Supporting Materials

Labeled EF-Tus for rapid kinetic studies of pre-translocation complex formation

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Calculations of FRET efficiency within TCs. For the calculations below, the simplifying assumptions are made that 1) the fluorescence of Phe-tRNA^{Phe}(Cy3) and tRNA^{Phe}(Cy3) are equal (Supplementary Figure S2a); and 2) that unlabeled EF-Tu^{AV} and EF-Tu$^{AV\text{-}Cy5}$ bind equally well to Phe-tRNA^{Phe}(Cy3) (Supplementary Figure S2b).

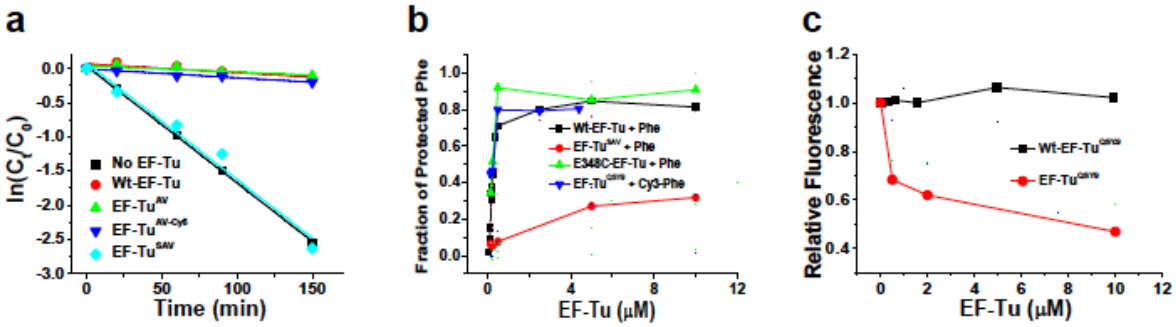
TC$^{QSY9/Cy3}$ FRET efficiency (Supplementary Figure S2a**).** The FRET efficiency within TC$^{QSY9/Cy3}$ was calculated from the fluorescence measurements presented in Supplementary Figure S2a, by comparing the Cy3 fluorescence for the same concentration of Phe-tRNA^{Phe}(Cy3) present in the TC and free in solution. The eluted TC fraction contained a total of 0.050 μM Phe-tRNA^{Phe}(Cy3). Based on the radioactivity found in the fractions immediately before and after the eluted TC, about 22% (0.012 μM) of Phe-tRNA^{Phe}(Cy3) dissociated and was present in the unbound form, so that the fraction contained 0.038 μM TC. Because the Phe-tRNA^{Phe}(Cy3) preparation was 61 % charged, the unbound [^{3}H]-Phe-tRNA^{Phe}(Cy3) solution containing 0.050 μM [^{3}H]-Phe-tRNA^{Phe}(Cy3) also contains 0.032 μM tRNA^{Phe}(Cy3), giving a total Cy3 concentration of 0.082 μM. Accordingly, the corrected free Phe-tRNA^{Phe}(Cy3) fluorescence is 0.46 of the measured value. Similarly, the corrected fluorescence value for the TC sample was obtained by subtracting the contribution of the 0.012 μM free Phe-tRNA^{Phe}(Cy3) that was present, giving a corrected value that was 0.55 of the measured value. The calculated FRET efficiency is 66%.

TC$^{AV\text{-}Cy5/Cy3}$ FRET efficiency (Supplementary Figure S2b**).** The results obtained show that a 4-fold excess of added EF-Tu.GTP suffices to convert all of Phe-tRNA^{Phe}(Cy3) into a TC. Two corrections to the results obtained were needed to calculate FRET efficiency. First, the contribution of uncharged tRNA^{Phe}(Cy3) fluorescence, which should be unaffected by EF-Tu.GTP addition, is subtracted from each of the traces. As the Phe-tRNA^{Phe}(Cy3) preparation used in this experiment was 46% charged, this amounts to subtraction of 54% of the Cy3 fluorescence measured for the sample in the absence of EF-Tu. Second, the EF-Tu$^{AV\text{-}Cy5}$ sample employed had a labeling stoichiometry of 0.85 so that the FRET efficiency resulting from the first correction was increased by 1/0.85, giving a final value of 76%.

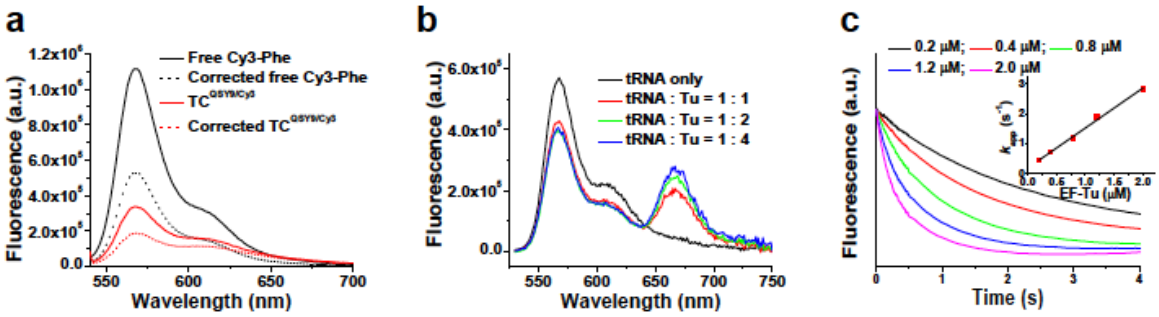
Supplementary Table 1. Labeling stoichiometries of EF-Tu variants[a].

EF-Tu variant	Label/EF-Tu
wt	0.5 QSY9 0.8 Cy3
E348C	1.5 QSY9 1.5 Cy3
C137A/C255V	0.2 Cy5
C137A/C255V/E348C	0.9 Cy5

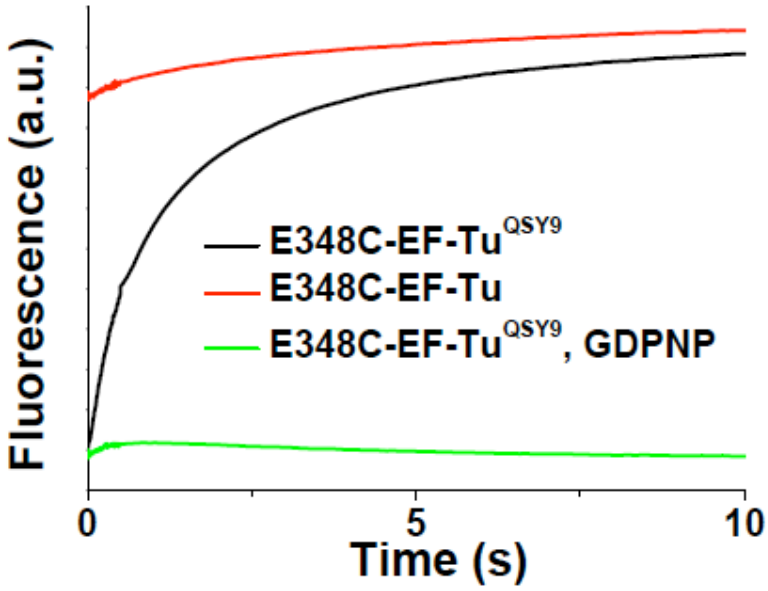
[a]Labeling conditions as described in Experimental.



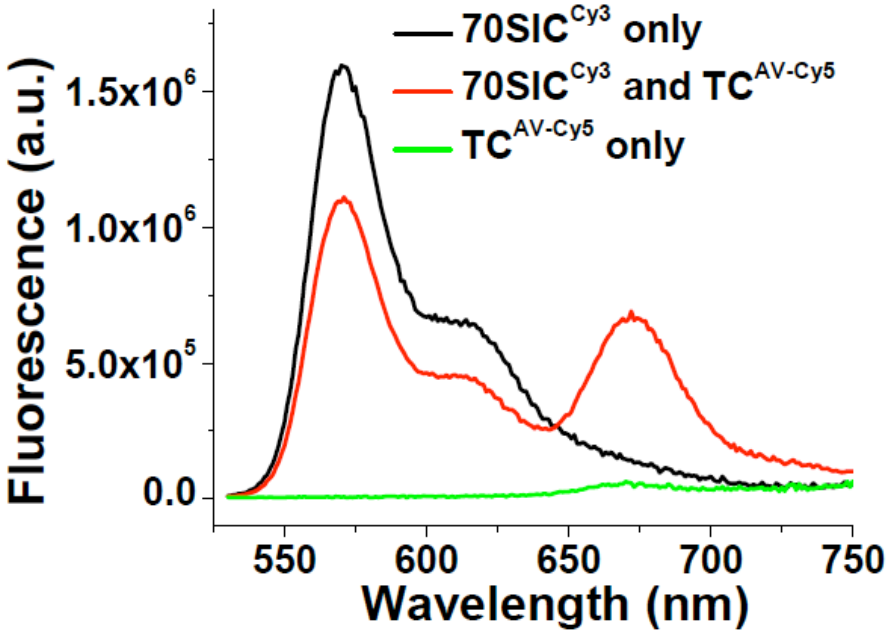
Supplementary Figure S1. Characterization of ternary complexes (TCs) formed by labeled EF-Tus. (a) Protection against spontaneous deacylation of [^{14}C]-Phe-tRNA^{Phe} by the indicated EF-Tu variants. C_t and C_o denote the concentrations of TC at times t and zero, respectively. (b) Protection of [^{3}H]-Phe-tRNA^{Phe} or [^{3}H]-Phe-tRNA^{Phe} (Cy3) (0.10 - 0.15 μM) from RNase A hydrolysis by the indicated EF-Tu variants. (c) Quenching of the fluorescence of Phe-tRNA^{Phe} (Cy3), 0.17 μM**,** by EF-Tu^{QSY9} or wt-EF-Tu^{QSY9}. The relatively high residual fluorescence at 10 μM EF-Tu is due to the presence of uncharged tRNA^{Phe} (Cy3).



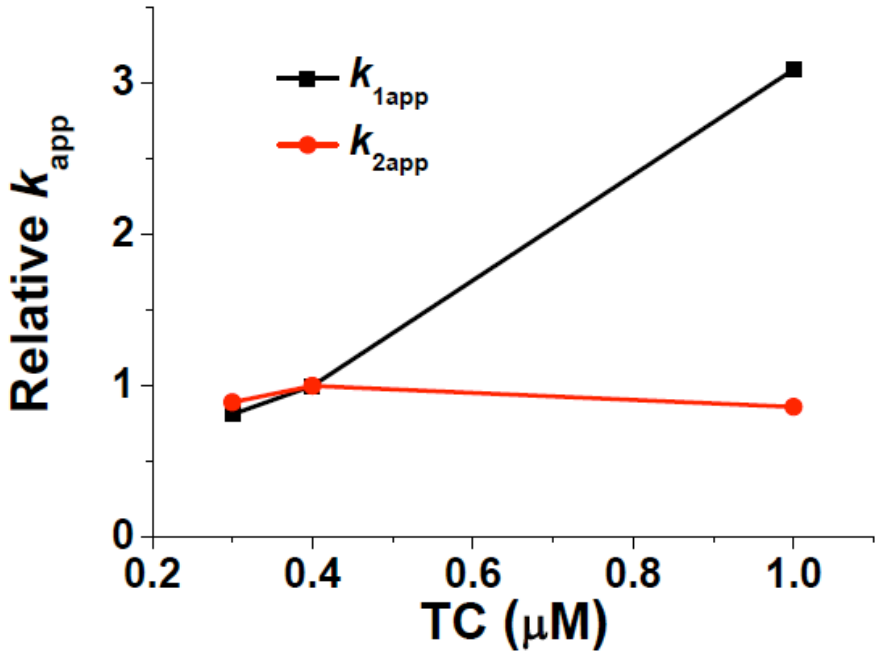
Supplementary Figure S2. FRET in TCs formed from labeled E348C-EF-Tu variants and Phe-tRNA^{Phe}(Cy3). (a) Quenching of the fluorescence of Phe-tRNA^{Phe} (Cy3) by EF-Tu^{QSY9} in a purified TC. The solid lines are observed traces at the same concentration of [^{3}H]-Phe-tRNA^{Phe}(Cy3), as measured by [^{3}H]-Phe, in the absence of EF-Tu (black) and for purified TC (red). The dotted lines are corrected traces that permit a FRET efficiency calculation of 66% (see Supplementary Material). (b) FRET between Phe-tRNA^{Phe}(Cy3) (0.1 µM) and EF-Tu$^{AV\text{-}Cy5}$, added at various ratios without purification of TC. A FRET efficiency of 76% was calculated (see Supplementary Material). (c) Kinetics of quenching of Phe-tRNA^{Phe}(Cy3) (0.1 µM) fluorescence following rapid mixing with various concentrations of EF-Tu^{QSY9}. A plot of k_{app} vs. EF-Tu gives a k_{on} value of 1.3 ± 0.1 µM^{-1}s^{-1}.



Supplementary Figure S3. Complete loss of fluorescence quenching due to FRET on rapid mixing of $TC^{QSY9/Cy3}$ (0.1 μM) and 70SIC (0.4 μM) in a stopped-flow spectrofluorometer, as shown by an increase in Cy3 fluorescence (black) on EF-Tu release from the ribosome. The red trace is a control showing that rapid mixing of TC^{Cy3} (0.1 μM) and 70SIC (0.4 μM), for which there is no FRET, gives a nearly constant fluorescence equal to the final fluorescence seen in the black trace. The green trace is for rapid mixing of $TC^{QSY9/Cy3}$ (0.1 μM) and 70SIC (0.4 μM) when GDPNP replaces GTP. This substitution blocks EF-Tu release, so the FRET interaction is maintained.



Supplementary Figure S4. Fluorescence spectrum resulting from addition of $TC^{AV\text{-}Cy5}$ (0.2 μM) to $70SIC^{Cy3}$ (0.1 μM) in the presence of GDPNP (1 mM), giving a stalled complex with EF-Tu bound in the A/T site.[31] . Concentrations are final. The labeling stoichiometries were EF-Tu (0.85); L11 (0.80). The FRET efficiency (0.55) corresponds to a Cy3-Cy5 distance of 58 Å, calculated according to the Förster equation {dye:dye distance = R_0 $[(1/E)-1]^{1/6}$},[27] assuming an R_0 of 60 Å and that both dyes rotate freely, i.e., κ^2 is equal to 0.67.



Supplementary Figure S5. Dependence of the apparent rate constants, k_{1app} and k_{2app} (equation 1), measuring changes in $70SIC^{Cy3}$ (0.1 μM) fluorescence (see Figure 5d, Tu-L11 assay), on rapid mixing with varying concentrations of the TC formed from $EF\text{-}Tu^{QSY9}$·GTP and the $Val\text{-}tRNA^{Val2B}$ T1 variant. Values are normalized to those found at 0.4 μM TC. The linear dependence (k_{1app}) and lack of dependence (k_{2app}) on TC concentration are consistent with Scheme 1.