Electronic Supplementary Information

A label-free and self-circulated fluorescent biosensor for sensitive detection of ten-eleven translocation 1 in cancer cells

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EXPERIMENTAL SECTION

Materials

All HPLC purified oligonucleotides were provided by TaKaRa Biotechnology Co., Lid. (Dalian, China), and their sequences are listed in Table S1. Ten-eleven translocation 1 protein (TET1) was provided by Epigentek Group Inc (New York, NY, USA). 10× NEBuffer 4 (500 mM potassium acetate, 200 mM Trisacetate, 100 mM magnesium acetate, 10 mM DTT, pH 7.9), T4 phage β-glucosyltransferase (T4-βGT), uridine diphosphate glucose (UDP-glucose), 10× CutSmart buffer (100 mM magnesium acetate, 200 mM Tris-acetate, 500 mM potassium acetate, 1 mg/mL BSA, pH 7.9), AbaSI, 10× ThermoPol reaction buffer (200 mM Tris-HCl, 100 mM $(NH_4)_2SO_4$, 100 mM KCl, 20 mM $MgSO_4$, 1% Triton X-100, pH 8.8), Bst DNA polymerase, 10× NEBuffer™ r3.1 (1M NaCl, 500 mM Tris-HCl, 100 mM $MgCl_2$, 1 mg/ml Recombinant Albumin, pH 7.9), Nb.BsmI, and deoxynucleotide solution mixture (dNTPs) were ordered from New England Biolabs (Ipswich, MA, USA). SYBR Gold and SYBR Green II were obtained from Invitrogen Co. (Carlsbad, CA, USA). N-Oxalylglycine, ammonium iron (II) sulfate hexahydrate ($(NH_4)_2Fe(SO_4)_2$), α-ketoglutaric acid disodium salt dehydrate (α-KG), DL-dithiothreitol (DTT), ascorbic acid, HEPES, $(NH_4)_2SO_4$, $MgCl_2$, and NaCl were ordered from Sigma-Aldrich Company (St. Louis, MO, USA). Human cervical cancer cell line (HeLa cells) and human neuroblastoma cell line (SK-N-BE (2) cells) were provided by Cell Bank of Chinese Academy of Sciences (Shanghai, China). Ultrapure water prepared by a Millipore filtration system (Millipore, Milford, MA, USA) was used throughout.

Table S1. Sequences of synthesized oligonucleotides [α]

note	sequence (5'-3')
sense substrate	TAC TTC TAG CTC mCGG TCA CGT ATC TAG TTC AAT CCG GTT C-NH_2
antisense substrate	GAA CmCG GAT TGA ACT AGA TAC GTG ACC GGA GCT AGA AGT A-NH_2
circular template	PO_4-CTT ACC TTG ATA CGA CCT CAG ATG ACC AGA ATG CAC GAG TAG ATA CGT GAC CGG AGC TAG AAG TA

[α] The letter mC in sense substrate and antisense substrate indicates the methylated cytosine, and the underlined letter in circular template indicates the nicking site of Nb.BsmI nicking endonuclease.

Preparation of stock solutions

All oligonucleotides were diluted with deionized water to prepare the stock solutions. The dsDNA substrates were prepared by incubating 10 μM sense substrate with 10 μM antisense substrate in annealing buffer (10 mM Tris-HCl, 1.5 mM $MgCl_2$, pH 8.0) at 95 °C for 5 min, followed by slowly cooling to room temperature. The obtained dsDNA substrates were stored at -20 °C for further use.

Detection of TET1

The detection of TET1 comprises of four sequential steps: (1) TET1-catalyzed conversion of 5mC to 5hmC in dsDNA substrates, (2) T4 β-glucosyltransferase (T4 β-GT)-assisted glucosylation of 5hmC to glucosyl-modified 5hmC (5ghmC) in dsDNA substrates, (3) cleavage of glucosylated dsDNA substrates catalyzed by AbaSI, (4) primers-activated PG-RCA reaction for the generation of abundant ssDNAs. 500 nM dsDNA substrates were incub ated with various concentrations of TET1 in the reaction buffer (2.5 mM DTT, 1 mM α-KG, 2 mM ascorbic acid, 75 μM $Fe(NH_4)_2(SO_4)_2$, 50 mM NaCl, and 50 mM HEPES) at 37 °C for 30 min, followed by the addition of 40 μM UDP-glucose, 3 U of T4-βGT, and 2 μL of 10×

NEBuffer 4 and incubation for another 2 h at 37 °C. Subsequently, 10 μL of glucosylated product was digested by 4 U of AbaSI in 1× CutSmart buffer at 25 °C for 30 min. Finally, the AbaSI digestion products (2 μL) were incubated with reaction solution (20 μL) containing 100 μM dNTPs, 5 nM circular template, 2 μL of 10× ThermoPol reaction buffer, 1.6 U of Bst DNA polymerase, 2 μL of 10× NEBuffer™ r3.1, and 4 U of Nb.BsmI to perform the PG-RCA reaction at 65 ℃ for 1 h. Finally, 20 μL of PG-RCA products (ssDNAs) and 6 μL of SYBR Green II (10×) were incubated in 34 μL of deionized water for 5 min at room temperature.

Fluorescent assay and gel electrophoresis

Fluorescence emission spectra were obtained at room temperature by using an F-7000 fluorescence spectrophotometer (Hitachi, Japan) equipped with a xenon lamp as the excitation source. The emission spectra from 500 to 650 nm were recorded at an excitation wavelength of 488 nm, with both emission and excitation slits being 5 nm. Data analysis was performed using the fluorescence intensity at 523 nm, with error bars showing the standard deviation of three experiments. The PG-RCA products were analyzed by 2 % agarose gel electrophoresis for 50 min at 110 V constant voltage in 1× TAE running buffer (2 mM EDTA and 40 mM Tris-ethylic acid). After staining with SYBR Gold, the gel was imaged using Bio-Rad ChemiDoc MP imaging system (California, U.S.A.).

Inhibition assay

To evaluate the effect of TET 1 inhibitor, different-concentration N-Oxalylglycine (NOG) was incubated with 500 nM dsDNA substrates and 10 ng/μL TET1 in the reaction buffer (2.5 mM DTT, 1 mM α-KG, 2 mM ascorbic acid, 75 μM $Fe(NH_4)_2(SO_4)_2$, 50 mM NaCl, and 50 mM HEPES) at 37 °C for 30 min. The TET1 activity was detected by this biosensor. The relative activity (R_A) of TET1 was calculated according to eq 1

$$R_A\,(\%) = \frac{C_i}{C_t} \times 100\% = 10^{(F_i - F_t)/948.51} \times 100\% \qquad (1)$$

where F_i is the fluorescence intensity generated by TET1 + NOG, and F_t is the fluorescence intensity generated by TET1. C_t and C_i were derived based on linear equation in Fig. 2B. The IC_{50} value of NOG was obtained from the inhibition curve.

Recovery assay

A total volume of 10 μL of reaction mixture containing 10% human serum spiked with various concentrations of TET1, 500 nM dsDNA substrates, 1× reaction buffer (2.5 mM DTT, 1 mM α-KG, 2 mM ascorbic acid, 75 μM $Fe(NH_4)_2(SO_4)_2$, 50 mM NaCl, and 50 mM HEPES), and incubated at 37 °C for 30 min. The subsequent reactions and measurement followed the above procedures.

Cell culture and preparation of cell extracts

Human tumorigenic N-type neuroblastoma cell line (SK-N-BE (2) cells) was cultured in 45 % MEM cell culture medium, and human cervical carcinoma cell line (HeLa cells) was cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, USA) with 5% CO_2 at 37 °C, with the addition of 10 % fetal bovine serum and 100 U/mL streptomycin and penicillin in culture medium. Prior to extraction, cell numbers were counted by a Countstar automated cell counter. The cells were harvested in the exponential growth phase with trypsin and washed twice with ice-cold phosphate buffered saline (137 mM NaCl, 2.7 mM KCl, 10 mM phosphate buffer, pH 7.4), and centrifuged at 800 rpm for 5 min. The nuclear proteins were collected by using a nuclear extract kit (Active Motif, Carlsbad, CA, U.S.A.), and the resultant cell extracts were transferred into a fresh tube and used for TET1 assay immediately.

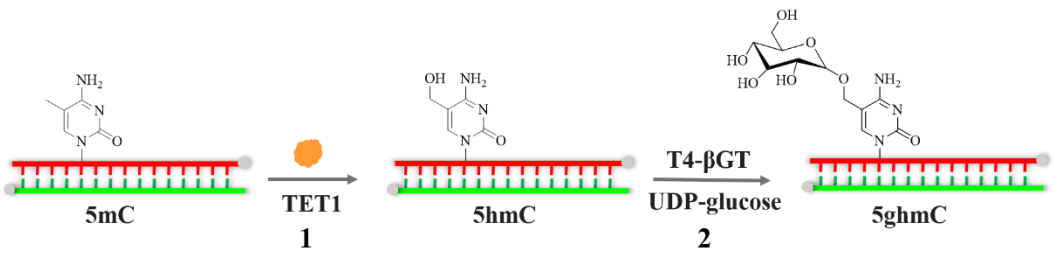

Fig. S1 Pathways of the hydroxymethylation of 5mC by TET1 and the glucosylation of 5hmC by T4 β-GT.

Optimization of experimental conditions

To achieve the best assay performance, we optimized a series of experimental parameters including the incubation time of TET1, the amount of AbaSI, the concentration of circular template, the amount of dNTP, the concentrations of Bst DNA polymerase and Nb.BsmI nicking endonuclease.

The incubation time of TET1 directly determines the yield of 5hmC in dsDNA substrate by catalyzing the oxidation reaction of 5mC. We investigated the effect of TET1 incubation time upon the assay performance. As shown in Fig. S2, the F/F_0 value enhances with the incubation time from 5 to 30 min, and levels off beyond 30 min, where F and F_0 are the fluorescence intensity in the presence and absence of TET1, respectively. Thus, 30 min of incubation time is selected for TET1 assay in the subsequent researches.

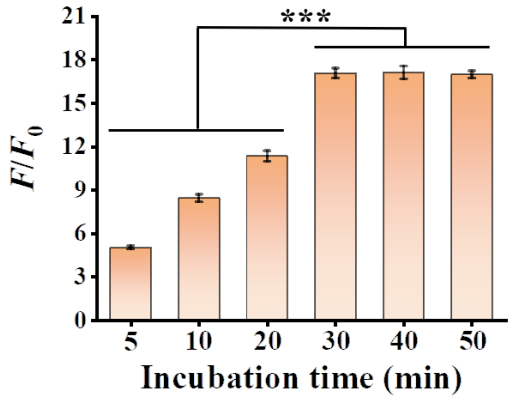

Fig. S2 Variance of the F/F_0 value with the incubation time. The TET1 concentration is 10 ng/μL.

***P<0.001. Error bars represent the standard deviation of three experiments.

AbaSI-mediated cleavage of glucosylated dsDNA substrate produces large numbers of primers to initiate the PG-RCA reaction. Therefore, the amount of AbaSI should be carefully optimized. As shown in Fig. S3, the F/F_0 value enhances with the increasing amount of AbaSI from 1 to 4 U, followed by the decrease beyond the amount of 4 U, where F and F_0 are the fluorescence intensity in the presence and absence of TET1, respectively. Thus, 4 U of AbaSI is selected for TET1 assay in the subsequent researches.

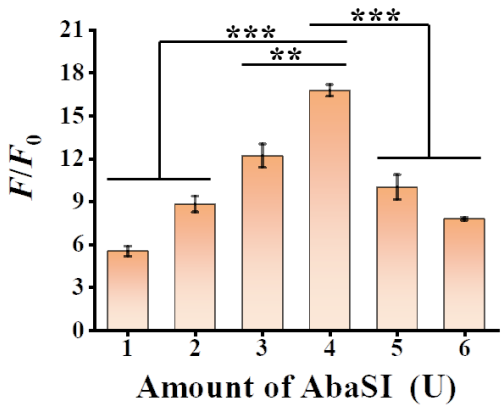

Fig. S3 Variance of the F/F_0 value with the amount of AbaSI. ***P<0.001, **P<0.01. Error bars represent the standard deviation of three experiments.

The concentration of circular template may affect the amplification efficiency of the PG-RCA. On one hand, the high-concentration circular template induces high amplification efficiency, but it might increase the background correspondingly. On the other hand, the low-concentration circular template decreases the background, but it may induce low amplification efficiency. Therefore, we monitored the variance of F/F_0 value with different concentrations of circular template. As shown in Fig. S4A, a maximum value of F/F_0 is obtained at the circular template concentration of 5 nM, where F and F_0 are

the fluorescence intensity in the presence and absence of TET1, respectively. Thus, 5 nM circular template is selected for TET1 assay in the subsequent researches.

We further investigated the effect of dNTP upon the amplification efficiency of the PG-RCA. As shown in Fig. S4B, the F/F_0 value enhances with the increasing amount of dNTP from 25 to 100 μM, follow by the decrease beyond the amount of 100 μM, where F and F_0 are the fluorescence intensity in the presence and absence of TET1, respectively. Thus, 100 μM dNTP is used in the subsequent researches.

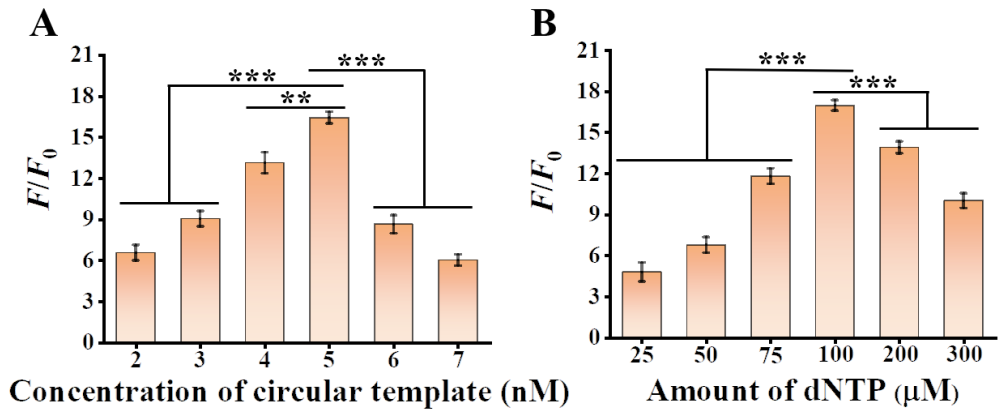

Fig. S4 (A) Variance of the F/F_0 value with the concentration of circular template. (B) Variance of the F/F_0 value with the amount of dNTP. ***P<0.001, **P<0.01. Error bars represent the standard deviation of three experiments.

In this assay, the amplification efficiency of PG-RCA relies on the cooperation of Bst DNA polymerase with Nb.BsmI nicking endonuclease. Therefore, the concentrations of Bst DNA polymerase and Nb.BsmI should be carefully optimized. We investigated the influence of Bst DNA polymerase upon the fluorescence signal. As shown in Fig. S5A, the F/F_0 value enhances with the increasing concentration of Bst DNA polymerase from 0.8 to 1.6 U, followed by the decrease beyond the concentration of 1.6 U, where F and F_0 are the fluorescence intensity in the presence and absence of TET1, respectively. Thus, 1.6 U of Bst DNA polymerase is used in the subsequent researches.

We further investigated the influence of Nb.BsmI upon the fluorescence signal. As shown in Fig. S5B, the F/F_0 value enhances with the increasing amount of Nb.BsmI from 1 to 4 U, followed by the decrease beyond the amount of 4 U, where F and F_0 are the fluorescence intensity in the presence and absence of TET1, respectively. Thus, 4 U of Nb.BsmI is used in the subsequent researches.

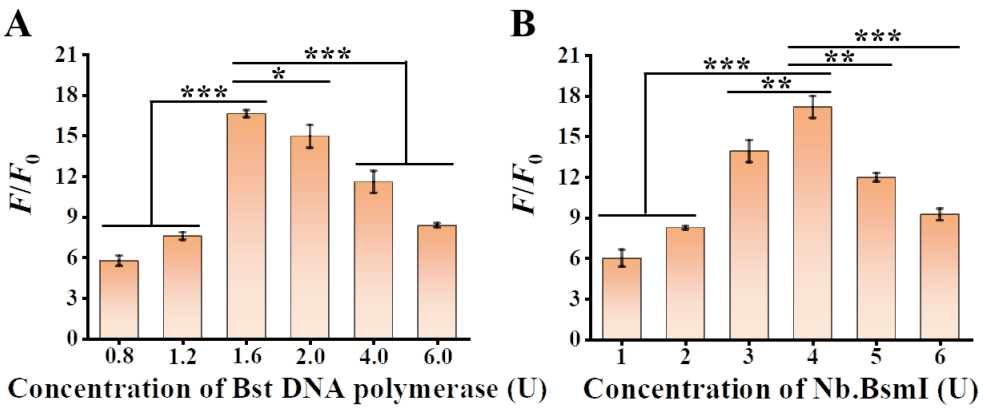

Fig. S5 (A) Variance of the F/F_0 value with the amount of Bst DNA polymerase. (B) Variance of the F/F_0 value with the amount of Nb.BsmI. ***P<0.001, **P<0.01, *P<0.05. Error bars represent the standard deviation of three experiments.

Specificity of the assay

We selected bovine serum albumin (BSA), uracil DNA glycosylase (UDG), fat mass and obesity-associated protein (FTO), and T4 polynucleotide kinase (PNK) as the negative controls to investigate the detection specificity. UDG is a DNA glycosylase that can excise uracil from the U:A base pair,[1] but it exhibits no activity toward 5mC. FTO is an RNA demethylase responsible for removing the methyl group from the RNA probe.[2] PNK enables phosphorylation of 5′-OH termini in oligonucleotides/nucleic acids.[3] BSA is a nonspecific protein. As expected, only target TET1 can produce a high fluorescence signal (Fig. 3A, line 1), which can be well distinguished from those produced by UDG (Fig. 3A, line 2), FTO (Fig. 3A, line 3), PNK (Fig. 3A, line 4), BSA (Fig. 3A, line 5), and without any target analytes (Fig. 3A, line

6, control). Notably, the fluorescence intensity generated by TET1 is 12.6, 12.5, 12.7, 11.7, and 17.0-fold higher than those generated by UDG, FTO, PNK, BSA, and control, respectively (Fig. 3B), suggesting good specificity of this biosensor toward TET1.

Kinetic analysis

The kinetic parameters of TET1 were further investigated. We determined the initial velocity (V) in response to 10 ng/μL TET1 plus different-concentration dsDNA substrate at 37 °C in 5-min reaction. The initial velocity of TET1 gradually improves with the increasing concentration of dsDNA substrate (Fig. 3C). Kinetic parameters of TET1 were measured based on Michaelis-Menten equation $V = V_{max}[S]/(K_m + [S])$, where K_m, V_{max}, and $[S]$ represent Michaelis-Menten constant, maximum initial velocity, and the dsDNA substrate concentration. The measured V_{max} is 308.72 nM/min, and the measured K_m of whole enzyme system is 572.35 nM, which is consistent with that measured by LC-MS/MS ($K_m = 0.38 \pm 0.10$ μM).[4]

Table S2. Comparison of the proposed biosensor with the reported methods for TET1 assay.

Method	Assay time	LOD (fg/μL)	Linear range (fg/μL)	Real samples	Ref.
LC-MS assay	not mentioned	not mentioned	1×10^{-7} – 3×10^{-7}	no	5
TET activity assay kit (fluorometric)	5 h	1×10^{6}	1×10^{6} – 1×10^{7}	Cells / tissues	Epigentek Group Inc.
Fluorescence assay based on ThT-induced conformation switch of G-quadruplexes	overnight	5.4×10^{6}	1.05×10^{7} – 5.25×10^{7}	no	6
Electrochemiluminescence biosensor based on gold nanoclusters-H_2O_2 system	16 h	3.7×10^{5}	1×10^{6} – 1×10^{7}	no	7
Electrochemical biosensor based on nanoparticles and Ru (III) redox recycling	20 h	3.3×10^{5}	3.5×10^{6} – 2.1×10^{7}	no	8
Fluorescent biosensor based on primer generation rolling circle amplification	4 h	0.068	1 – 1×10^{7}	live cells serum samples	This work

Table S3. Recovery ratio of TET1 spiked in human serum (10 %) samples

Added (fg/μL)	Measured (fg/μL)	Recovery (%)	RSD (%)
10	9.93	99.32	1.43
100	99.63	99.63	0.84
1000	1001.10	100.10	0.29

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