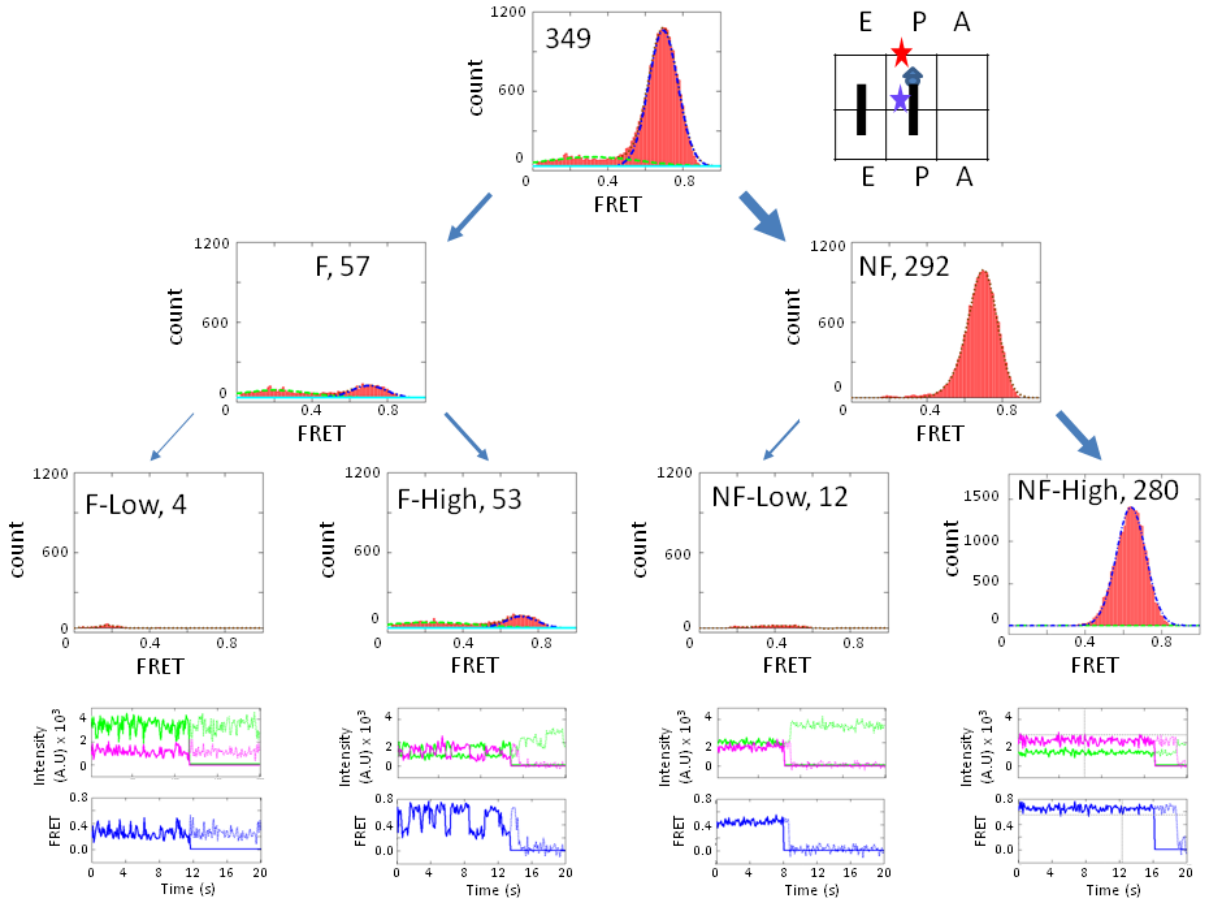
The typical ribosome traces were classified by their different dynamics. (a) a non-fluctuating ribosome trace; (b) a fluctuating ribosome trace that only samples FRET values lower than 0.6; (c) a fluctuating ribosome trace that samples FRET value higher than 0.6. The legends are shown in the plot. The fluorescence intensities of donor and acceptor are green and magenta respectively. FRET values are calculated as $I_{acceptor}/(I_{acceptor}+I_{donor})$ and plotted separately in blue. The original data are displayed in dotted lines and data truncated before the bleaching points are displayed in solid lines.

Supporting material Figure. S3.



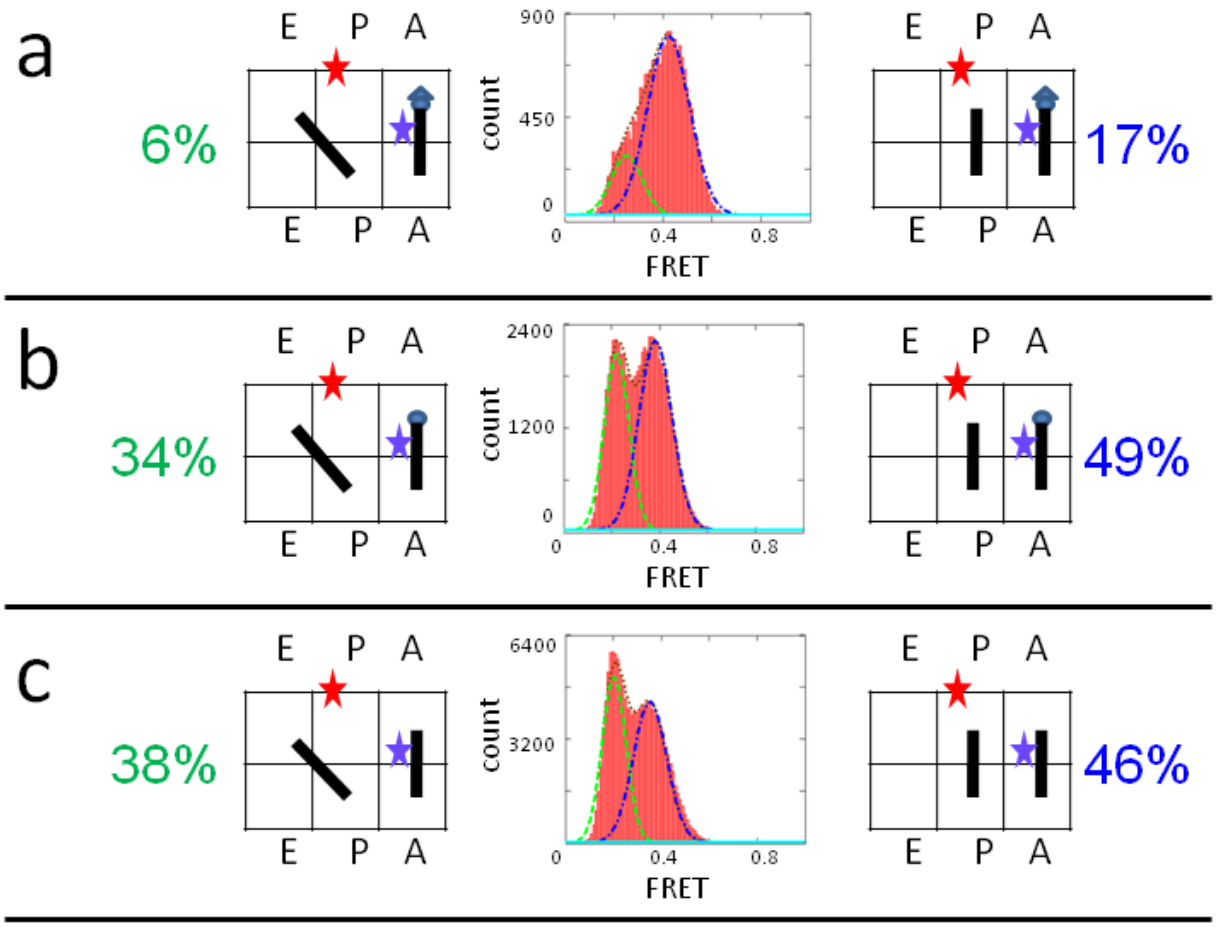
FRET efficiency histograms of POST-Complex. Four horizontally arranged plots are shown. The top tier plots show the FRET histogram of the total ribosomes (743 particles as shown in the parenthesis) and the cartoon for POST-complex. The second tier plots show the FRET histogram of the ribosomes separated as being fluctuating (F, 265 particles) or non-fluctuating (NF, 478 particles). The third tier plots show the FRET histogram of the ribosomes being further separated as being fluctuating below/above FRET value of 0.6 (F-low, 97 particles/F-high, 168 particles). The similar thresholds (as discussed in the Data process part above) are applied for the NF-ribosomes to separate them as being in stable FRET state below/above 0.6 (NF-low, 124 particles/NF-high, 354 particles). The Fourth tier plots are the representative fluorescence of donor and acceptor and the corresponding FRET traces (both the original and the truncated data) from each of the 4 subpopulations. The percentage of translocation under this experimental condition is ~ 50%.

Supporting material Figure. S4.



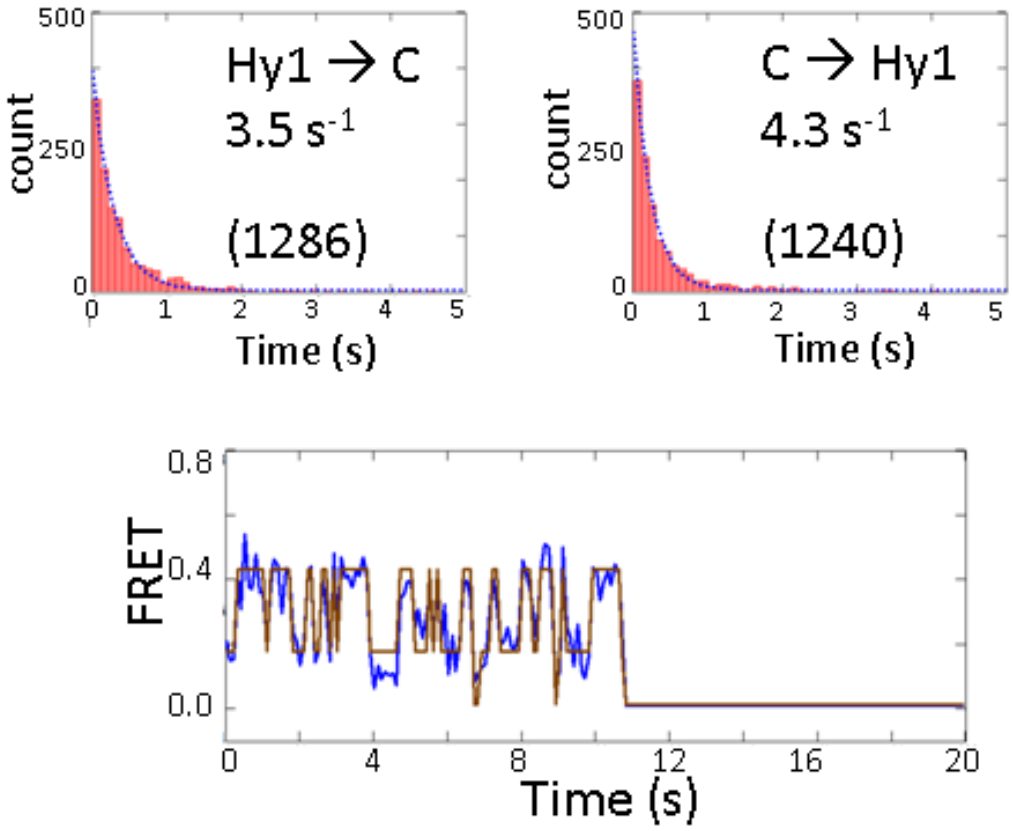
FRET efficiency histograms of POST-Complex generated in the presence of large molar excess of EF-G (0.2 μM PRE-Complex incubated with 10 μM EF-G and 4 mM GTP). All of the other descriptions are the same as in Figure S3. In this case, the percentage of translocation achieved 80.2%.

Supporting material Figure. S5.



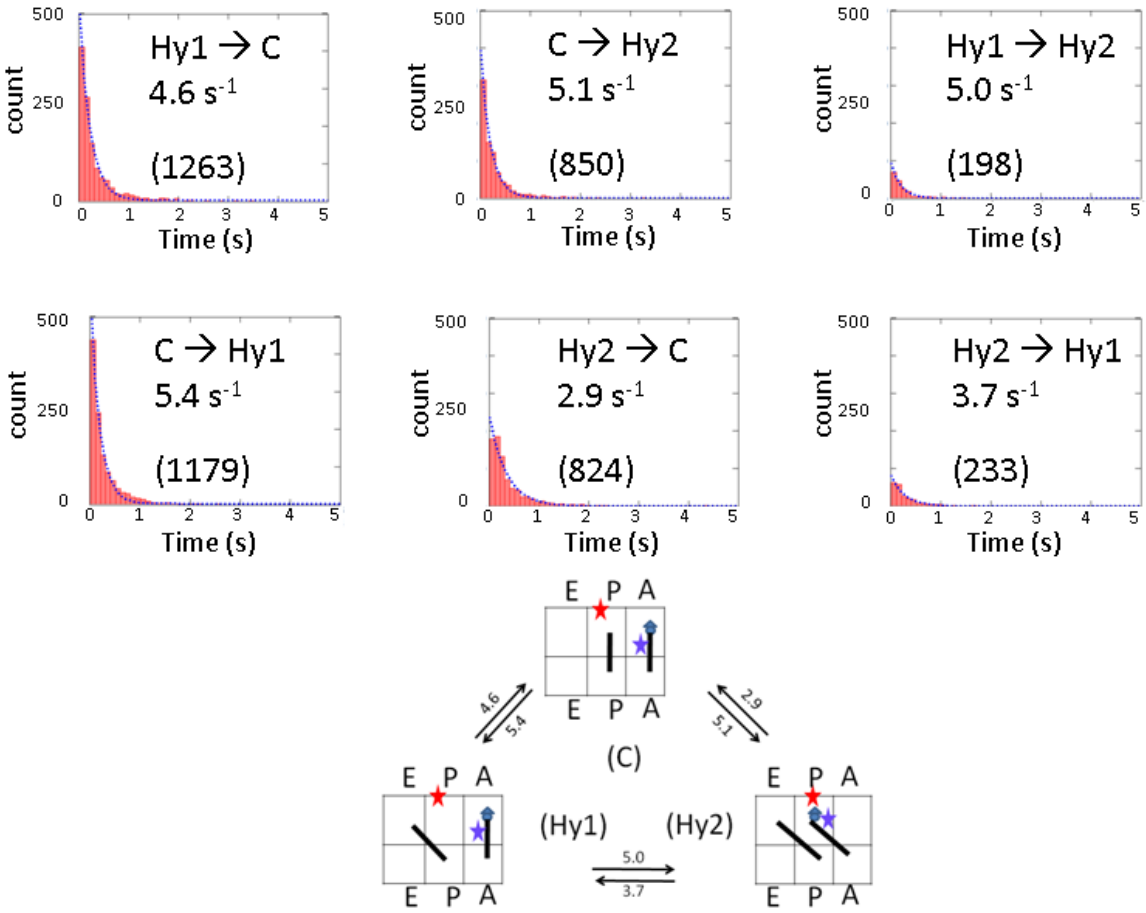
Comparison of NF-Low subpopulations in PRE-1 (a), PRE-2 (b) and PRE-3 (c). PRE-1 complex is formed via incubation of ribosome initiation complex (associated with 032 mRNA and charged fMet-tRNA$^{formyl\text{-}Met}$) and Phe-tRNA^{Phe} ·EF-Tu·GTP ternary complex. PRE-2 complex is formed via incubation of ribosome complex (associated with 032 mRNA and uncharged fMet-tRNA) and Phe-tRNA^{Phe} ·EF-Tu·GTP ternary complex. PRE-3 complex is formed via incubation of ribosome complex (associated with 032 mRNA and uncharged fMet-tRNA) and uncharged Phe-tRNA. The resulting PRE-1, PRE-2 and PRE-3 complexes contain Dipeptidyl-, Aminoacyl- and uncharged tRNAs, respectively, at the A-site. The triangle stands for formyl methionine and the circle stands for phenylalanine. The 0.2 FRET is assigned as Hy1 state (hybrid 1 state), with the conformation scheme and its percentage showing on the left side of the histogram graph. The 0.44 FRET state is assigned as C state (classical state), with the conformation scheme and its percentage showing on the right side of the histogram graph. With decreased ability of A/P formation, the Hy1 state (A/A - P/E) formation increased from (a) to (b) and from (b) to (c). There were small shifts of the 0.44 FRET state due to the slight differences on the tRNA CCA ends. These results support the assignment of Hy1 state.

Supporting material Figure. S6.



Kinetic fitting of the Hy1<==> C transitions in F-Low ribosomes. The FRET traces from 250 ribosomes are idealized and fitted using the HaMMy analysis package (6). The transitions and the rates are show in the plots. Numbers in the parenthesis are the total numbers of transitions to generate the dwell time histograms. The bottom panel is one example of the HaMMy idealized FRET transitions (brown) overlaid with the FRET trace (Blue).

Supporting material Figure. S7.



Kinetic fitting of the transitions among Hy1, C, Hy2 in F-High ribosomes of PRE-Complex. The FRET traces from 250 ribosomes are idealized and fitted using the HaMMy analysis package. The transitions and the rates are show in the plots. Numbers in the parenthesis are the total numbers of transitions to generate the dwell time histograms.

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