Electronic Supplementary Information

CRISPR/Cas12a-enhanced single-molecule counting for sensitive detection of flap endonuclease 1 activity at single-cell level

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EXPERIMENTAL SECTION

Chemicals and Materials.

All DNA oligonucleotides (Table S1) were synthesized and HPLC purified by Sangon Biotech Co., Ltd. (Shanghai, China), and the crRNAs were synthesized and HPLC purified by Accurate Biotechnology Co., Ltd. (Hunan, China). Thermostable FEN1, EnGen Lba Cas12a (Cpf1), uracil DNA glycosylase (UDG), Exonuclease III (Exo III), BssHII, human apurinic/apyrimidinic endonuclease (APE1), bovine serum albumin (BSA), Lambda exonuclease (λ exo) and streptavidin-coated magnetic beads were obtained from New England Biolabs (Ipswich, MA, USA). Diethylpyrocarbonate (DEPC)-treated water was obtained from Sangon Biotech Co., Ltd. (Shanghai, China). Aurintricarboxylic acid (ATA) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Human breast adenocarcinoma cell line (MCF-7 cells), human hepatic cell line (LO2 cells), human hepatocellular carcinoma cell lines (HepG-2 cells), human lung adenocarcinoma A549 cell line (A549 cells) and human cervical carcinoma cell line (HeLa cells) were bought from Cell Bank, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China). All other reagents were of analytical grade and used as received without further purification. All the solutions for reactions were prepared with DEPC-treated water.

Table S1. Sequence of synthesized oligonucleotides

note	sequence(5′-3′)
HP1	AGT TAG GGT TAG GGT TAG GGG AAA ACT GTT TCC CCT AAC CCT AAC CCT AAC TTT TTT GTG TCG CGC GAT GTA GCT GG
HP2	Biotin-TTT TTC CAG CTA CAT CGC GCG ACA CAC CAG TTC CTC GAT CTT GTC TCC CTG ACA AGA TCG AGG AAC TGG T
crRNA	UAA UUU CUA CUA AGU GUA GAU CCC UAA CCC UAA CCC UAA CU
Signal probe	Cy5-TTATT- BHQ2
PAGE-signal probe	Cy5-CAG GCA ATG TCC AAC ACG AA-BHQ2

Preparation of HP1/HP2@MB nanoprobes.

50 μL of 4 mg/mL streptavidin-coated magnetic beads solution was washed twice with 1×B & W buffer (5 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 1 M NaCl), and the magnetic beads were resuspended in 50 μL of 2 × B&W buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 2 M NaCl). Subsequently, 50 μL of 1 μM HP1 / HP2 were added into the solution, respectively, and incubated for 30 min at room temperature with rotation. The mixture was then washed three times with 50 μL of 1× B&W buffer to remove the uncoupled probes. Finally, the remaining HP1/HP2@MB nanoprobes were dispersed in 50 μL of DEPC-treated water.

FEN1 detection.

10 μL of reaction system containing 1 × ThermoPol buffer, 2 μL of HP1/HP2@MB nanoprobes, and different-concentration FEN1 was incubated at 60 ℃ for 40 min. After

magnetic separation, 0.2 μL of supernatants was added into 20 μL of reaction system containing 100 nM crRNA, 100 nM Cas12a and 100 nM signal probe, and incubated at 37 ℃ for 1 h.

FEN1 inhibition assay.

For the FEN1 inhibition assay, different-concentration ATA was incubated with FEN1 and HP1/HP2@MB nanoprobe in 1 × ThermoPol buffer for 5 min at 60 °C, The measurement of Cy5 counts follows same procedure described above. The relative activity (*RA*) of FEN1 was measured according to equation 1.

$$RA\ (\%) = \frac{C_i}{C_t} \times 100\% = 10^{(Ni-Nt)/38.59} \times 100\% \quad (1)$$

where N_t represents the Cy5 counts in the presence of 1 U of FEN1, and N_i represents the Cy5 counts in the presence of 1 U of FEN1 + ATA inhibitors. C_i and C_t were obtained according to the linear correlation equation (Fig. 3A), respectively.

$$N_t = 216.59 + 38.59\ lgC_t \quad (2)$$

$$N_i = 216.59 + 38.59\ \lg C_i \quad (3)$$

The IC_{50} value of inhibitor was obtained from the curve-fitting equation.

Fluorescence measurement.

The fluorescence spectra were detected by a FLS-1000 fluorescence spectrophotometer (Edinburgh Instruments Ltd., Livingston, United Kingdom) at an excitation wavelength of 635 nm, and the fluorescence intensity at 670 nm was employed for date analysis.

Gel electrophoresis.

To analyze the products of FEN1 and Cas12a-mediated cleavage reaction, 12% nondenaturing polyacrylamide gel electrophoresis (PAGE) was performed in 1 × TBE buffer (9 mM Tris-HCl,

PH 7.9, 9 mM boric acid, 0.2 mM EDTA) at a 110 V constant voltage for 60 min at room temperature. The gel was imaged by ChemiDoc MP Imaging System (Hercules, CA, USA).

Single-molecule detection and data analysis.

Prior to single-molecule detection, the reaction products were diluted 80-fold with the imaging buffer (5 mM $MgCl_2$, 1 mM trolox, 50 mM KCl, 10 mM Tris-HCl, pH 8.0). The 10 μL of sample was dropped onto the coverslip for total internal reflection fluorescence microscopy (TIRF, Nikon, Ti-E, Japan) imaging. The Cy5 molecules were excited by 640 nm laser (50 mW, Coherent, USA) via the total internal reflection. A 100 × objective (Olympus, Japan) was used to collect the photons from Cy5, and an Andor Ixon DU897 EMCCD was employed to image Cy5 molecules with an exposure time of 500 ms. Image J software was used to count Cy5 molecules in a region of interest of 500 × 500 pixels.

Kinetic Analysis.

To evaluate the enzyme kinetic parameters of FEN1, we measured the initial velocity in the presence of 6.4 U FEN1 and different-concentration hairpin probe in 5 min reaction at 65 °C. The kinetic parameter is fitted to the Michaelis-Menten equation:

$$V = \frac{V_{max}+[S]}{K_m+[S]} \qquad (4)$$

where V_{max} is the maximum initial velocity, and [S] is the concentration of hairpin probe, and K_m is the Michaelis-Menten constant.

Cell culture and preparation of cell extracts.

MCF-7 cells, HepG-2 cells and LO_2 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin in 5% CO_2 incubator at 37°C. At the exponential phase of growth, the cells were collected with trypsinization, washed

twice with ice-cold phosphate buffered saline, and pelleted at 200 rcf at 4 C for 5 min. The nuclear extracts were prepared by using a nuclear extract kit (Active Motif, Carlsbad, CA, USA). The obtained supernatants were subjected to FEN1 activity assay immediately or stored at -80 °C until use.

RESULTS AND DISCUSSION

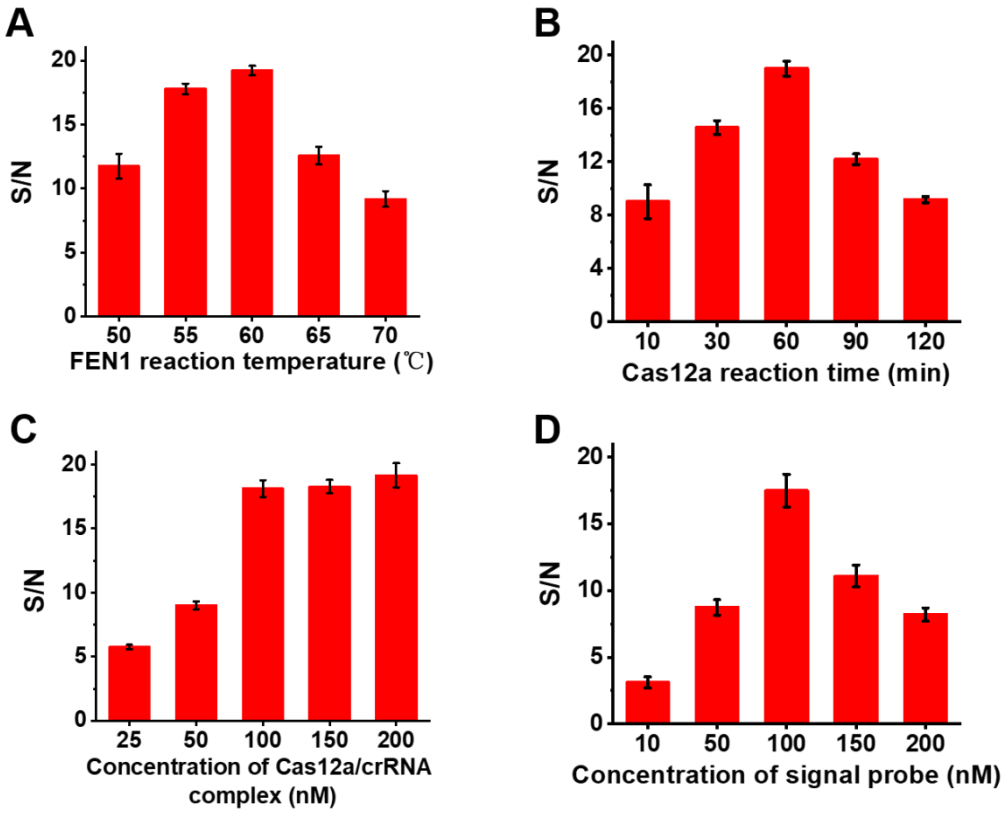

Fig. S1. Variance of signal-to-noise ratio (S/N) with FEN1 reaction temperature (A), Cas12a reaction time (B), the concentration of Cas12a/crRNA complex (C), and the concentration of signal probe (D), respectively. Error bars represent the standard deviation of three experiments.

To achieve the best assay performance, we optimized several experimental conditions including the reaction temperature of FEN1, the concentration of signal probe, the reaction time of Cas12a,

and the concentration of Cas12a/crRNA complex. The signal to noise ratio (*S/N*) was used to evaluate the assay performance, and it represents the ratio of Cy5 signal in the presence of FEN1 to that in the absence of FEN1. As shown in Fig. S1, the maximum *S/N* value is obtained at the FEN1 reaction temperature of 60 °C, the Cas12a reaction time of 60 min, 100 nM Cas12a/crRNA complex, and 100 nM signal probe, which are set as the optimal experimental conditions.

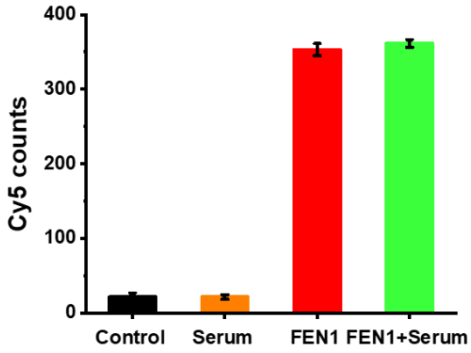

Fig. S2. (A) Measurement of Cy5 counts in response to reaction buffer (control), 10% normal human serums, FEN1, FEN1+10% serum samples.

To demonstrate the feasibility of this biosensor for complex sample analysis, we measured FEN1 spiked in 10% normal human serums. Negligible signal is detected in response to control group with only reaction buffer (Fig. S2A, black column) and 10% serum samples (Fig. S2A, yellow column). The addition of FEN1 to the 10% normal human serum generates a high Cy5 signal (Fig. S2, green column), which is identical to that generated by FEN1 alone (Fig. S2A, red column). We further measured the recovery ratios by spiking various concentrations of FEN1 (0.01–1 U) in 10% human serum. The recovery ratio of 98.11–103.15% with a relative standard deviation (RSD) of 0.61–1.60% is obtained (table S3), suggesting the feasibilty of this biosensor for complex sample analysis.

Table S2. Comparison of the proposed biosensor with the reported FEN1 assays.

Strategy	Signal model	Linear range	LOD	Reaction time	Ref.
Poly dA_{20}-based nanoprobe	Fluorescence	0.05 – 2 U	0.007 U	2.5 h	1
Fluorescent nanoprobe based on DNA-silver nanoclusters	Fluorescence	0.8 – 40 U	0.4 U	8 h	2
Nanocomposite formed by graphene oxide and dye-labeled DNA.	Fluorescence	0.008 – 4 U	0.015 U	50 h	3
Nt.BstNBI-induced tandem signal amplification	UV−vis	0.03 – 1.5 U	0.01 U	5 h	4
	Fluorescence	0.001 – 1.5 U	0.00075 U	4 h	
Nicking enzyme-assisted signal amplification based on ZIF-8	Fluorescence	0.05 – 2 U	0.007 U	3.5 h	5
DNA nanosphere	Fluorescence	0 – 3 U	0.016 U	7 h	6
Hyperbranched rolling circle amplification	ECL	6.5×10^{-2} – 6.5×10^{3} U/L	2.2×10^{-2} U/L	5 h	7
Rolling circle amplification	Fluorescence	0.0008 – 0.32 U	0.0006 U	1.5 h	8
Ligation-promoted hyperbranched rolling circle amplification	Fluorescence	2.0×10^{-5} – 2.0×10^{-3} U	1.51×10^{-5} U	5 h	9
Magnetic separation-assisted cascade hybridization chain reaction amplification	Fluorescence	0.002 – 0.25 U	0.0018 U	10 h	10
CRISPR/Cas12a-enhanced single-molecule counting	Single-molecule detection	1.0×10^{-5} – 1 U	2.25×10^{-5} U	100 min	This work

Table S3. Detection of FEN1 spiked in 10% serum samples.

Sample	Added (U)	Detected (U)	Recovery ratio (%)	RSD (%, n=3)
1	1.00	0.98	98.11	0.61
2	0.10	0.10	101.62	1.40
3	0.01	0.01	103.15	1.60

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