ONLINE METHODS

Preparation of pre-translocation ribosome complexes. Pre-translocation complexes with labeled L1 and P-site tRNA were prepared exactly as previously described[13,14]. Complexes with labeled A-site tRNA were prepared in the same manner, but with unlabeled $tRNA^{fMet}$ (or $tRNA^{Phe}$) and $tRNA^{Phe}$(Cy3-acp^{3}U47) (or $tRNA^{Lys}$(Cy3-acp^{3}U47)). All tRNAs were purchased (Sigma), fluorescently labeled, and purified by hydrophobic chromatography as described[13,15,16].

Preparation of fluorescently labeled EF-G. For enzymatic fluorescent labeling via the Sfp phosphopantetheinyl transferase reaction, EF-G was cloned into the pET-SUMO vector (Invitrogen) with a 12-residue peptide (amino acid sequence: GDSLSWLLRLLN) fused at

the C terminus[34]. The EF-G-peptide fusion was over-expressed in *E. coli* BL21(DE3) and purified by Ni chromatography. The pET29-Sfp plasmid, encoding the Sfp protein was provided by Christopher Walsh (Harvard University). The 6His-tagged Sfp was over-expressed in *E. coli* BL21(DE3), and purified by Ni chromatography. Cy5-maleimide (GE Healthcare) was conjugated to Coenzyme A (CoA) (Sigma) according to a published protocol[35]. Cy5-CoA was purified by HPLC on a C18 reverse-phase column (Varian). EF-G was labeled essentially according to the published protocol[35]. In a buffer containing 50 mM HEPES pH 7.5 and 10 mM $MgCl_2$, 17 μM EF-G, 34 μM Cy5-CoA and 8 μM Sfp were incubated for 1 hr at room temperature. Unbound Cy5-CoA and Sfp were removed by Ni chromatography. The 6His-SUMO tag was removed from EF-G by proteolytic cleavage.

Acquisition and analysis of smFRET data. All experiments were performed in Tris Polymix Buffer (50 mM Tris-OAc pH 7.5, 100 mM KCl, 5 mM NH_4OAc, 0.5mM $Ca(OAc)_2$, 0.1 mM EDTA, 5 mM putrescine, 1 mM spermidine and 50 mM BME) with 15 mM $Mg(OAc)_2$, a cocktail of triplet state quenchers (1 mM trolox, 1 mM nitrobenzyl alcohol and 1 mM cyclooctatetraene), and an enzymatic oxygen scavenging system[52,53]. The antibiotics viomycin (US Pharmacopeia), fusidic acid (Alexis Biochemicals) and spectinomycin (MP Biomedicals) were used at 200 μM, 50 μM and 5 mM, respectively. All fluorescently labeled complexes were surface-immobilized on quartz microscope slides, and mounted on a total internal reflection fluorescence microscope as described[15,16]. Movies were acquired using Metamorph (Molecular Probes) at 10 ms time resolution in the case of complexes with labeled L1 and P-site tRNA, and 100 ms time resolution in the case of labeled EF-G and A-site tRNA. Imaging under conditions in which photobleaching was insignificant was performed at 500 ms time resolution. Trajectories were extracted using custom made Matlab (MathWorks) software. Kinetic analysis of smFRET trajectories acquired from complexes with labeled L1 and P-site tRNA was performed first through idealization to the five-state model displayed in **Supplementary Figure 4** using the segmental *k*-means algorithm in QuB[31]. In contrast to previous studies of the same complexes in which each trajectory was analyzed separately[13,14], here a single set of average rate constants were determined for each data set through maximum likelihood optimization[32]. The reason for this distinction is that under the present high-illumination intensity conditions each trajectory did not display enough transitions to support individual analysis. smFRET trajectories acquired from the delivery of labeled EF-G to complexes with labeled A-site tRNA were analyzed by manually placing a threshold at 0.3 FRET. The resulting dwell times above and below this threshold were compiled into histograms, and fit to exponential functions in Origin (OriginLab). All figures were prepared in Matlab (MathWorks) and Origin. The structural renderings in **Figure 1** were generated in PyMol.