Supplementary Methods

Ribosomes, buffers, translation factors and mRNAs

Tight-coupled 70S ribosomes were purified from *Escherichia coli* MRE600 S30 as reported by Noller *et al.* [1] and Wintermeyer, *et al.* [2]. 30S and 50S subunits were purified from 70S ribosomes by dialysis against Tris-polymix buffer (see below) at 1 mM Mg^{2+} followed by sucrose gradient ultracentrifugation in the same buffer.

The composition of our buffer system has been adjusted to optimize the activity of purified ribosomes using *in vitro* translation assays as described [3]. The optimal buffer conditions for *in vitro* translation was 50 mM Tris-OAc ($pH_{25°C}$ = 7.5), 100 mM KCl, 5 mM NH_4OAc, 0.5 mM $Ca(OAc)_2$, 5 mM $Mg(OAc)_2$, 6 mM β-mercaptoethanol, 5 mM putrescine and 1 mM spermidine. Single-molecule experiments were performed in the same buffer system at 15 mM $Mg(OAc)_2$. Single-molecule experiments were performed in the same buffer system at 15 mM $Mg(OAc)_2$.

Initiation factors IF-1, IF-2 and IF-3 from *E. coli* were purified from overexpressing strains (a kind gift from Prof. Claudio Gualerzi University of Camerino, Italy) as reported [4,5].

Elongation factors, EF-Tu, EF-Ts and EF-G, were PCR cloned from *E. coli* C600 genomic DNA into the pProEX HT-b plasmid system[6]. All mRNAs were chemically synthesized and contained a 5 -biotin followed by a 25 nucleotide spacer region, a strong (UAAGGA) Shine-Dalgarno ribosomal binding site and twelve codons derived from the open reading frame of T4 gene product 32. mRNAs differ only in the sequence identity of the second codon: UUU (cognate), CUU (near-cognate) and AAA (non-cognate).

tRNA aminoacylation and formylation.

Plasmids encoding *E. coli* methionyl tRNA synthetase and formylmethionyl-tRNA formyltransferase (a kind gift from Prof. Sylvain Blanquet, CNRS-Ecole Polytechnique, Palaiseau Cedex, France) were over-expressed and purified as described [7]. The formyl donor, 10-formyltetrahydrofolate, was prepared as described [8]. Aminoacylation and formylation of $tRNA^{fMet}$ and $tRNA^{fMet}$(Cy3-s^4U) was achieved as reported[9] and aminoacylation of $tRNA^{Phe}$ was achieved following standard protocols [10].

Fluorescent labeling of tRNAs.

Labeling of $tRNA^{fMet}$ with Cy3-maleimide at the s^4U8 position and labeling of $tRNA^{Phe}$ with Cy5-NHS ester at the acp^3U47 position were performed following published procedures[10-13]. Met-$tRNA^{fMet}$, dye labeled at the α-amino group of methionine was prepared by aminoacylation of $tRNA^{fMet}$ as previously described [7], and subsequent labeling with Cy3- or Cy5-NHS ester following published procedures[14]. Dye-labeled tRNAs were purified from unlabeled tRNA by HPLC using a TSK-phenyl 5-PW column.

70S complex formation.

70S complexes were initiated on gene32-derived mRNA *in vitro* in Tris-polymix buffer at 5 mM $Mg(OAc)_2$ using slight modifications of previouisly reported protocol [15]. Initiation complexes were purified by sucrose density ultracentrifugation in Tris-polymix buffer at 20 mM $Mg(OAc)_2$.

The EF-Tu(GTP)aa-tRNA complex.

Phe-tRNA^{Phe} or Phe-tRNA^{Phe}(Cy5-acp^{3}U) was complexed with EF-Tu(GTP) in three steps. (1) A 10 mM GTP charging mixture was prepared by incubating 10mM GTP, 30 mM phosphoenolpyruvate and 12 U/mL pyruvate kinase in Tris-polymix buffer at 15 mM $Mg(OAc)_2$. (2) Nucleotide exchange was accomplished by addition of 10 mM GTP charging mixture to a solution of 12 μM EF-Tu and 12 μM EF-Ts in Tris-polymix buffer (excluding putrescine and spermidine) to a final concentration of 1 mM GTP. (3) 10 μM Phe-tRNA^{Phe} was added to the reaction to achieve a final concentration of 1.5 μM in Phe-tRNA^{Phe}.

Total Internal Reflection Microscopy.

Preparation of quartz microscope slides and glass coverslips for use in total internal reflection fluorescence microscopy was derived from Ha et al.[16] The flow-cell constructed from the quartz slide and glass coverslip was passivated with a solution of 10 μM bovine serum albumin and 10 μM duplex DNA (14 base pairs) in order to prevent nonspecific binding of ribosome complexes and EF-Tu(GTP)Phe-tRNA^{Phe}(Cy5-acp^{3}U) ternary complex.

A lab-built, prism-based total internal reflection (TIR) apparatus, based on an inverted microscope, was used for all experiments. Cy3- or Cy5-labeled molecules were excited using a diode-pumped 532 nm laser or a 635 nm diode laser. Fluorescence emission was collected by a 1.2 NA/60X water-immersion objective and imaged onto a cooled, back-illuminated CCD camera with 9 pixel-binning at 100 ms exposure time. Stopped-flow delivery was achieved using a custom-built, motor-driven syringe injection system where the dead time for complete mixing following delivery of substrates is estimated at ~500 ms.

To extend the lifetimes and reduce the noise of Cy3 and Cy5 fluorescence for fluorescence microscopy, an oxygen scavenging system composed of 1% β-D-glucose, 25 U/mL glucose oxidase and 250 U/mL catalase was added to all samples.

Missing event analysis

Two missing-event corrections were applied to the near-cognate aa-tRNA data set. The first missing event correction was applied to the number of near-cognate aa-tRNAs transitioning from 0.35 FRET to 0 FRET. A histogram of the time spent by near-cognate aa-tRNA in the 0.35 FRET state before transitioning to 0 FRET follows a single exponential decay with a lifetime of 55 ms ± 1.6 (**Supplementary Fig. 9a** online). A Monte-Carlo simulation reveals that, given the 100 ms recording time per image of our video camera, approximately 81% of events with a lifetime of 55 ms would not yield a FRET signal ≥ 0.25 (the noise threshold used for data analysis) and would thus be missed. Applying this missing event correction, the 126 *observed* near-cognate aa-tRNAs progressing from 0.35 to 0 FRET actually correspond to approximately 674 *real* events (**Supplementary Table 1** online).

The second missing event correction involves the number of near-cognate aa-tRNAs transitioning from 0.35 FRET to FRET ≥ 0.5. In addition to the 35 events that are directly observed to transition from 0.35 FRET to FRET ≥ 0.5, we observe 46 events that progress directly from FRET ≤ 0.25 to a FRET value ≥ 0.5 without an explicit data point at 0.35 FRET. We assume that these 46 events *must* have passed through 0.35 FRET and were added to the 35 events explicitly observed to transition from 0.35 FRET to FRET ≥ 0.5. Therefore, 81 total events transition from 0.35 FRET to FRET ≥ 0.5 and we calculate

that a total of 11% of near-cognate aa-tRNAs advance past the initial selection step (**Supplementary Table 1** online).

Missing event corrections similar to those made for the near-cognate aa-tRNA data set can be applied to the cognate aa-tRNA data set. In this case, the majority of events at 0.35 FRET advance to FRET ≥ 0.5, only 10 events are observed to transition from 0.35 FRET to 0 FRET. Using this limited data set, a histogram of the time spent by cognate aa-tRNA in the 0.35 FRET state before transitioning to 0 FRET follows a single exponential decay with a lifetime of 72 ms ± 8.8 (**Supplementary Fig. 9b** online). However, a better lifetime fit is obtained by using the larger cognate EF-Tu(GDPNP)aa-tRNA data set; this leads to a lifetime of 55 ms ± 6 (**Supplementary Fig. 8** online). Once again, Monte-Carlo simulations using a lifetime of 55 ms reveal that approximately 81% of the events are missed. Applying this correction, the 10 observed cognate aa-tRNA transitioning from 0.35 FRET to 0 FRET actually correspond to approximately 53 *actual* events (**Supplementary Table 1** online).

Fidelity calculations

In the near-cognate data set, 674 events are estimated to have been rejected at the initial selection step, transitioning from 0.35 FRET to 0 FRET, whereas an estimated 81 events transition from 0.35 FRET to FRET states ≥ 0.5. Conservatively assuming that we did not undercount the number of events transitioning to FRET ≥ 0.5, we observe a total of 755 attempts for near-cognate aa-tRNAs to advance past the initial selection step. Of these, 3 events advance past both the initial selection and proofreading steps and stably accommodate at the 0.75 FRET state. Dividing the number of stably accommodated near-

cognate aa-tRNAs by the total number of attempts yields an overall accommodation ratio of 4×10^{-3}. By comparison, approximately 53 events in the cognate data set transition from 0.35 FRET to 0 FRET and are rejected at the initial selection step, while 97 events are estimated to progress from 0.35 FRET to FRET ≥ 0.5. This yields a total of 150 attempts for cognate aa-tRNAs to progress past initial selection. Of these, 84 events advance past both the initial selection and proofreading steps to accommodate at a stable 0.75 FRET state, yielding an overall accommodation ratio of 0.56. The overall error frequency of aa-tRNA selection in our system at 15 mM Mg^{2+} and 25 °C is obtained by dividing the near-cognate over the cognate accommodation ratios and yields a value of 7.1×10^{-3}.

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