Supporting Information for "Single-molecule, real-time measurement of enzyme kinetics by alternating-laser excitation fluorescence resonance energy transfer"

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Preparation of samples

Wild type (active 8-17): 5'- AACCTCTTCTCCGAGCCGGTCGAAATAGTGGATA – 3'

Mutant (inactive 8-17): 5'- AACCTCTTCTCCGA**T**CCGGTCGAAATAGTGGATA – 3'

Substrate: 5'- TATCCACTAT(rA)GGAAGAGGTT – 3', where rA denotes a ribonucleotide.

Wild type 8-17 deoxyribozyme[1] (*wild type*) and inactively mutated 8-17 deoxyribozyme (*mutant*) were ordered from Integrated DNA Technologies (Coralville, Iowa, USA). Substrate (*substrate*), labeled with carboxytetramethylrhodamine (TMR) at the 5'-end and modified with an amino-group at the 3' end, was ordered from Operon Technologies (Cologne, Germany). We labeled the 3'-end of the substrate with Alexa 647 (Molecular Probes, Eugene, OR) through the amino-modifier and purified it using 18% denaturing polyacrylamide gel electrophoresis, which resulted in a labeling efficiency of higher than 95%.

Single-molecule experimental setup and data analysis

The instrumentation, data acquisition, and data analysis of alternating laser excitation fluorescence resonance energy transfer (ALEX-FRET) have been well described in other papers.[2,3] We used 532-nm (solid state green laser, DPGL-20P, World Star Tech) and 633-nm (HeNe laser, 25-LHP-925, Melles-Griot) excitations, which were alternated using the combination of polarizers and electro-optic modulators (EOM, Conoptics) with a period of 120 μs. The excitation intensities were 100 μW and 27 μW for the 532-nm and 633-nm lights, respectively. Both lights were circularly polarized by λ/4 waveplates and coupled through a dichroic mirror (560DRLP, Omega Optical). The coupled lights were directed to an inverted microscope (IX71, Olympus), reflected on a dichroic mirror (Z488-533-633RPC, Chroma), and focused 20 μm from the surface of a coverslip through a water-immersion objective (60×, 1.2 NA, UPLAPO, Olympus). Fluorescence emissions, collected through the objective and the dichroic mirror, were focused through a 100 μm pinhole, and refocused using a biconvex lens onto silicon avalanche photodiode detectors (APD) (SPCM AQR-14, EG&G Perkin Elmer). After the biconvex lens, fluorescence was separated into two streams by a beamsplitter and filtered in front of each APD (for TMR, HQ600/40; for Alex647, HQ665LP; both from Chroma). All data analysis was performed using homemade LABVIEW software (National Instruments). ALEX-FRET generates four streams of photon counts.[2] From the 600-μs binned time traces, we

selected bursts (single molecules passing through the excitation volume) using a start/stop criterion on photon counts from acceptor-detection channel during acceptor-excitation time (threshold ~20 photons) and each burst was registered in all four streams of photon counts. From these photon counts for a burst, we calculated stoichiometric ratio (***S***) and FRET efficiency (***E***) for each molecule. In the discontinuous assay format, we collected fluorescent data from the mixture of the 8-17 deoxyribozyme and substrates for 10 minutes. The uncleaved substrates and cleaved ones were discriminated by ***S*** or in two-dimensional ***E-S*** diagram. In the continuous single measurement format, we collected fluorescent data for 1 hour and binned the bursts according to the detection time (reaction time) by the interval of 3 to 5 minutes, and obtained the ratio of uncleaved substrates.

Activity measurement by ensemble FRET

We measured the fluorescence intensity of a donor (TMR) and an acceptor (Alexa 647) using a spectrofluorometer (QM-4/2005SE, PTI) (excitation wavelength = 545 nm, emission wavelength = 555 ~ 730 nm, slit width = 1 nm resolution, and scanning time = 17 s). We used a 100-μL ultra-micro quartz cuvette with 1-cm path length (Hellma Cells, 105.250.QS10). For the measurement, we used 140 μL of the sample mixture (2 μM *wild type*, 10 nM *substrate*, 50 mM pH 7 Tris-HCl, 100 mM NaCl, and 0.01 mM EDTA, followed by the addition of a specific concentration of $MgCl_2$ ranging from 0 to 20 mM to initiate the cleavage reaction). As soon as we added $MgCl_2$ to the mixture, we mixed it thoroughly and started to measure the change of fluorescence intensity. The fluorescent intensities of the donor and the acceptor were calculated by integrating the fluorescent intensity from 565 to 595 nm for the donor, and from 650 to 680 nm for the acceptor. According to the measured time, the intensities were fit by a single-exponential function, which yielded the cleavage rate constant. The increase in the donor emission and the decrease in the acceptor emission gave the same cleavage rate constants within the fitting error. For control experiments, we carried out the same process using the inactive 8-17 deoxyribozyme (*mutant*) and without Mg^{2+}. In both cases, no change in the fluorescent intensities of the donor and the acceptor was observed (data not shown). All experiments were performed in the single-turnover condition where the enzyme is in excess over the substrate and a single cleavage event takes place. The observed rate constants were plotted as a function of the Mg^{2+} concentration, which was fit well with a hyperbolic function (Figure 2a, solid black line): $k_{obs} = k_{max}$ [Mg^{2+}] / (K_d + [Mg^{2+}]), where k_{max} denotes the cleavage rate constant at the saturating Mg^{2+} and K_d denotes the apparent dissociation constant of Mg^{2+}.

Quenching assay format for the activity measurement by ALEX-FRET

We prepared a 10 μL mixture of 1μM 8-17 deoxyribozyme (*wild type*), 2.5 nM *substrate*, 50

mM pH 7 Tris-HCl, 100 mM NaCl, and 0.01 mM EDTA, and incubated it at 22℃ for 30 minutes. Then the cleavage reaction of 8-17 deoxyribozyme was initiated by adding $MgCl_2$ to the mixture (final concentration, 0 ~ 20 mM). At each time point, we took out 1 μL of the reaction mixture and diluted it with 25 ~ 50 μL of quenching buffer (EDTA with a half concentration of added $MgCl_2$, 50 mM pH 7 Tris-HCl, 250 mM NaCl, 5 % glycerol, and 1 mM mercaptoethylamine), which resulted in 50 ~ 100 pM substrate concentration for single-molecule measurement. Using our ALEX-FRET setup, we obtained a 2D ***E-S*** diagram for each diluted sample through 10 minutes of data acquisition. From the 2D ***E-S*** diagram, we obtained the fraction of the uncleaved substrate (F = [(number of G-R)/(number of G-R + number of R)]) at various reaction times. The decay of F was fitted with single exponential decay function, which yielded the observed cleavage rate (Figure 1b). We performed the same process using the inactive 8-17 deoxyribozyme and without Mg^{2+} in our control experiments, which showed no change in the fraction of the uncleaved substrates (data not shown).

Continuous assay format for the activity measurement by ALEX-FRET

We prepared a 10 μL mixture of 2.5 μM 8-17, 2.5 nM *substrate*, 50 mM pH 7 Tris-HCl, 100 mM NaCl, and 0.01 mM EDTA, and incubated it at 22℃ for 30 minutes. Then we initiated the cleavage reaction of 8-17 deoxyribozyme by diluting 1 μL of the mixture using 25 ~ 50 μL of reaction buffer (various concentrations of $MgCl_2$, 50 mM pH 7 Tris-HCl, 250 mM NaCl, 5 % glycerol, and 1 mM mercaptoethylamine). We immediately started measuring the fluorescence from the sample using ALEX-FRET for 1 hour. The uncleaved fraction was obtained by binning the data at appropriate time points (Figure 2c). By fitting the uncleaved fraction according to reaction time with the single exponential decay function, we obtained the catalytic rate of 8-17 deoxyribozyme at various Mg^{2+} concentrations. The observed rate constants were plotted as a function of Mg^{2+} concentration, which was fitted well to a hyperbolic function (Figure 2d).

Figures

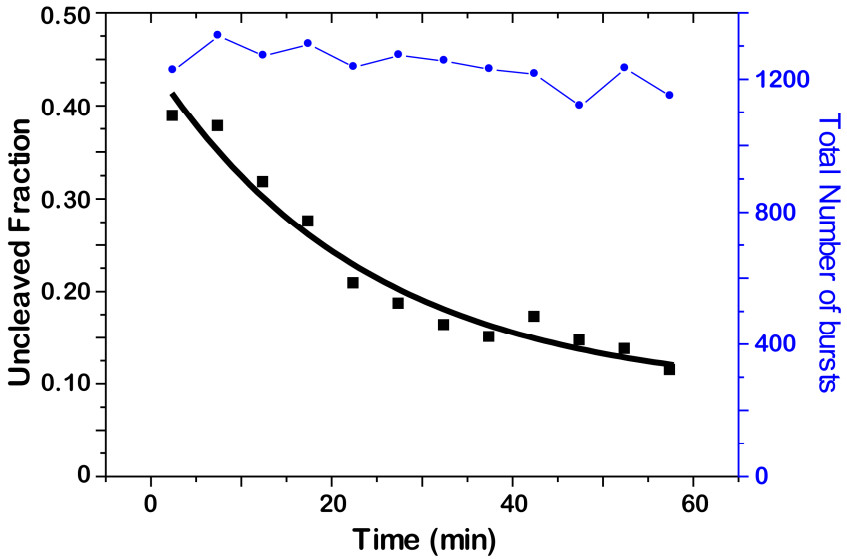

Fig. S1 The total number of red dyes (blue circles) over time shown against the fraction of uncleaved substrates (black squares) in single continuous ALEX-FRET measurement at 2 mM Mg^{2+}. The decrease in the number of red dyes is seen negligible during the measurement, which supports that ALEX-FRET suffers little from fluorophore photobleaching.

Reference

(1) **Li, J.; Zheng, W. C.; Kwon, A. H.; Lu, Y.** ***Nucleic Acids Res.*** **2000,** ***28*****, 481-488.**

(2) **Lee, N. K.; Kapanidis, A. N.; Wang, Y.; Michalet, X.; Mukhopadhyay, J.; Ebright, R. H.; Weiss, S.** ***Biophys. J.*** **2005,** ***88*****, 2939-2953.**

(3) **Lee, N. K.; Kapanidis, A. N.; Koh, H. R.; Korlann, Y.; Ho, S. O.; Kim, Y.; Gassman, N.; Kim, S. K.; Weiss, S.** ***Biophys. J.*** **2007,** ***92*****, 303-312.**