Electronic Supplementary Information

Integration of single-molecule detection with endonuclease IV-assisted signal amplification for sensitive DNA methylation assay

Yan Zhang,[‡a] Jinping Hu,[‡a] Xiaoran Zou,[‡a] Fei Ma,[*b] Jian-Ge Qiu,[*c] and Chun-yang Zhang[*a]

[a] College of Chemistry, Chemical Engineering and Materials Science, Collaborative Innovation Center of Functionalized Probes for Chemical Imaging in Universities of Shandong, Key Laboratory of Molecular and Nano Probes, Ministry of Education, Shandong Provincial Key Laboratory of Clean Production of Fine Chemicals, Shandong Normal University, Jinan 250014, China

[b] School of Chemistry and Chemical Engineering, Southeast University, Nanjing 211189, China.

[c] Academy of Medical Sciences, Zhengzhou University, Zhengzhou, 450000, China.

‡ These authors contributed equally.

* Correspondence authors. E-mail: cyzhang@sdnu.edu.cn, fei@seu.edu.cn, jiangeqiu@zzu.edu.cn.

EXPERIMENTAL SECTION

Materials.

All oligonucleotides (Table S1) were synthesized and HPLC purified by Sangon Biotechnology Co. Ltd. (Shanghai, China). HpaII, 10 × Cutsmart buffer (500 mM KAc, 200 mM Tris-Ac, 100 mM $Mg(Ac)_2$, 100 μg/mL bovine serum albumin (BSA), pH 7.9), endonuclease IV, 10 × NEB buffer 3 (1 M NaCl, 500 mM Tris-HCl, 100 mM $MgCl_2$, 10 mM DTT, pH 7.9) and NEBNext dsDNA Fragmentase were obtained from New England Biolabs (Beverly, MA, USA). The streptavidin-coated magnetic beads (Dynabeads®M-280 Streptavidin, Dynal) were obtained from Invitrogen (California, CA, USA). Nuclear extract kit was brought from Active Motif (Carlsbad, CA, U.S.A.). All other reagents were of analytical grade and used without further purification. Ultrapure water was prepared by a Millipore filtration system (Millipore, Milford, MA, USA).

Table S1. Sequences of the oligonucleotides[a]

note	sequences (5'–3')
S-Methylated p16	GCT GCT TC^{m}C GGC TGG TGC
A-Methylated p16	GCA CCA GC^{m}C GGA AGC AGC
S-Unmethylated p16	GCT GCT TCC GGC TGG TGC
A-Unmethylated p16	GCA CCA GCC GGA AGC AGC
Signal probe	Biotin-AAA AAA AAA AGC ACC AGC CXG AAG CAG C-Cy5
S-Random 1	GAT CAG AGC ACG TCC TGA
A-Random 1	TCA GGA CGT GCT CTG ATC
S-Random 2	AGG TAA TGC GGT TTT GTT

A-Random 2	AAC AAA ACC GCA TTA CCT

[a] In the signal probe, the "X" indicates the AP site.

Detection of DNA methylation.

The indicated concentrations of target DNA, 1 μL of 10 × Cutsmart buffer and 8 U of HpaII were incubated in 10 μL of reaction mixture at 37 ºC for 80 min. Then 6 μL of signal probes (1 μM) were added into the system with a total volume of 20 μL, followed by incubation at 95 °C for 5 min and slowly cooling to room temperature to form the S-DNA-signal probe duplexes. Subsequently, 60 μL of 5 mg/mL streptavidin-coated magnetic beads were added into the reaction mixture, followed by incubation for 15 min on a roller mixer at room temperature. The mixture was then washed three times by magnetic separation using 1 × B&W buffer (5 mM Tris-HCl, pH 7.5, 500 μM EDTA, 1 M NaCl) to remove the uncoupled probes. The beads were re-suspended in 20 μL of reaction mixture containing 2 μL of 10 × NEB buffer 3, 5 U of Endo IV, followed by incubation at 37 °C for 1 h. The unreleased signal probes were removed by magnetic separation, and the supernatant solution was subjected to single-molecule detection.

Single-molecule detection and data analysis.

The reaction products were diluted 10000-fold with the buffer (10 mM Tris-HCl, 50 mM KCl, 5 mM $MgCl_2$, 1 mM Trolox, pH 8.0). Then 10 μL of samples were spread on a glass coverslip for imaging. The images of the single molecules were acquired by TIRF microscopy (Nikon, Ti-E, Japan). The 640 nm laser was used to excite the Cy5 fluorescence. The photons were collected using an oil immersion objective (CFI Apochromat TIRF 100 ×). The Cy5 fluorescence was imaged onto an EMCCD camera (Photometrics, Evolve 512). For data analysis, a region of 600 ×

600 pixels was selected for Cy5 molecule counting using Image J software. The average Cy5 counts were obtained by calculating ten frames.

Gel electrophoresis and fluorescence measurement.

The products were analyzed by 20% nondenaturing polyacrylamide gel electrophoresis (PAGE) in 1 × TBE buffer (9 mM Tris-HCl, 9 mM boric acid, 0.2 mM EDTA, pH 7.9) at a 110 V constant voltage for 100 min at room temperature. The gel was imaged by a ChemiDoc MP Imaging System (Bio-Rad, Madrid, Spain). The fluorescence spectra of Cy5 were recorded by a Hitachi F-7000 fluorometer at an excitation wavelength of 635 nm, and the spectra were recorded in the range of 650 – 800 nm. Both the excitation and emission slits were set to 5.0 nm. The fluorescence intensity at the emission wavelengths of 665 nm was used for analysis.

Detection of DNA methylation level in the mixture.

To investigate the capability of proposed biosensor for accurate measurement of DNA methylation level in the mixture, we prepared a series of artificial mixtures by mixing methylated and unmethylated DNA at different ratios. The total concentration of methylated and unmethylated DNA is 100 pM, and the mixtures contain 0.01%, 0.1%, 1%, 10%, and 100% methylated DNA, respectively. The measured methylation level is calculated on the basis of eqn S1.

$$\text{Methylation level (\%)} = \frac{M}{M+U} \times 100\% \qquad \text{S1}$$

Where M is the quantity of methylated DNA measured by the proposed biosensor and U is the quantity of unmethylated DNA.

Cell culture and extraction of genomic DNA.

Human cervical carcinoma cell line (HeLa cells), human lung adenocarcinoma cell line (A549 cells), human hepatoma cell line (Hep G2 cells), human breast cancer cell line (MCF-7 cells),

human breast cancer cell line (MDA-MB231 cells) and human breast epithelial cells (MCF-10A cells) were obtained from Cell Bank, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China). HeLa cells, A549 cells, Hep G2 cells, MDA-MB231 cells and MCF-7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, GrandIsland, NY, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, GrandIsland, NY, USA) and 1% penicillin-streptomycin Invitrogen (California, CA, USA). MCF-10A cells were cultured in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12, Gibco, GrandIsland, NY, USA) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. All cell lines were cultured in a humidified incubator containing 5% CO_2 at 37°C. The genomic DNAs from the cells were extracted by using a QIAamp DNA micro kit (Qiagen, Germany) according to the manufacturer's protocol. Genomic DNAs were digested by dsDNA Fragmentase to 50 – 200 bp following the manufacturer's protocol. The concentration of genomic DNA was determined using NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, U.S.A.).

Supplementary Results

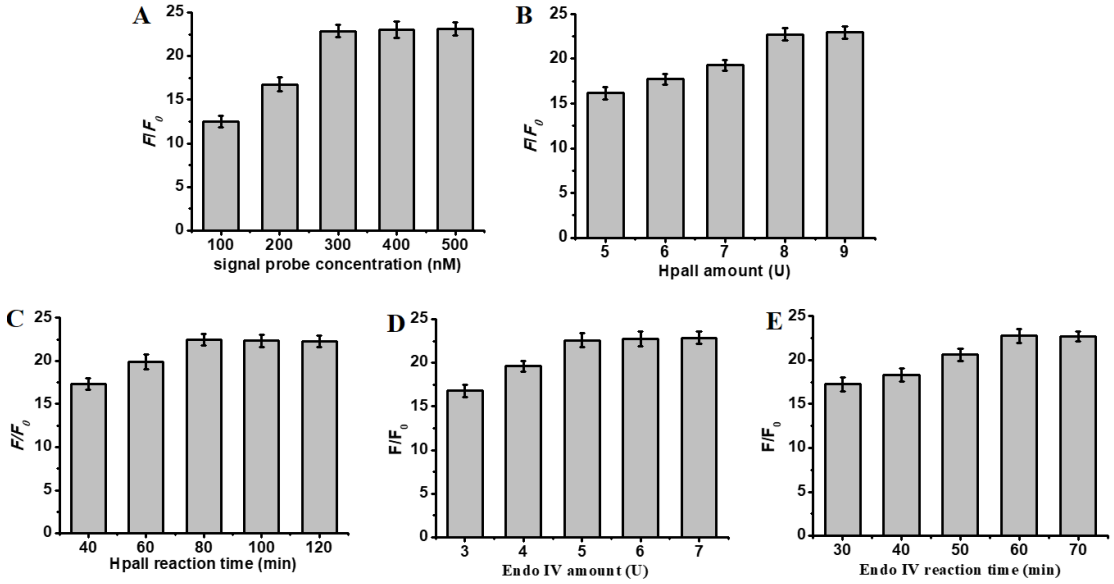

Fig. S1 (A) Variance of the F/F_0 value with different concentrations of signal probes. (B) Variance of the F/F_0 value with different amounts of HpaII. (C) Variance of the F/F_0 value with HpaII reaction time. (D) Variance of the F/F_0 value with different amounts of Endo IV. (E) Variance of the F/F_0 value with Endo IV reaction time. F and F_0 are the Cy5 counts in the presence and absence of methylated DNA, respectively. The concentration of target methylated DNA is 100 nM. Error bars show the standard deviation of three experiments.

To achieve the best assay performance, we optimized the experimental conditions including the concentration of signal probes, the amount of HpaII and its reaction time, the amount of Endo IV and its reaction time, respectively. The F/F_0 value is used for evaluating the assay performance, where F and F_0 are the Cy5 counts in the presence and absence of methylated DNA, respectively. As shown in Fig. S1A, the F/F_0 value enhances with the increasing concentration of signal probes from 100 to 300 nM, and reaches a plateau at the concentration of 300 nM. This can be explained by the fact that the high-concentration signal probes may result in higher hybridization efficiency

of target S-DNA-signal probe hybrids and consequently higher cleavage efficiency of Endo IV and the generation of more Cy5 counts. However, when the signal probe concentration is saturated for the cyclic cleavage-hybridization reaction, there is no significant improvement in Cy5 counts. Thus, 300 nM signal probe is used in the subsequent research.

In this biosensor, the methylation-sensitive restriction endonuclease HpaII is used to discriminate methylated DNA from unmethylated one. As shown in Fig. S1B, the F/F_0 value enhances with the increasing amount of HpaII from 5 U to 8 U and reach the maximum value at the amount of 8 U. As shown in Fig. S1C, the F/F_0 value enhances with the reaction time, and reaches a plateau at the reaction time of 80 min (F and F_0 are the Cy5 counts in the presence and absence of methylated DNA, respectively). Thus, 8 U of Hpa II and the HpaII reaction time of 80 min are used in the subsequent experiments.

Endo IV is the core enzyme for signal amplification in this biosensor. As shown in Fig. S1D, the F/F_0 value enhances with the increasing amount of Endo IV, and reaches the maximum value at the amount of 5 U. As shown in Fig. S1E, the F/F_0 value enhances with the reaction time from 30 to 60 min and reach a plateau beyond 60 min. Thus, 5 U of Endo IV and the Endo IV reaction time of 60 min are used in the subsequent experiments.