Supporting Information

Single-Molecule Photoactivation FRET: A General and Easy-to-Implement Approach to Break the Concentration Barrier

Sijia Peng, Ruirui Sun, Wenjuan Wang, and Chunlai Chen*

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Methods and Materials

Materials

CAGE 552 (1 mg, NHS ester) and CAGE 552 (1 mg, maleimide) were purchased from Abberior (Göttingen, Germany). CAGE FAM (CMNB-Caged Carboxyfluorescein, Succinimidyl Ester, 1 mg) and Alexa Fluor® 488 (1 mg, NHS ester) were purchased from Invitrogen. Cyanine3 (1 mg, NHS ester) and Cyanine5 (1 mg, NHS ester) were purchased from Lumiprobe. UltraPure™ 1M Tris-HCl pH 7.5 was obtained from Invitrogen. Other common materials and reagents were purchased from Sigma or Amresco.

Fluorescence measurement

Cy3 and CAGE 552 fluorescence signals were recorded using a plate reader (Molecule Devices) with Ex 532 nm / Em 585 nm. Alexa 488 and CAGE FAM fluorescence were recorded with Ex 488 nm / Em 525 nm.

Labeled DNA preparation

All DNA oligonucleotides (Table S4) were purchased from Sangon Biotech (Shanghai, China). The Cy3, CAGE 552, Alexa 488, and CAGE FAM labeled DNA strands were prepared via covalently conjugating N-hydroxysuccinimido (NHS) group of fluorescent dyes to an amino group on DNA following procedures from manufacturers. In brief, fluorophores and DNA strands were mixed at 20:1 molar ratio and kept at room temperature (~23 °C) for two hours. Labeled DNAs were separated from excess free fluorophores through a desalting Sephadex G-25 column (Nap-5, GE).

Biotinylated DNA strands were needed for surface immobilization. DNA duplexes used in sm-FRET and sm-PAFRET were prepared by mixing fluorescent dyes labeled D-strand, Cy5 labeled A-strand and biotinylated strand at equal molar ratio and heating to 55°C for 15 minutes followed by cooling to room temperature overnight. Duplexes formed using unlabeled A-strands were used to measure and compare fluorescence properties of Cy3, CAGE 552, Alexa 488, and CAGE FAM when no Cy5 was present.

Ribosome preparation

70S ribosomes from MRE600, *E. coli* initiation factors 1, 2, and 3, elongation factor G, and elongation factor Tu were prepared according to published procedures.[1] mRNA MVF (GGG AAU UCA AAA AUU UAA AAG UUA AUA AGG AUA CAU ACU AUG GUG UUC CGU UAU GAA UAU GAA UAU, underlined sequences highlight region from initiator codon, Val and Phe codons) were purchased as 5’-biotinylated derivatives (Dharmacon RNAi Tech.) and used as received.

Initiation complexes (ICs) were formed by mixture of 1 µM 70S ribosome with 2 µM mRNA, 1.5 µM initiation factors 1/2/3, and 1.5 µM *E. coli* tRNA^{Met} (MP Biomedicals) in TAM$_{15}$ buffer (15 mM MgAc$_2$, 50 mM Tris-HCl pH 7.5, 30 mM NH$_4$Cl, 70 mM KCl, and 1 mM dithiothreitol) at 37 °C for 25 minutes.
**E. coli tRNA^Phe and tRNA^Val preparation and labeling**

To overexpress *E. coli* tRNA^Phe, full-length *E. coli* tRNA^Phe DNA containing flanking restriction sites was prepared by heating tRNA^Phe primer A and B (Table S4) at 95°C for 5 minutes followed by cooling to room temperature overnight, which was cloned into the expression vector pBSTNAV\(^\text{[2]}\) \(\text{(a gift from Luc Ponchon, Addgene Plasmid #45801)}\) between PstI and EcoRI sites. Constructed *E. coli* tRNA^Phe expression plasmid was transformed in BL21(DE3) cells and selected under 50 µg/ml ampicillin. Cells transformed with our constructed plasmid were grown at 37 °C in TB medium with 50 µg/ml ampicillin until OD600 = ~1.3. Total cellular tRNA was extracted as previous described.\(^\text{[3]}\)

According to previous method, to purify tRNA^Phe from total tRNA, streptavidin-agarose beads (Sigma) coated with biotinylated single stranded DNA (tRNA^Phe hander, Table S4) complementary to part of tRNA^Phe were used to isolate tRNA^Phe through specific RNA/DNA heteroduplex formation.\(^\text{[4]}\) tRNA^Phe bound on the beads was released by heating at 65°C and concentrated through ethanol precipitation.

tRNA^Phe was labeled by Cy3 or CAGE FAM through reaction of NHS ester with the primary aliphatic amino group of the 3-(3-aminoo-3-carboxypropyl)-uridine at position 47 (acp3\(^\text{U47}\)) according to previous method.\(^\text{[5]}\) Briefly, tRNA^Phe and fluorophore were mixed at 1:20 molar ratio in 50 mM HEPES pH 8.0, 0.9 M NaCl. Mixture was kept at 30°C for 8 hours followed by additional 12 hours at 4 °C. Labeled tRNA^Phe was separated from unlabeled tRNA^Phe by reverse-phase HPLC using a DeltaPak C4 Column (Waters, 300Å, 15 µm, 3.9 mm × 300 mm). The tRNA mixture was applied to the column equilibrated with buffer A (20 mM NH4Ac pH 7.0, 10 mM MgAc2 and 400 mM NaCl) and the labeled tRNAs were eluted with a linear gradient of buffer A containing 30% (v/v) ethanol.

*E. coli* tRNA^Val (Sigma) was labeled by Cy5-NHS and purified through the same procedure.

**Aminoacyl tRNA and ternary complexes preparation**

*E. coli* Phe and Val tRNA synthetase, whose plasmids were constructed using vector pET-28a, were overexpressed in *E. coli* BL21 (DE3) cells and purified on a Ni-NTA column (Qiagen).

Aminoacylation mixture containing 25 µM tRNA, 100 µM L- amino acid, 12.5 µM tRNA synthetase, 10 mM ATP, 3 mM dithiothreitol, 0.005 unit/µL Thermostable Inorganic Pyrophosphatase (NEB) in 100 mM Tris-HCl pH 7.8, 50 mM MgCl2, and 2.5 mM EDTA were kept at 37°C for 30 minutes. Aminoacyl tRNA was purified by phenol-chloroform extraction, Nap-5 column (GE) desalting, and ethanol precipitation as previous described.\(^\text{[1d,6]}\)

Ternary complexes were formed by incubating 8 µM EF-Tu, 1 µM aminoacyl tRNA, 3 mM GTP, 1.3 mM phosphoenolpyruvate, and 5 µg/mL pyruvate kinase in TAM15 buffer for 15 minutes at 37 °C.

**Plasmid construction, protein purification and labeling of elongation factor G (EF-G)**

A cysteine-free mutant of *E. coli* EF-G (C113D, C265A, C397S)\(^\text{[7]}\) in vector pET24b was constructed according to QuikChange site-directed mutagenesis using Q5® High-Fidelity DNA Polymerase (NEB). Ser692 of the cysteine-free mutant was mutated to Cys and confirmed by DNA sequencing. All primers (Beijing Zixi bio tech) used for mutagenesis were described in Table S4. The EF-G S692C mutant protein was overexpressed in *E. coli* BL21 (DE3) cells and purified on a Ni-NTA column (Qiagen) with EF-G storage buffer (50 mM Tris-HCl pH 7.5, 70 mM NH4Cl, 30 mM KCl, 7 mM MgCl2, 1 mM TCEP).
CAGE 552 maleimide and EF-G S692C were mixed at 5:1 molar ratio in EF-G storage buffer and kept at room temperature (~23°C) for two hours. Reactions were quenched by adding 10 mM DTT. Labeled EF-G was separated from excess free fluorophores through a desalting Sephadex G-25 column (Nap-5, GE).

Preparation of PEG-passivated slides

PEG-passivated slides were prepared according to previous procedure with minor modifications.[8] In brief, slides and coverslips were sonicated at 40 °C in the order of acetone (10 minutes), 0.2 M KOH (20 minutes), and ethanol (10 minute). Cleaned slides and coverslips were treated with amino-silane reagents (1ml 3-aminopropyltriethoxysilane, 5 ml acetic acid, and 94 ml methanol) at room temperature overnight and then incubated with polyethylene glycol (PEG from Laysan Bio, Inc., containing 20% w/w mPEG-Succinimidy l Valerate, MW 2,000 and 1% Biotin-PEG-SC, MW 2,000) in 0.1 M sodium bicarbonate (pH 8.3) for 3 h. Slides and coverslips were dried by clean N2, put in 50 mL falcon tubes, vacuum sealed in food saver bags, and stored it at -20 °C.

Acquisition of smFRET data

PEG-passivated slides were incubated with 0.05 mg/ml streptavidin for 3 minutes. Then DNA duplexes and initiation complexes (ICs) were specifically attached to microscope flow cells via biotinylated DNA or mRNA to PEG-passivated slides, decorated with biotin-PEG and streptavidin. smFRET experiments were performed at 23 °C in TAM15 buffer (15 mM MgAc2, 50 mM Tris-HCl pH 7.5, 30 mM NH4Cl, 70 mM KCl, and 1 mM dithiothreitol) with an oxygen scavenging system, containing 3 mg/mL glucose, 100 µg/mL glucose oxidase (Sigma-Aldrich), 40 µg/mL catalase (Roche), 1 mM cyclooctatetraene (COT, Sigma-Aldrich), 1 mM 4-nitrobenzylalcohol (NBA, Sigma-Aldrich), 1.5 mM 6-hydroxy-2,5,7,8-tetramethyl-chromane-2-carboxylic acid (Trolox, Sigma-Aldrich – added from a concentrated DMSO stock solution).

Single molecule fluorescence and FRET measurements were performed on a home-built objective-type TIRF microscope, based on a Nikon Eclipse Ti-E with an EMCCD camera (Andor iXon Ultra 897), and solid state 405 nm, 488 nm, 532 nm, and 640 nm excitation lasers (Coherent Inc. OBIS Smart Lasers) which can be modulated using digital signals from the EMCCD camera. For single molecule photoactivation FRET (sm-PAFRET), alternating laser excitation (ALEX) between 405 nm and 532 nm (or 488 nm) excitation was achieved by synchronizing laser on-off switch with EMCCD frames. Fluorescence emission from the probes was collected by the microscope and spectrally separated by interference dichroic (T550lpxr for Alexa 488/Cy5 FRET pair or T635lpxr for Cy3/Cy5 FRET pair, Chroma) and bandpass filters, ET525/50m (for Alexa 488 and CAGE FAM, Chroma) or ET585/65m (for Cy3 and CAGE 552, Chroma), and ET700/75m (for Cy5, Chroma), in a Dual-View spectral splitter (Photometrics, Inc., Tucson, AZ). All single molecule movies were collected using Cell Vision software (Beijing Coolight Technology).

Collected movies were analyzed by a custom-made software program developed as an ImageJ plugin (http://rsb.info.nih.gov/ij). Fluorescence spots were fitted by a 2-D Gaussian function within a 9-pixel by 9-pixel area, matching the donor and acceptor spots using a variant of the Hough transform.[9] The background subtracted total volume of the 2-D Gaussian peak was used as raw fluorescence intensity I.

Three or more replicates were performed for each experiments. Standard error of mean (SEM)
was displayed as error bar and variation.

**FRET efficiency correction**

Actual FRET efficiency was calculated via equation,

$$E = \left(1 + \frac{I_D}{I_A - \chi I_D} \right)^{-1}$$  \hspace{1cm} (Eq. S1)

where $I_D$ is raw fluorescence intensity of donor, $I_A$ is raw fluorescence intensity of acceptor, and $\chi$ is the cross-talk of the donor emission into the acceptor channel. $\gamma$ accounts for the differences in quantum yield and detection efficiency between the donor and the acceptor and is calculated as the ratio of change in the acceptor intensity ($\Delta I_A$) to change in the donor intensity ($\Delta I_D$) upon acceptor photobleaching ($\gamma = \Delta I_A / \Delta I_D$).[^10]

**Estimation of reaction rates shown in Figure 3E**

Percentages of the ribosomes going through one of three parallel translocation pathways shown in Figure 3E under certain EF-G concentration can be expressed as:

$$P_1 = \frac{k_{b1}[\text{EF-G}]}{k_{b1}[\text{EF-G}]+k_{12}}$$  \hspace{1cm} (Eq. S2)

$$P_2 = \frac{k_{12} k_{b2}[\text{EF-G}]}{k_{b1}[\text{EF-G}]+k_{12} k_{b2}[\text{EF-G}]+k_{23}}$$  \hspace{1cm} (Eq. S3)

$$P_3 = \frac{k_{23}}{k_{b1}[\text{EF-G}]+k_{23} k_{b2}[\text{EF-G}]+k_{23}}$$  \hspace{1cm} (Eq. S4)

in which, $P_1$, $P_2$, and $P_3$ are percentages of the ribosomes going through pathway 1 (red), pathway 2 (green), and pathway 3 (blue), respectively; [EF-G] is the concentration of EF-G; $k_{12}$ and $k_{23}$ are transition rates from PRE(I) to PRE (II) and from PRE(II) to PRE(III), respectively; $k_{b1}$ and $k_{b2}$ are binding rates of EF-G onto PRE(I) and PRE (II), respectively. Previous studies have shown that EF-G can interact with different ribosomal PRE states to promote translocation.[^14,11] We assume both $k_{b1}$ and $k_{b2}$ are the same (150 μM$^{-1}$s$^{-1}$).[^12] $P_1$, $P_2$, and $P_3$ under five different [EF-G] were extracted from FRET distributions shown in Figure 3D and applied to Eqs. S2-S4 to perform a global fitting, which estimated values of $k_{12}$ and $k_{23}$ to be 19±3 s$^{-1}$ and 6±1 s$^{-1}$, respectively.

Apparent dwell times ($t_{app}$) of EF-G bound states were weighted sum of dwell times from all parallel pathways:

$$t_{app} = P_1 \frac{1}{k_{dG1}} + P_2 \frac{1}{k_{dG2}} + P_3 \frac{1}{k_{dG3}}$$  \hspace{1cm} (Eq. S5)

in which, $k_{dG1}$, $k_{dG2}$ and $k_{dG3}$ are dissociation rates of EF-G from EF-G bound intermediate states in pathway 1 (red), pathway 2 (green), and pathway 3 (blue), respectively. $t_{app}$, $P_1$, $P_2$, and $P_3$ under five different EF-G concentrations were extracted from dwell time and FRET distributions shown in Figures 3B and 3D and applied to Eq. S5 to estimate values of $k_{dG1}$, $k_{dG2}$ and $k_{dG3}$ to be 10±1 s$^{-1}$, 6±1 s$^{-1}$, and 2.8±0.1 s$^{-1}$, respectively.
Reference:

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Figure S1. Visualization of individual immobilized Cy3 labeled DNA duplex formed from Cy3 labeled D-strand-I, unlabeled A-strand-I, and biotinylated hander-I in the presence of free Cy3 or CAGE 552 in solution under 1.99 μW μm⁻² 532 nm laser illumination and 25 ms per frame. Non-activated CAGE 552 drastically reduced the fluorescence background.
**Figure S2.** Visualization of individual immobilized Alexa 488 labeled DNA duplex formed from Alexa 488 labeled D-strand-II, unlabeled A-strand-II, and biotinylated hander-II in the presence of free Alexa 488 or CAGE FAM in solution under 0.68 μW μm$^{-2}$ 488 nm laser illumination and 50 ms per frame. Non-activated CAGE FAM drastically reduced the fluorescence background.
Figure S3. Distributions of signal-to-noise ratio (SNR) of Cy3 in the presence of free Cy3 or CAGE 552 in solution. Single molecule fluorescence trajectories of Cy3 were collected using Cy3 labeled DNA duplex formed from Cy3 labeled D-strand-I, unlabeled A-strand-I, and biotinylated hairer-I under 1.99 μW μm\(^{-2}\) 532 nm laser illumination and 25 ms per frame. SNR of a single molecule trajectory was calculated as the ratio of the average intensity and its standard deviation before a single-step photobleaching.
Figure S4. Distributions of apparent FRET between Cy3/Cy5 on dual-labeled DNA duplex in the presence of free Cy3 or CAGE 552 in solution under 1.99 μW μm$^{-2}$ 532 nm laser illumination and 25 ms per frame. Duplex was formed from Cy3 labeled D-strand-I, Cy5 labeled A-strand-I, and biotinylated hander-I. Apparent FRET of each frame calculated as the ratio of acceptor intensity over total intensity was contributed as a single count to the distribution.
Figure S5. Distributions of signal-to-noise ratio (SNR) of Alexa 488 in the presence of free Alexa 488 or CAGE FAM in solution. Single molecule fluorescence trajectories of Alexa 488 were collected using Alexa 488 labeled DNA duplex formed from Alexa 488 labeled D-strand-II, unlabeled A-strand-II, and biotinylated hander-II under 0.68 μW μm⁻² 488 nm laser illumination and 50 ms per frame. SNR of a single molecule trajectory was calculated as the ratio of the average intensity and its standard deviation before a single-step photobleaching.
**Figure S6.** Distributions of apparent FRET between Alexa 488/Cy5 on dual-labeled DNA duplex in the presence of free Alexa 488 or CAGE FAM in solution under 0.68 μW μm$^{-2}$ 488 nm laser illumination and 50 ms per frame. Duplex was formed from Alexa 488 labeled D-strand-II, Cy5 labeled A-strand-II, and biotinylated hander-II. Apparent FRET of each frame calculated as the ratio of acceptor intensity over total intensity was contributed as a single count to the distribution.
Figure S7. Photoactivation curves of CAGE FAM under various activation laser (405 nm) power. CAGE FAM was attached through duplex formed from CAGE FAM labeled D-strand-II, unlabeled A-strand-II, and biotinylated hander-II.
Figure S8. Distributions of corrected FRET between Cy3/Cy5, CAGE 552/Cy5, Alexa 488/Cy5, and CAGE FAM/Cy5. Single molecule FRET signals were measured using duplexes formed from Cy3, CAGE 552, Alexa 488, or CAGE FAM labeled D-strand-II with Cy5 labeled A-strand-II and biotinylated hander-II. 0.20 μW μm$^{-2}$ 532 nm laser illumination or 0.17 μW μm$^{-2}$ 488 nm laser illumination was used with 100 ms per frame collecting rate. Corrected FRET of each frame was calculated according to Eq. S1 and contributed as a single count to the distribution. CAGE 552 and CAGE FAM were activated by 0.14 μW μm$^{-2}$ 405 nm laser for 10 seconds before collecting FRET signals.
Figure S9. sm-FRET measured reaction rates of aa-tRNA accommodation using Cy3/Cy5 FRET pair. (A) Cartoon illustrates experimental design. (B) Logarithms of the number of appeared FRET events plotted as a function of time and (C) reaction rates of aa-tRNA accommodation under various concentrations of Cy5 labeled tRNA. Number of events in the first bin were normalized to 1. Curves in (B) represent single-exponential fittings.
Figure S10. Time-dependent FRET contour plots constructed from sm-PAFRET trajectories recorded while delivering CAGE FAM labeled aa-tRNA to ribosomal POST complexes with Cy5-tRNA in the P-site to capture process shown in Figure 2D. 0.84 μW μm$^{-2}$ (A) or 1.4 μW μm$^{-2}$ (B) 405 nm laser were used for activation with 0.68 μW μm$^{-2}$ 488 nm laser excitation and 25 ms per frame collecting rate. All sm-PAFRET traces are aligned to the beginning of FRET events as t = 0, when both CAGE FAM and FRET signals appear simultaneously. Average FRET values at each time point were calculated and used to plot the black curves, from which transition rates from the initial low FRET to the stable high FRET were fitted by single exponential decay and listed.
Table S1. Signal-to-noise ratio (SNR) of Cy3, apparent FRET efficiency ($E_{app}$) and sigma of FRET distribution between Cy3/Cy5 under different concentrations of free fluorophores.

| Free Dyes   | Concentration/ nM | SNR a | $E_{app}$ b | sigma of FRET distribution b |
|-------------|-------------------|-------|-------------|-------------------------------|
| Cy3         | 0                 | 8.52±0.04 | 0.558±0.001 | 0.064±0.001 |
|             | 3                 | 8.46±0.04 | 0.556±0.001 | 0.065±0.001 |
|             | 10                | 7.89±0.03 | 0.555±0.001 | 0.069±0.001 |
|             | 30                | 6.91±0.04 | 0.556±0.001 | 0.083±0.001 |
|             | 100               | 4.74±0.05 | 0.554±0.001 | 0.148±0.001 |
|             | 300               | NA      | 0.512±0.001 | 0.181±0.001 |
| CAGE 552    | 0                 | 8.34±0.04 | 0.553±0.001 | 0.062±0.001 |
|             | 3                 | 8.42±0.05 | 0.555±0.001 | 0.063±0.001 |
|             | 10                | 8.35±0.03 | 0.558±0.001 | 0.064±0.001 |
|             | 30                | 8.91±0.05 | 0.557±0.001 | 0.063±0.001 |
|             | 100               | 9.03±0.05 | 0.559±0.001 | 0.065±0.001 |
|             | 300               | 9.40±0.05 | 0.559±0.001 | 0.064±0.001 |
|             | 1000              | 8.57±0.05 | 0.556±0.001 | 0.065±0.001 |
|             | 3000              | 8.66±0.04 | 0.556±0.001 | 0.066±0.001 |
|             | 10000             | 7.13±0.04 | 0.560±0.001 | 0.075±0.001 |
|             | 30000             | 5.53±0.05 | 0.575±0.001 | 0.114±0.001 |
|             | 100000            | 3.23±0.05 | N.A.         | N.A.            |

Single molecule trajectories were collected under 1.99 μW μm$^{-2}$ 532 nm laser illumination and 25 ms per frame.

a SNR of Cy3. Cy3 labeled D-strand-I were immobilized on microscope slide by forming duplex with unlabeled A-strand-I and biotinylated hander-I. Images of Cy3 under different concentrations of free fluorophores were presented in Figure S1. Distributions of Cy3 SNR were shown in Figure S3.

b FRET between Cy3 and Cy5 was measured from surface attached DNA duplex formed from Cy3 labeled D-strand-I, Cy5 labeled A-strand-I and biotinylated hander-I. Distributions of Cy3/Cy5 FRET were shown in Figure S4.
Table S2. Signal-to-noise ratio (SNR) of Alexa 488, apparent FRET efficiency ($E_{\text{app}}$) and sigma of FRET distribution between Alexa 488/Cy5 under different concentrations of free fluorophores.

| Free Dyes   | Concentration/nM | SNR $^a$ | $E_{\text{app}}$ $^b$ | Sigma of FRET distribution $^b$ |
|-------------|------------------|----------|------------------------|----------------------------------|
| Alexa 488   | 0                | 8.7±0.2  | 0.387±0.001            | 0.060±0.001                      |
|             | 3                | 9.0±0.2  | 0.391±0.001            | 0.060±0.001                      |
|             | 10               | 7.5±0.1  | 0.387±0.001            | 0.062±0.001                      |
|             | 30               | 6.8±0.1  | 0.386±0.001            | 0.066±0.001                      |
| CAGE FAM    | 0                | 8.3±0.2  | 0.388±0.001            | 0.062±0.001                      |
|             | 3                | 8.3±0.2  | 0.388±0.001            | 0.060±0.001                      |
|             | 10               | 8.3±0.2  | 0.386±0.001            | 0.059±0.001                      |
|             | 30               | 8.1±0.1  | 0.389±0.001            | 0.060±0.001                      |
|             | 100              | 8.1±0.1  | 0.389±0.001            | 0.060±0.001                      |
|             | 300              | 8.2±0.1  | 0.394±0.001            | 0.071±0.001                      |
|             | 1 000            | 7.6±0.1  | 0.383±0.001            | 0.122±0.001                      |
|             | 3 000            | 5.4±0.4  | 0.368±0.004            | 0.137±0.004                      |

Single molecule trajectories were collected under 0.68 µW µm$^{-2}$ 488 nm laser illumination and 50 ms per frame.

$^a$ SNR of Alexa 488. Alexa 488 labeled D-strand-II were immobilized on microscope slide by forming DNA duplex with unlabeled A-strand-II and biotinylated hander-II. Images of Alexa 488 under different concentrations of free fluorophores were presented in Figure S2. Distributions of Alexa 488 SNR were shown in Figure S5.

$^b$ FRET between Alexa 488 and Cy5 was measured from surface attached DNA duplex formed from Alexa 488 labeled D-strand-II, Cy5 labeled A-strand-II and biotinylated hander-II. Distributions of Alexa 488/Cy5 FRET were shown in Figure S6.
Table S3. Photoactivation rates of CAGE 552 and CAGE FAM under different activation laser (405 nm) power

| Dyes    | Power / μW μm⁻² | Photoactivation rate / s⁻¹ |
|---------|-----------------|---------------------------|
|         | 1.4             | 45±2                      |
| CAGE 552| 0.42            | 11.4±0.4                  |
|         | 0.14            | 3.46±0.07                 |
|         | 0.042           | 0.758±0.006               |
|         | 0.014           | 0.246±0.001               |
|         | 0               | 9.51±0.05 ×10⁻⁴           |
|         | 1.4             | 18±1                      |
| CAGE FAM| 0.42            | 5.8±0.2                   |
|         | 0.14            | 1.93±0.03                 |
|         | 0.042           | 0.92±0.01                 |
|         | 0.014           | 0.364±0.001               |
|         | 0               | 4.24±0.05 ×10⁻³           |

CAGE 552 and CAGE FAM were covalently attached on D-strand-I, which were immobilized on microscope slide by forming DNA duplex with unlabeled A-strand-I and biotinylated hander-I.
| Name           | Sequence (from 5' to 3')                                                                 |
|---------------|-----------------------------------------------------------------------------------------|
| Hander-I      | Biotin-CTAGATGTATGCTGC                                                                  |
| D-strand-I    | NH$_2$C$_6$-GCATCGAGC                                                                   |
| A-strand-I    | NH$_2$C$_6$-GCTCGATGC TACATC GCAGCATACATCTAG                                            |
| Hander-II     | Biotin-CCCTGGTCCGGTGGTGCCGCTGCTGCTGCCC                                                |
| D-strand-II   | GACGAGCAGACGCAATAAGATGT(NH$_2$C$_6$)GACGCTGATATTTCAG                                   |
| A-strand-II   | AGGCGGACCACCAGACCAGGGTCGAAT(NH$_2$C$_6$)ACCAGCG                                       |
| EFG(C113D)    | TGTTACGATGCGATTACGTTGCGTTGCTGATCN                                                     |
| EFG(C113D) rev| CTGCATCTGAAACCATTACCAGCGCC                                                             |
| EFG(C265A)    | GGTAACCAGCTGTCTGCTGCCTCAAGAGCA                                                        |
| EFG(C265A) rev| GAACCAGCGGTACAGAGATTTCCGTTG                                                           |
| EFG(C397S)    | CCTGTCTGACCACGGGTGCGGCC                                                               |
| EFG(C397S) rev| GGGTCAACACGGGTGCTACGGGACAAGG                                                         |
| EFG(S692C)    | GCGGTGAACGTTGCTCAGGCC                                                                |
| EFG(S692C) rev| CGTTACACGGCGCTTCATCATCAGTTCC                                                        |
| tRNA$^\text{Phe}$ primer A | AATTCGCCGGATAGCTCGAGCGGTAGACGAGGAGGTTGAAAA TCCCCGGTTCTTGGTTGATTCGAGTCCGGGCAACA CTGCA |
| tRNA$^\text{Phe}$ primer B | GTGGTGCCGGACTCGGAATCGAACCAAGGAGGAGGGGGATTGAAAA |
| tRNA$^\text{Phe}$ hander | TTTCAATCCCGCTCGCTACCGACTGAGTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT- Biotin |