Supplementary Information 1

Real-time assay for testing components of protein synthesis

SUPPLEMENTARY RESULTS

1. EmGFP concentration and fluorescence calibration curve.

EmGFP concentrations were estimated using a calculated molecular mass of 31,475 Da for the full-length cloned protein plus N-terminal and C-terminal extensions, and the Bradford assay as previously described for another variant of EmGFP(51). By comparison, a molar extinction coefficient (ε_{280} = 28,880 $M^{-1}cm^{-1}$ calculated as described http://web.expasy.org/protparam/), gave EmGFP concentrations that were 20% lower. Purified EmGFP served as a standard for obtaining a calibration curve of fluorescence vs. total EmGFP concentration in order to quantify the total EmGFP produced in the CFPS experiments, making the assumption that the EmGFP which is overexpressed in bacteria and purified using a His-tag affinity column has the same fluorescence intensity per unit weight of protein as that synthesized using the CFPS kit. Fluorescence intensities of serial dilutions of purified EmGFP, from 8 nM to 2 μM, were measured in the plate reader (λ_{ex} = 486 nm, λ_{em} = 535 nm), except for experiments using Phe-tRNA^{Phe}(Cy3) (λ_{ex} = 450 nm, λ_{em} = 510 nm). In this range, fluorescence intensity is linear with EmGFP concentration (Fig. S1, correlation coefficient $R^2 = 0.99 \pm 0.03$).

2. The effects of CFPS kit manipulations and modifications on EmGFP synthesis.

2A. Comparing the translational activities of MRE600 and AM77^{L11} ribosomes. In the EmGFP time traces, the length of the lag phase, t_L, corresponds to processes preceding EmGFP maturation, including the translation time of the full-length EmGFP. The results presented in Fig. S2, examine the observed lag phases on addition of either MRE600 or AM77^{L11} ribosomes to a CSPS$^{-ribosome}$ kit (see also Fig. 2B). Simulations were performed for experimentally determined concentrations of added active ribosomes: MRE600 (0.48 μM); AM77^{L11} (0.15 μM). All other simulated parameters were the same for both simulations. The close agreement between the simulated and observed t_L suggests that both ribosomes have similar translational rates. This indicates that the differences between the MRE600 and AM77^{L11} are primarily as result of differences in the fractions of the ribosomes that are active, rather than differences in the intrinsic activity per active ribosome.

2B. Addition of initiation and elongation factors. In order to determine whether preparation of the CFPS^{-Rib} kit might involve the partial removal of protein factors that are not fully restored when AM77^{L11} S100P ribosomes are added back, we determined the effect on EmGFP synthesis of adding IF1, IF2, IF3, EF-Tu, and EF-G, one at a time, at two different concentrations, 0.3 μM and 3.0 μM, corresponding to 20% and 200% of the total ribosome concentration. The results (Fig. S3A-B) demonstrate that the major effects are seen with the initiation factors (IFs) at the higher concentration, with the largest increase in EmGFP synthesis (1.6-fold) recorded for IF2, whereas adding EF-Tu or EF-G (or EF-P or RbbA - data not shown) at either concentration had little effect. Titration

experiments for each of the initiation factors showed that addition of 15-20 μM gives optimal results, with 20 μM IF2 giving a 1.9-fold increase. Simultaneous addition of 20 μM of each IF leads to 2.5-fold increase in EmGFP produced (data not shown). Progressive depletion of IFs might account for the differences in the ribosome activities observed when different centrifugation methods are utilized for exogenous ribosome preparation.

2C. Phe depletion within CFPS kit is dependent on protease and PheRS inhibition. Initial attempts to deplete the aminoacid Phe from the CFPS lysate included dialysis of the lysate against ice-cold TAM_{15} buffer supplemented with 5% PEG-8000 (to match the osmotic pressure between the lysate and the buffer). Following the dialysis step, all other CFPS components were added to the lysate, excluding Phe, and the reaction was initiated with addition of the EmGFP plasmid. As seen in Fig. S3C, Phe-depletion was incomplete since some EmGFP formation is observed (red curve). When this partially Phe-depleted CFPS mixture was incubated on ice for two hours prior to addition of the EmGFP plasmid, an even higher rate of EmGFP formation is observed (Fig. S3C, green line), from which we concluded that additional Phe and Phe-$tRNA^{Phe}$ is generated during the incubation period, possibly due to production of new Phe by proteolysis and Phe-RS dependent recharging of $tRNA^{Phe}$. Inclusion of both protease inhibitor cocktail (Halt, Pierce) and a specific Phe-RS inhibitor (N-benzyl-2-phenylethylamine, Acros) during the dialysis step and all following steps completely abolished EmGFP formation (Fig. 3C, black line).

These two treatments result in formation of the $CFPS^{-Phe}$ kit. Addition of either yeast Phe-$tRNA^{Phe}$ (10 μM) or Phe (80 μM) results in substantial EmGFP synthesis, amounting to about 40% of that obtained with the commercial undepleted CFPS kit (Fig. S3D). The empirical parameters describing the curves in Fig. S3D are summarized in Table S1.

2D. Determining the amount of initial endogenous Phe-$tRNA^{Phe}$ per ribosome in the CFPS kit. The ribosome concentration in the *E. coli* CFPS kit averaged 1.5 ± 0.2 μM in various batches, as determined by high-speed pelleting of the kit solution, resuspension of the pellet in TAM_{15} buffer, and measuring absorption, assuming 26 pmol/A_{260}. The initial endogenous Phe-$tRNA^{Phe}$ concentration was determined by allowing EmGFP formation by the CFPS kit, induced using a circular plasmid, to proceed to completion in the presence of protease inhibitor cocktail and N-benzyl-2-phenylethylamine, which together prevent regeneration of Phe-$tRNA^{Phe}$. Approximately 1.6 μM of total EmGFP was produced, corresponding to 22.4 μM initial endogenous Phe-$tRNA^{Phe}$ (14 Phe residues per EmGFP molecule). This amounts to 15 Phe-$tRNA^{Phe}$/ribosome, ~ 45 Phe-$tRNA^{Phe}$/active ribosome, or ~3 full-length EmGFP turnovers/active ribosome.

2E. Addition of deacylated $tRNA^{Phe}$. Increasing unlabeled or labeled Phe-$tRNA^{Phe}$ concentration in the lysate-based cell-free system also results in significant increase in the concentration of unlabeled or labeled deacylated $tRNA^{Phe}$ (Table 3). We tested the effect of added unlabeled yeast $tRNA^{Phe}$ on EmGFP formation in the presence of protease and PheRS inhibitors to prevent $tRNA^{Phe}$ aminoacylation during the course of reaction. The results obtained (Fig. S3E) show a biphasic response. Added 2.3-6.9 μM $tRNA^{Phe}$

(corresponding to 1-3 μM Phe-$tRNA^{Phe}$) leads to a marginal increase in EmGFP formation, whereas added $tRNA^{Phe}$ above 13.8 μM (corresponding to 6 μM Phe-$tRNA^{Phe}$) is inhibitory. Such inhibition might be due to competition by $tRNA^{Phe}$ for Phe-$tRNA^{Phe}$ binding to the ribosomal A-site, or to effects of deacylated tRNA binding to the ribosomal E-site.

2F. Addition of EF-Tu to a $CFPS^{-Phe}$ kit. Elongation factor Tu (EF-Tu) binds and delivers charged tRNA to the ribosome. We measured fluorescent EmGFP synthesis on addition of 10 μM yeast Phe-$tRNA^{Phe}$ to a $CFPS^{-Phe}$ kit ± added 10 μM EF-Tu. Since addition of EF-Tu markedly decreased fluorescent EmGFP formation (Fig. S3F), no exogenous EF-Tu was added in further experiments.

2G. Reproducibility of EmGFP synthesis results on Phe-$tRNA^{Phe}$ addition to the $CFPS^{-Phe}$ kit. Addition of increasing concentrations of either yeast or *E. coli* Phe-$tRNA^{Phe}$ in the range of 1 - 10 μM led to progressive, reproducible increases in EmGFP synthesis as shown in Fig. S4.

3. Overcoming the spectral overlap of EmGFP and the CyDyes

Real time, continuous detection of EmGFP accumulation in a CFPS system involves plate reader fluorescence detection with very high sensitivity, using an excitation wavelength of 486 nm with a wide bandpass emission filter centered at 535 nm. These conditions are advantageous as long as EmGFP is the sole fluorophore in the system. However, experiments containing Cy3 cannot be conducted using the same filters due to a spectral overlap between these two fluorophores. We have therefore identified recording conditions that minimize contribution of Cy3 fluorescence to the apparent EmGFP fluorescence. As shown in Fig. S5A, EGFP (which has similar spectral properties to EmGFP) shows an absorption/excitation maximum at 488 nm (dashed green line) which strongly overlaps with the excitation peak of Cy3 (dashed blue line), and less so with Cy5 (dashed red line). Shifting the EmGFP excitation wavelength to 450 nm (light blue bar), strongly decreases Cy3 and Cy5 excitation, and using an emission filter of 510 nm (light green bar) further assures that the fluorescence signal is dominated by the EmGFP fluorescence (solid green line), while rejecting contributions from Cy3 emission (solid blue line) and Cy5 emission (solid red line).

In order to experimentally test this point, 0.2 μM EmGFP was mixed with 5 μM Cy3-Phe-$tRNA^{Phe}$ and emission spectra were recorded in the fluorometer using various excitation wavelengths that corresponded to the available plate reader excitation filters. The data presented in Fig. S5B shows that the emission intensity at the 510 nm (EmGFP) peak is relatively independent of the excitation wavelength over the range 450 - 486 nm when the 510 nm emission filter is employed. Lowering the excitation wavelength to 420 nm causes a significant loss in EmGFP sensitivity. We have therefore chosen the 450 nm excitation wavelength, which is a good trade-off between minimizing Cy3 excitation and sensitivity to EmGFP fluorescence.

4. Numerical simulations of EmGFP translation using a Matlab Algorithm.

4A. Characteristics and inputs

Mature EmGFP formation using a $CFPS^{-Phe}$ kit, supplemented with added Phe-$tRNA^{Phe}$, was simulated using a numerical integration algorithm written and performed in MATLAB V7.5. As described in the main text, the kinetic model includes 315 steps which proceed in series (3 initiation steps, 310 EmGFP elongation steps, corresponding to 40 residues in the N-terminal His-tag sequence, 239 residues of EmGFP and 31 residues in the C-terminal sequence, and two maturation steps). The number of rate constants needed to simulate this model was kept to a minimum (five) by assigning the same rate constant, k_{in}, to each of the transcription initiation, transcription elongation and translation initiation steps and a second rate constant, k_f, to both fluorophore maturation steps. The three other constants are k_1 (296 non-Phe elongation steps); k_2 (14 Phe elongation steps using endogenous Phe-$tRNA^{Phe}$); and k_2* (14 Phe elongation steps for exogenously added Phe-$tRNA^{Phe}$). k_{in}, k_1, and k_f are pseudo first-order constants; k_2 and k_2* are 2nd order rate constants.

Good simulation of the experimental curves of EmGFP fluorescence required, in addition to the five rate constants described above, estimation of several additional parameters based on three assumptions. The first assumption is that ribosomes are functionally heterogeneous in translation elongation. This assumption accounts, in part, for the small observed threshold value of exogenously added Phe-$tRNA^{Phe}$ (~ 1 μM) that has to be exceeded in order to detect EmGFP fluorescence (Fig. 3C). If all ribosomes were equally active, we would expect small amounts of Phe-$tRNA^{Phe}$ to be consumed by synthesis of incomplete, non-fluorescent chains leading to a considerably higher threshold Phe-$tRNA^{Phe}$ concentration (Fig. S6E). The assumption of ribosome heterogeneity reduces the fraction of incomplete chains, since the more active ribosomes outcompete less active ribosomes for the limited supply of Phe-$tRNA^{Phe}$, thus decreasing the apparent threshold Phe-$tRNA^{Phe}$ concentration. To simulate functional heterogeneity, the ribosomes are separated into a number of equal-sized bins having different activities. For the simulations reported in Figs. 3 and 4, five bins having relative activities of 0.5, 0.7, 1.0, 1.4, 2.0 (i.e., a relative heterogeneity factor value of 1.4 between bins) gave adequate fits to the results presented in Fig. 3A. Poorer fits were obtained using fewer bins or a narrower range of relative activities.

The second assumption, which also reduces the apparent threshold concentration of added Phe-$tRNA^{Phe}$, is that endogenous Phe-$tRNA^{Phe}$ is not completely removed in our preparation of the $CFPS^{-Phe}$ kit. Even though EmGFP synthesis with this kit is totally dependent on added Phe-$tRNA^{Phe}$, some endogenous Phe-$tRNA^{Phe}$ could still be present, as long as it fell below the threshold needed for synthesis of measurable amounts of full-length EmGFP. The third and final assumption is that protease and Phe-RS activities are not completely inhibited in the $CFPS^{-Phe}$ kit. These residual activities would allow a continual slow regeneration of the limiting reagent, Phe-$tRNA^{Phe}$, resulting in the slow second phase of EmGFP formation observed.

A large number of simulations were performed to generate the simulated curves in Fig. 3A that well match the observed results (see also Fig. S6). The parameter values used for these curves were: k_i 0.017 s^{-1}, k_f 0.0056 s^{-1}, residual endogenous Phe-$tRNA^{Phe}$, 2 μM, and steady-state concentration of regenerated Phe-$tRNA^{Phe}$, 0.01 μM. In addition, ribosomal heterogeneity was attributed to the existence of five equal pools of ribosomes, with a four-fold range of relative protein synthesis activities (0.5, 0.7, 1.0, 1.4, 2.0), where the relative value of 1.0 corresponds to rate constants k_1 and k_2* having values of 0.5 s^{-1} and 0.05 $s^{-1}\mu M^{-1}$ for either yeast or *E. coli* Phe-$tRNA^{Phe}$.

4B. Calculations

The evolution of concentrations is calculated by

$$C_{t+\Delta t} = C_t \cdot e^{M \cdot \Delta t}$$

In which Δt is evolution step (typically 1 s - increasing time resolution to 10 ms intervals gave essentially identical results), $C_{t+\Delta t}$ and C_t are concentration matrices of all species at time t+Δt and t, respectively. M is a transfer matrix calculated from reaction rates through

$$m_{i,i} = -k_i$$

$$m_{i,i+1} = k_i$$

$m_{i,i}$ and $m_{i,i+1}$ are elements of transfer matrix M, k_i is the reaction rate constant for the i*th* step.

4C. Utilizing the algorithm

The algorithm is provided as a separate MATLAB (version R2007b) file, denoted Supplementary Information File 2. The output of the simulation is a group of fifteen 60 min time courses of mature EmGFP formation per ribosome, calculated as a function of varying added exogenous Phe-$tRNA^{Phe}$ concentration. Using a PC with a 3.07 GHz processor (Intel core i7), one such group of simulations with 1 s sampling usually takes 4-6 hours. To vary inputs, up to 11 lines of code can be changed, as shown in Table S2.

Sample simulations are shown in Fig. S6 for variations in k_2, k_{in}, k_f, k_1, steady-state regenerated Phe-$tRNA^{Phe}$ concentration, endogenous Phe-$tRNA^{Phe}$ concentration, ribosome concentration, heterogeneity factor, and number of heterogeneity bins. The effects of changes in these parameters on empirical measures of EmGFP formation (t_L, S_1, A_1 and S_2) are summarized in the Table S3. Three results are most noteworthy. First, severely reducing k_2 induces apparent biphasic EmGFP formation, even in the absence of steady-state regenerated Phe-$tRNA^{Phe}$. Second, different values for k_{in} and k_1 primarily affect the length of the lag phase, whereas changes in k_f affect the overall shape of the curve. Third, the heterogeneity factor and the number of heterogeneity bins strongly affect all parameters. A heterogeneity factor set to 1.4 using 5 bins adequately reproduced the results presented in Figs. 3 and 4. Parameter values may vary for different CFPS kit preparations. Constant values for k_{in}, k_f, k_1, endogenous Phe-$tRNA^{Phe}$ concentration, ribosome concentration, heterogeneity factor and heterogeneity bins were used for the simulations performed as a function of added exogenous Phe-$tRNA^{Phe}$. Values for k_2 and

the steady-state regenerated Phe-tRNA^{Phe} concentration were adjusted so as to give the lowest reduced chi-square.

Table S1. Empirical parameters for different variants of the CFPS kit[a]

	Kit		
Parameter	CFPS	$CFPS^{-Phe}$ + Phe-$tRNA^{Phe}$ (10 μM)	$CFPS^{-Phe}$ + Phe- (80 μM)
Lag, t_L	1.0	1.20	0.87
Phase 1 slope, S_1	1.0	0.49	0.48
Phase 1 formation, A_1	1.0	0.39	0.36
Phase 2 slope, S_2	1.0	0.36	0.22

[a] based on results in Fig. S3D. Data normalized to the unmodified CFPS data.

Table S2. Inputs to the simulation program

Input	Code line	Default parameters (meaning)	Alternative parameters (meaning)
15 values of exogenous added Phe-$tRNA^{Phe}$ concentration	11	`Ct=0:14` (0 – 14 μM in 1 μM increments)	`Ct=0:0.3:4.2` (0 – 4.2 μM in 0.3 μM increments)
k_2^* value	12	`Rr=1` ($k_2 = k_2$*)	`Rr=5` ($k_2 = 5k_2$*)
Heterogeneity factor	14	`hete=1.4` (H = 1.4)	`hete=1.2` (H = 1.2)
# of activity bins	15	`hgroup=5` (5 bins)	`hgroup=1` (homogenous sample)
endogenous Phe-$tRNA^{Phe}$ concentration	33	`Cnt=2` (2 μM)	`Cnt=0.5` (0.5 μM)
active ribosome concentration	35	`Cr=0.6` (0.6 μM)	`Cr=0.3` (0.3 μM)
steady-state regenerated Phe-$tRNA^{Phe}$ concentration	39	`Ctbaseline=0.100` (0.1 μM)	`Ctbaseline=0.050` (0.05 μM)
k_{in} value	29	`t1=60` ($k_{in} = 1/60\ s^{-1}$)	`t1=10` ($k_{in} = 1/10\ s^{-1}$)
k_1 value	42	`t3=2` ($k_1 = 1/2\ s^{-1}$)	`t3=0.5` ($k_1 = 2\ s^{-1}$)
k_2 value	32	`t2=2` ($k_2 = 1/20\ s^{-1}\mu M^{-1}$)	`t2=0.5` ($k_2 = 1/5\ s^{-1}\mu M^{-1}$)
k_f value	45	`t4=180` ($k_f = 1/180\ s^{-1}$)	`t4=10` ($k_f = 1/10\ s^{-1}$)

Table S3 - Summary of qualitative effects of parameters on phenomenological measures characterizing time-dependence of EmGFP formation

	Lag t_L	Phase-1-slope S_1	Phase-1-formation A_1	Phase-2-slope S_2
k_2	-	-	+	+
k_{in}	+	-	-	-
k_f	+	+	+	+
k_1	++	+	+	+
Steady state regeneration	-	-	-	++
Endogenous [Phe-tRNA^{Phe}]	-	++	++	+
Active [Ribosome]	-	++	++	+
Heterogeneity factor	+	+	+	-
Heterogeneity bins	+	+	+	+

The notations -, +, and ++ correspond to: no effect, moderate effect, and prominent effect, respectively.

SUPPLEMENTARY FIGURE LEGENDS

Figure S1. EmGFP fluorescence calibration curve. The fluorescence intensities of EmGFP samples (10 µl) ranging from 8 nM to 2 µM were measured in a plate reader using with λ_{ex} = 486 nm and λ_{ex} = 535 nm. The highest value shown corresponds to 1 µM EmGFP for clarity.

Figure S2. Simulations of the lag phase. The data presented in Fig. 2B was simulated using the active MRE (0.48 µM) and AM77^{L11} (0.15 µM) concentrations added to the CFPS$^{-ribosome}$ kit. The dashed lines are derived from the numerical simulation model using the default parameter values (16 µM endogenous Phe-tRNA^{Phe}, no exogenous and no steady-state Phe-tRNA^{Phe}, heterogeneity factor = 1.4, heterogeneity bins = 5, k_i: 0.017 s^{-1}, k_f: 0.0056 s^{-1}, k_1 = 0.5 s^{-1} k_2 = 0.05 $\mu M^{-1}s^{-1}$).

Figure S3. The effects of CFPS kit alterations on EmGFP synthesis. CFPS^{-Rib} was supplemented with 1.5 µM AM77^{L11} ribosomes and 0.3 µM (A) or 3.0 µM (B) of each of the indicated factors, added one at a time. (C) Depletion of Phe from the CFPS lysate. The CFPS lysate was dialyzed and reconstituted in the absence of Phe, with (green) or without (red) a 2 hr preincubation at 0 ^{o}C prior to initiation of EmGFP formation by addition of EmGFP plasmid. Repeating the procedure in the presence of protease inhibitor cocktail (Halt, Pierce, 1:100 dilution) and N-benzyl-2-phenylethylamine (Acros, 60 µM) PheRS inhibitor completely suppressed EmGFP formation (black). The decrease in fluorescence intensity at the beginning of the reaction is likely due to bleaching of background fluorescence. (D) Modified vs. unmodified CFPS mixtures. Comparison of EmGFP synthesis by the unmodified CFPS kit (green) with modified CFPS^{-Phe} kit supplemented with either 10 µM Phe-tRNA^{Phe} (black) or 80 µM Phe (red). (E) The effect of added deacylated tRNA^{Phe} on the translational activity. The cell-free mixture was supplemented with the indicated concentrations of deacylated tRNA^{Phe}. (F) The effect of added EF-Tu on translational activity. CFPS^{-Phe} was supplemented with 10 µM yeast Phe-tRNA^{Phe} with (red) or without (black) added 10 µM EF-Tu. Fluorescence is recorded either in a fluorometer (Fluorolog-3, Horiba Jobin Yvon) or a plate reader (EnVision, Perkin-Elmer). In all cases EmGFP synthesis was initiated with 130 ng of the EmGFP plasmid per 10 µl reaction volume.

Figure S4. Reproducibility of EmGFP synthesis as a function of Phe-tRNA^{Phe} addition to the CFPS^{-Phe} kit. CFPS^{-Phe} (black) was supplemented with the indicated concentrations of (A) yeast Phe-tRNA^{Phe} or (B) *E. Coli* Phe-tRNA^{Phe}. The active ribosome concentrations were 0.58 µM (A) and 0.3 µM (B). The triplicate traces demonstrate the reproducibility of the assay using a given kit preparation.

Figure S5. Overcoming the spectral overlap of EmGFP and the CyDyes. (A) The spectral properties of GFP (green), Cy3 (blue) and Cy5 (red). Excitation and emission spectra are normalized and represented as dotted and solid lines, respectively. To achieve spectral resolution between the EmGFP and the CyDyes, an excitation filter of λ_{ex} = 450 nm with

8 nm bandpass (light blue bar), and an emission filter with λ_{em} = 510 with 10 nm bandpass (light green bar) have been used. All spectra were acquired from Fluorescence SpectraViewer, at http://www.invitrogen.com/site/us/en/home/support/Research-Tools/Fluorescence-SpectraViewer.html (B) Emission spectra of Cy3-Phe-$tRNA^{Phe}$ and EmGFP as a function of excitation wavelength. A mixture of 200 nM EmGFP and 5 µM Cy3-Phe-$tRNA^{Phe}$ was analyzed. Fluorometer (Fluorolog-3, Horiba Jobin Yvon) traces were obtained using the indicated excitation wavelengths. The emission wavelength range was 495-650 nm. The EmGFP and Cy3 peaks are centered at 510 and 570 nm, respectively. In the excitation range 450 – 486 nm, Cy3 emission (at 570 nm) is much more sensitive to excitation wavelength than EmGFP emission (at 510 nm).

Figure S6. Simulated EmGFP formation as a function of varying parameter values. All simulated curves had the following input parameters, unless indicated otherwise: [Active ribosome] = 0.3 µM; [Endogenous Phe-$tRNA^{Phe}$] = 2 µM, [Added exogenous Phe-$tRNA^{Phe}$] = 2 µM; steady-state concentration of regenerated Phe-$tRNA^{Phe}$ = 0 µM; k_i = 0.017 s^{-1}, k_f = 0.0056 s^{-1} [consistent with results reported in ref. (26)] ; heterogeneity factor = 1.4, number of heterogeneity bins = 5; values of rate constants k_1 and k_2 = 0.5 s^{-1} and 0.05 $\mu M^{-1}s^{-1}$, respectively. Variation of: (A) k_2 ($\mu M^{-1}s^{-1}$); (B) k_i (s^{-1}); (C) k_f (s^{-1}); (D) k_1 (s^{-1}); (E) Steady-state regenerated Phe-$tRNA^{Phe}$ concentration; (F) Endogenous Phe-$tRNA^{Phe}$ concentration; (G) Ribosome concentration. Notably, when Phe-$tRNA^{Phe}$ is limiting, lower ribosome concentration leads to higher concentration of fully elongated EmGFP. (H) Heterogeneity factor. In all cases a relative activity of 1.0 corresponds to k_1 = 0.5 s^{-1} and k_2 = 0.05 $\mu M^{-1}s^{-1}$. The two sets of lines correspond to low (3 µM) and high (12 µM) added Phe-$tRNA^{Phe}$; (I) Number of heterogeneity bins (3, 5, or 7) for added Phe-$tRNA^{Phe}$ concentrations of 3 µM (black lines), 6 µM (red lines) and 9 µM (green lines).

SUPPLEMENTARY REFERENCES

51. Teerawanichpan, P., Hoffman, T., Ashe, P., Datla, R. and Selvaraj, G. (2007) Investigations of combinations of mutations in the jellyfish green fluorescent protein (GFP) that afford brighter fluorescence, and use of a version (VisGreen) in plant, bacterial, and animal cells. *Biochimica et biophysica acta*, **1770**, 1360-1368.