Electronic Supplementary Information

A DNA walker powered by endogenous enzymes for imaging uracil-DNA glycosylase activity in living cells

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Experimental Section

Materials. Hydrogen tetrachloroaurate (Ⅲ) trihydrate ($HAuCl_4 \cdot 3H_2O$), sodium citrate and tris(2-carboxyethyl)phosphine hydrochloride (TCEP) was purchased from Sigma-Alrich (St. Louis, MO, USA). Tris base, boric acid, ethylenediaminetetraacetic acid tetrasodium (EDTA) and 40% (w/v) acrylamide/bis-acrylamide solution (19:1) were purchased from Sangon Biotech Co., Ltd. (Shanghai, China). Uracil-DNA glycosylase (UDG), uracil glycosylase inhibitor (UGI), 8-oxoguanine DNA glycosylase (hOGG1), humman alkyladenine DNA glycosylase (hAAG) Apurinic/apyrimidinic endonuclease IV (endo IV) and apurinic/apyrimidinic endonuclease 1 (APE1) were purchased from New England Biolabs Ltd. (Ipswich, MA, USA). SYBR Gold nucleic acid gel stain was bought from Invitrogen. Phosphate buffered saline (8 mM Na_2HPO_4, 2 mM KH_2PO_4, 136 mM NaCl, 2.6 mM KCl, pH 7.4) was bought from Biological Industries (Cromwell, CT, USA). All other reagents were of analytical grade and used as received. Ultra-pure water (18.2 MΩ•cm) obtained from a UP water purification system was used throughout the experiment. Lipofectamine 2000 and Opti-MEM were purchased from Invitrogen (Carlsbad, CA, USA).

DNA oligonucleotides were synthesized in Sangon Biotech Co., Ltd. (Shanghai, China). The sequences are listed in Table S1.

Table S1. Sequences of DNA oligonucleotides used in this study.[a]

Name	Sequence (5'-3')
Walking strand (58-mer)	SH-TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT **TTT CCT AAG C**
8-Track strand (U)	SH-TTT TTT TTT T**GC TUA GGA**T-FAM
8-Track strand (T)	SH-TTT TTT TTT T**GC TTA GGA** T-FAM
Track strand (Poly T)	SH-TTT TTT TTT T**TT TTT TTT** T-FAM
Walking strand (61-mer)	SH-TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TT **ATC CTA AGC AA**
11-Track strand (U)	SH-TTT TTT TT**T TGC TUA GGA T**-FAM
11- Track strand (T)	SH-TTT TTT TT**T TGC TTA GGA T**-FAM
Walking strand (55-mer)	SH-TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TT **CT AAG**
5-Track strand (U)	SH-TTT TTT TTT TG**C TUA G**GAT-FAM
5-Track strand (T)	SH-TTT TTT TTT TG**C TTA G**GAT-FAM

[a] The purple letters represent the complementary bases between the walking strand and the track strand, and the red letters indicate the uracil bases or substituted thymine bases. The number in the name of track strands suggests the number of complementary bases between the walking strand and the track strand. The concentrations of oligonucleotides were determined according to the UV absorbance at 260 nm and the extinction coefficients of sequences.

Small interfering RNA (siRNA) oligonucleotides were synthesized in Genepharma Co., Ltd. (Shanghai, China). The sequences are listed in Table S2.

Table S2. Sequences of siRNA oligonucleotides used in this study.[a]

Name	Sequence (5'-3')
siUDG-1	CAUCAAGCCAACUCUCAUATT UAUGAGAGUUGGCUUGAUGTT
siUDG-2	CUGUGAGCUUUAUCAGAUATT UAUCUGAUAAAGCUCACAGTT
siUDG-3	CCUUGAUCUUGUUAGCAAUTT AUUGCUAACAAGAUCAAGGTT
siUDG-4	GGGACAGGAUCCAUAUCAUTT AUGAUAUGGAUCCUGUCCCTT
siAPE1	GUCUGGUACGACUGGAGUATT UACUCCAGUCGUACCAGACCU

[a] siUDG means the siRNA against UDG gene and siAPE1 means the siRNA against APE1 gene. A pool of four individual siUDGs (siUDG1 to siUDG4) is transfected into HeLa cells for knockdown of UDG, and individual siAPE1 is transferred into HeLa cells for knockdown of APE1. The siRNAs were synthesized and annealed in its duplex form.

Instrumentation. UV-vis absorption spectra were recorded on a U-2910 spectrophotometer (Hitachi, Japan). Fluorescence spectra were measured on an F-7000 fluorescence spectrophotometer (Hitachi, Japan). The excitation and emission wavelength were set at 488 nm and 520 nm respectively. Both the excitation slit and emission slit were 10 nm and the voltage of the photomultiplier was 700 V. The scan speed was 240 nm/min and the response time was 2 s. Transmission electron microscopy (TEM) measurements were made on a JSM-6700F transmission electron microscope operated at an accelerating voltage of 200 kV (JEOL, Japan). Dynamic light scattering (DLS) measurements were performed on a Zetasizer Nano ZS (Malvern, UK). The samples for DLS characterization were adjusted to AuNP concentration of 10 nM, and were transferred to 50-μL disposable cuvettes (Sarstedt, Germany) for measurements. The polyacrylamide gel was imaged by GelDocTM XR^+ imaging system (Bio-RAD Laboratories Inc., USA). Fluorescence images were obtained with an Axio Observer A1 inverted fluorescence microscope (Carl Zeiss, Germany) with an objective lens of 63×. Flow cytometry was measured on an ACEA

NovoCyte flow cytometer (Bucher Biotec AG, Switzerland).

Synthesis of AuNPs. Gold nanoparticles (13 nm diameter) were synthesized by reduction of $HAuCl_4$ by sodium citrate.[1] Briefly, a solution of sodium citrate (10 mL, 38.8 mM) was rapidly added to a vigorously stirred boiling aqueous solution of $HAuCl_4$ (100 mL, 1 mM). After a continuous boiling for 10 min, the mixed solution was stirred for additional 15 min. The solution was then cooled to room temperature, filtered through a 0.22 μm filter and stored in a refrigerator at 4 °C before use. The concentration of synthesized AuNPs was determined according to the absorbance at 520 nm and the corresponding molar extinction coefficient.[2]

Functionalization of AuNPs with DNA. The synthesized AuNPs were functionalized with thiolated DNA according to the previous report[3] with modifications. Prior to the functionalization, thiolated DNA was incubated with TCEP in a 1:70 molar ratio for 2 h to reduce the disulfide bond of DNA. 2 μL of 50 μM TCEP-reduced walking strands and 20 μL of 100 μM TCEP-reduced track strands were added into 500 μL of 9 nM AuNPs, and the molar ratio of walking strands versus track strands was 1:20. The mixture was incubated at room temperature for 16 h in the dark. Then 50 μL of ultrapure water that contains 1% Tween 20 was added and the mixture was incubated for 10 min. Subsequently, 80 μL of 2 M NaCl was gradually added to the above mixture, through the way of adding 10 μL each time with a time interval of 40 min. The solution was further incubated at room temperature for 12 h in the dark. After the incubation, the solution was centrifuged at 12,000 rpm for 30 min at 4 °C to separate DNA-functionalized AuNPs from the excess reagents. The DNA-AuNP conjugates were washed three times by 1 × PBS buffer, diluted with 200 μL PBS buffer and stored in dark at 4 °C. The DNA walker was obtained when functionalizing AuNPs with walking strands and track strands that contain uracil, and the control DNA-AuNP conjugate that substitute thymine for uracil is obtained when functionalizing AuNPs with walking strands and track strands that contain thymine. Control DNA-AuNP conjugate was also prepared by functionalizing AuNPs with only track strands, or by functionalizing AuNPs with walking strands and poly-thymine track strands.

Determination of amount of track strands on AuNPs. The amount of track strands on AuNPs was determined by the DTT-displacement method.[4] Equal volumes of DNA-AuNP conjugates and 1.0 M of DTT were mixed and the mixture was incubated overnight, followed by the centrifugation at 12,000 rpm for 20 min at 4 °C. The supernatant containing the displaced track strands was then carefully transferred to measure fluorescence. Calibration curves were meanwhile established by mixing known concentrations of track strands with DTT under the same procedure to measure fluorescence. The concentration of track strands was obtained according to the fluorescence of supernatant and the calibration curve. The number of track strands per AuNP was calculated by dividing the track strand concentration by the AuNP concentration.

Feasibility study of the operation of DNA walker. To study the feasibility of the operation of DNA walker, 40 μL of mixture containing 0.5 nM DNA walker, 1 U/mL UDG and 1× Cutsmart buffer was incubated at 37 ℃ for 1 h. Then 30 U/mLAPE1 was added and the mixture was incubated for another 1 h. Finally, the solution was centrifuged at 12,000 rpm for 30 min to separate the DNA walker from the unreacted reagents, followed by the fluorescence measurement. Control experiment was conducted under otherwise identical conditions except using control DNA-AuNP conjugates instead of the DNA walker.

To analyze the products during the DNA walker operation, native polyacrylamide gel electrophoresis (PAGE) were performed. After the reaction, AuNP-conjugated DNA, including walking strands and track strands, were displaced by incubation with DTT. The solution was then centrifuged and the supernatant that contained displaced DNA were loaded as samples for the PAGE analysis. The PAGE gel (15%) was run at 30 mA constant current for 1.5 h at 15 °C in 1× TBE buffer. The gel was then stained with 1× SYBR Gold for 40 min in the dark and imaged by the imaging system.

Detection of UDG activity by the DNA walker. 40 μL of mixture containing 0.5 nM DNA walker, various concentrations of UDG (0 U/mL, 0.1 U/mL, 0.2 U/mL, 0.3 U/mL, 0.6 U/mL, 0.8 U/mL and 1.0 U/mL) and 1× Cutsmart buffer was incubated at 37 ℃ for 1 h. Then 30 U/mLAPE1 was added and the mixture was incubated for

another 1 h. Finally, the solution was centrifuged at 12,000 rpm for 30 min to separate the DNA walker from the unreacted reagents, followed by the fluorescence measurement.

Investigation of the specificity of the DNA walker. 1 U/mL UDG, hAGG, hOGG1 and endo IV were respectively added to the reaction solution that contained 0.5 nM DNA walker and 1× Cutsmart buffer. The obtained mixture was incubated at 37 °C for 1 h. Then 30 U/mL APE1 was blended into above mixture followed by incubation at 37 °C for 1 h. After that, the fluorescence was measured. Relative fluorescence intensities were calculated by dividing the fluorescence intensity in the presence of tested enzymes by the fluorescence intensity in the presence of UDG. The experiments were conducted three times in parallel.

Investigation of the UGI inhibition to the DNA walker operation. 0.5 nM DNA walker and various concentrations of UGI were incubated, followed by the addition of 1 U/mL UDG. After the incubation in 1× Cutsmart buffer at 37 °C for 1 h, 30 U/mL APE1 were blended into the above solution. The mixture was further incubated at 37 °C for 1 h, followed by fluorescence measurements. The relative fluorescence intensity, calculated by dividing the fluorescence intensity in the presence of UGI by the fluorescence intensity in the absence of UGI, is used to demonstrate the inhibition effect of UGI to the DNA walker operation. The experiments were conducted three times in parallel.

Investigation of the resistance of DNA walker against nonspecific nuclease degradation. 2.5 μU DNase I and target enzymes were respectively mixed with the 0.5 nM DNA walker. Time-dependent fluorescence changes were then recorded. The experiments were conducted three times in parallel.

Intracellular fluorescence imaging. HeLa cells were cultured in a flask in RPMI medium 1640 (GIBCO) supplemented with 10% fetal calf serum and 1% dual antibody at 37 °C in a humidified atmosphere containing 5% CO_2. HeLa cells were inoculated on the confocal dish and growth for 24 h. The DNA walker or its control DNA-AuNP conjugate with a final concentration of 1.5 nM was added and incubated with cells for 3.0 h, followed by imaging with fluorescence microscope. For

fluorescence imaging of HepG2 and MCF-7 cells, the same procedures were performed as that for HeLa cells except the culture and inoculation of HepG2 and MCF-7 cells, respectively.

siRNA transfection. 5×10^3 HeLa cells were plated the 35 mm Petri dish (Cellvis, Mountain View, CA, USA) and allowed to attach overnight. siRNA was transfected into HeLa cells with Lipofectamine 2000 according to the manufacturer's instruction.

For siUDG transfection, equal volume of 20 μM siUDG-1, 20 μM siUDG-2, 20 μM siUDG-3 and 20 μM siUDG-4 were mixed to obtain a pool of 20 μM siUDGs. 2.5 μL of 20 μM siUDG pool was diluted in 50 μL Opti-MEM, and 1.5 μL Lipofectamine 2000 was diluted in 50 μL Opti-MEM. After 5-min incubation, the diluted siUDG and diluted Lipofectamine 2000 were mixed gently and incubated for 20 min at room temperature. Then 25 μL siUDG-Lipofectamine complex was added to the confocal dish containing cells and culture medium (no antibiotics included). The cells were incubated for 6 h before the change of medium. Then the cells were further incubated for 48 h for knockdown of UDG. The DNA walker or its control DNA-AuNP conjugate with a final concentration of 1.5 nM was added and incubated with cells for 3.0 h, followed by imaging with fluorescence microscope.

For siAPE1 transfection, 1 μL of 20 μM siAPE1 was diluted in 50 μL Opti-MEM, and 1 μL Lipofectamine 2000 was diluted in 50 μL Opti-MEM. After 5-min incubation, the diluted siAPE1 and diluted Lipofectamine 2000 were mixed gently and incubated for 20 min at room temperature. Then 25 μL siAPE1-Lipofectamine complex was added to the confocal dish containing cells and culture medium (no antibiotics included). The cells were incubated for 6 h before the change of medium. Then the cells were further incubated for 48 h for knockdown of APE1. The DNA walker or its control DNA-AuNP conjugate with a final concentration of 1.5 nM was added and incubated with cells for 3.0 h, followed by imaging with fluorescence microscope.

For siUDG and siAPE1 transfection, 2.5 μL of 20 μM siUDG pool and 1 μL of 20 μM siAPE1 were diluted in 50 μL Opti-MEM, and 2.1 μL Lipofectamine 2000 was diluted in 50 μL Opti-MEM. After 5-min incubation, the diluted siUDG pool/siAPE1

and diluted Lipofectamine 2000 were mixed gently and incubated for 20 min at room temperature. Then 25 μL siUDG pool/siAPE1-Lipofectamine complex was added to the confocal dish containing cells and culture medium (no antibiotics included). The cells were incubated for 6 h before the change of medium. Then the cells were further incubated for 48 h for knockdown of both UDG and APE1. The DNA walker or its control DNA-AuNP conjugate with a final concentration of 1.5 nM was added and incubated with cells for 3.0 h, followed by imaging with fluorescence microscope.

Flow cytometry. HeLa cells were seeded in a 6-well plate at a density of 4×10^5 cells/ well at least 24 h. The cells were then washed with PBS to remove antibiotics and serum, and the DNA walker or its control DNA-AuNP conjugate with a final concentration of 1.5 nM was added. After the incubation at 37 °C for 3 h, the cells were washed with PBS and analyzed by flow cytometer. Samples of 1×10^4, 4×10^4 and 8×10^4 cells were counted, and fluorescence was measured with laser excitation at 488 nm during the recording of events.

Additional Figures.

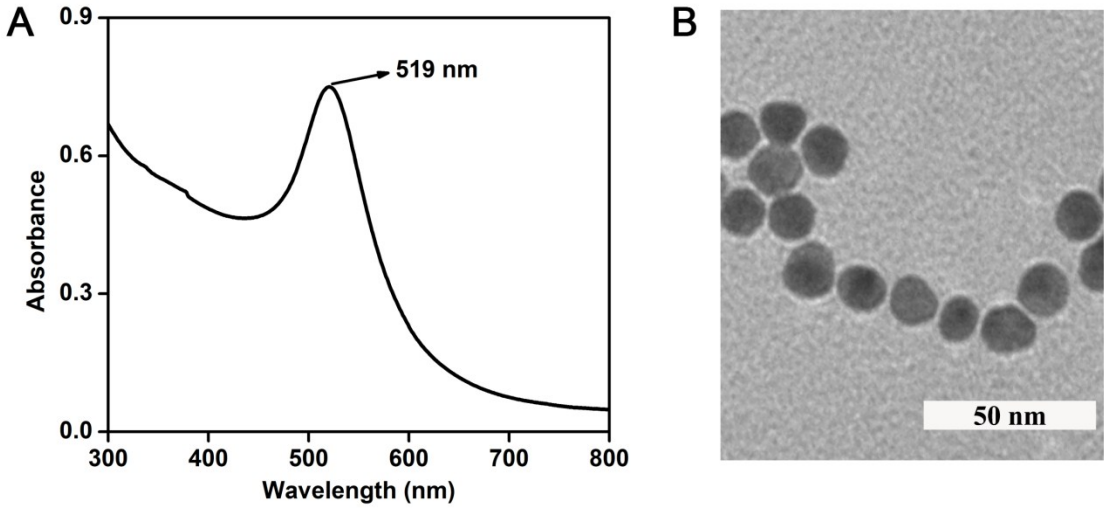

Fig. S1 Characterization of synthesized gold nanoparticles: UV-vis absorption spectra (A) and TEM image (B).

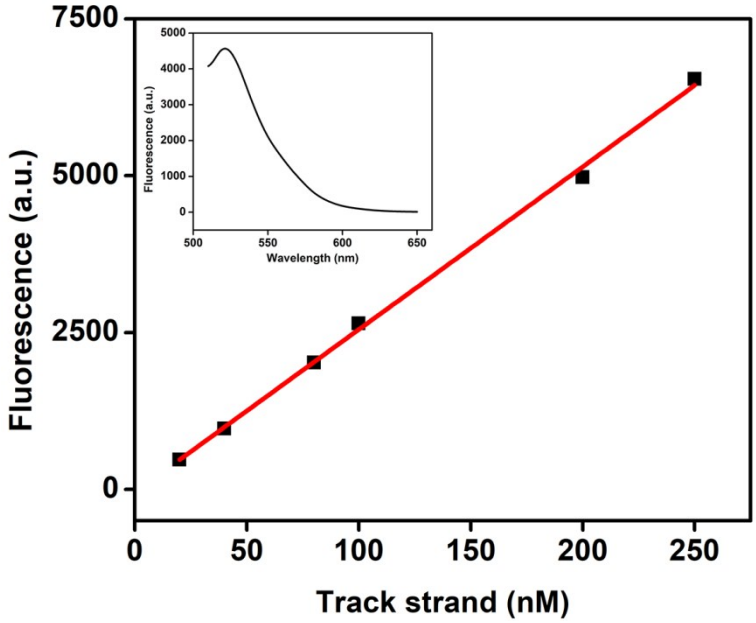

Fig. S2 Quantification of the amounts of track strands on AuNP surface: calibration curve of fluorescence versus known concentrations of track strands and the fluorescence spectra of the supernatant from DTT-incubated DNA walker.

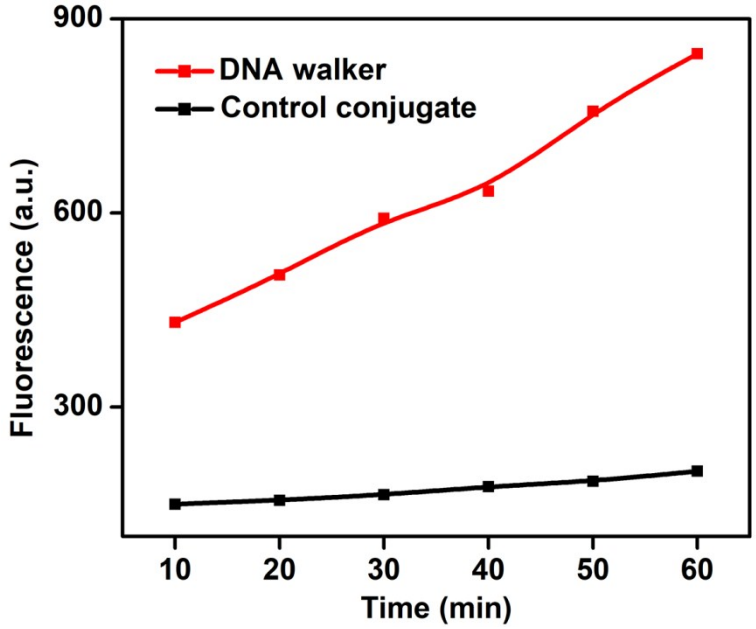

Fig. S3 Time-dependent fluorescence changes of the DNA walker and the control DNA-AuNP conjugate that substitutes thymine for uracil in the presence of UDG (1 U/mL) and APE1 (30 U/mL).

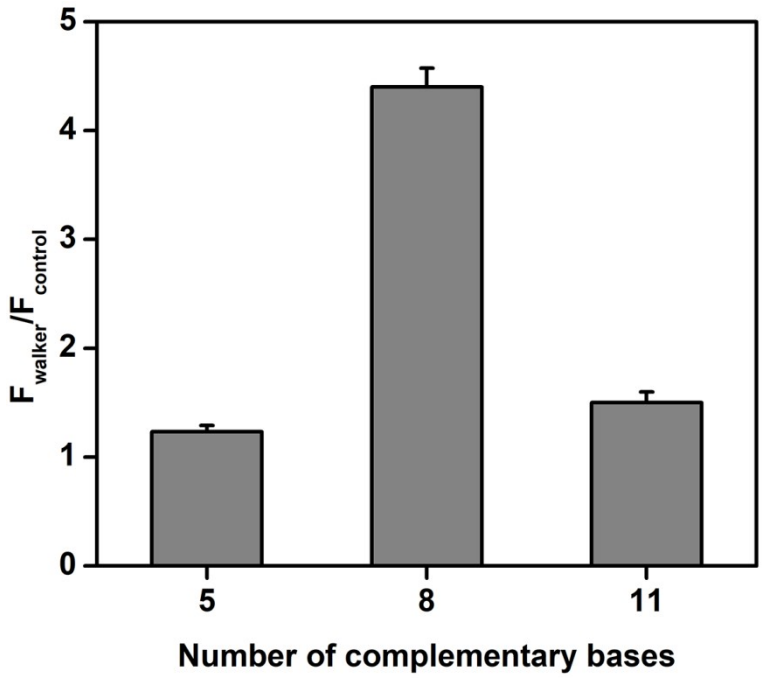

Fig. S4 Optimization of number of complementary bases between the walking strand and the track strand for the DNA walker operation. F_{walker} and $F_{control}$ represent the generated fluorescence of the DNA walker and the control DNA-AuNP conjugate that substitutes thymine for uracil in the presence of UDG (1 U/mL) and APE1 (30 U/mL), respectively.

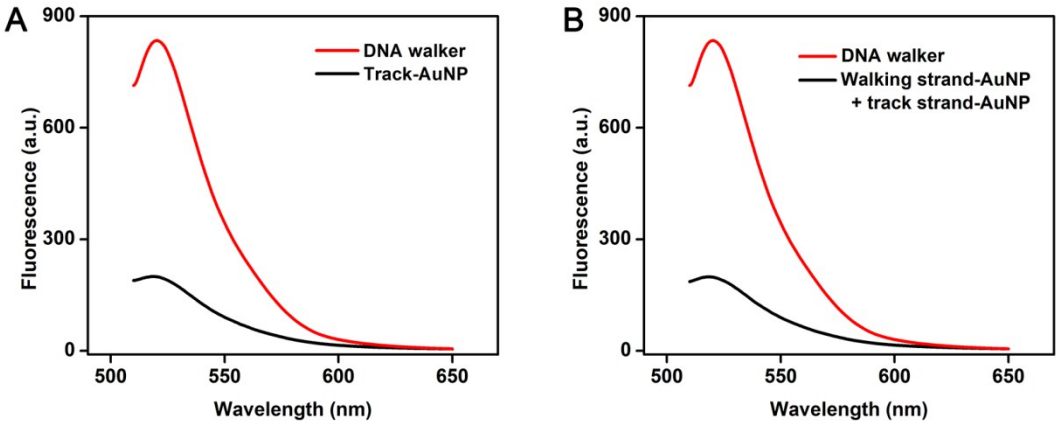

Fig. S5 Fluorescence spectra of the DNA walker versus control track strand-AuNP conjugate (A) and the DNA walker versus the mixture of walking strand-AuNP and track strand-AuNP conjugates (B) in the presence of UDG (1 U/mL) and APE1 (30 U/mL).

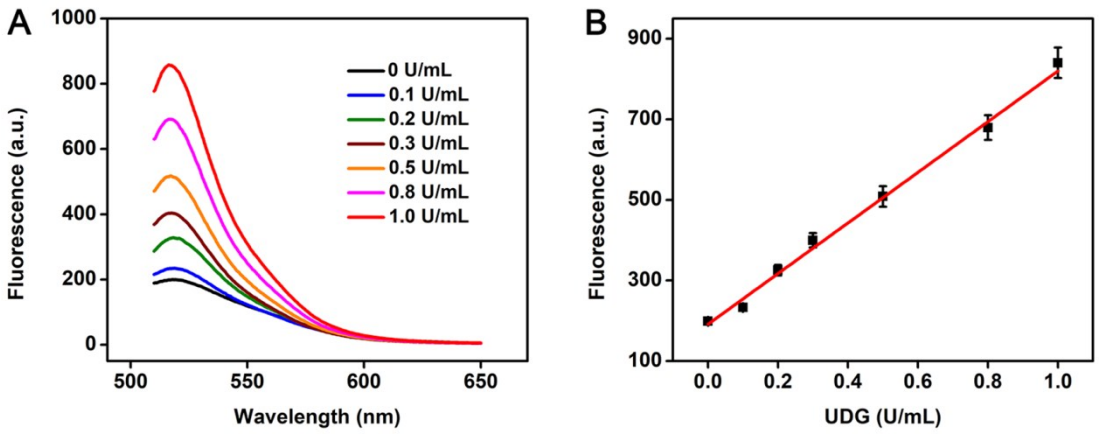

Fig. S6 (A) Fluorescence spectra of the DNA walker that operates under the action of UDG with different concentrations (0 to 1 U/mL) and APE1 (30 U/mL). (B) Linear relationship between the fluorescence intensity at 520 nm and the concentration of UDG. Error bars show the standard deviation from three independent parallel experiments.

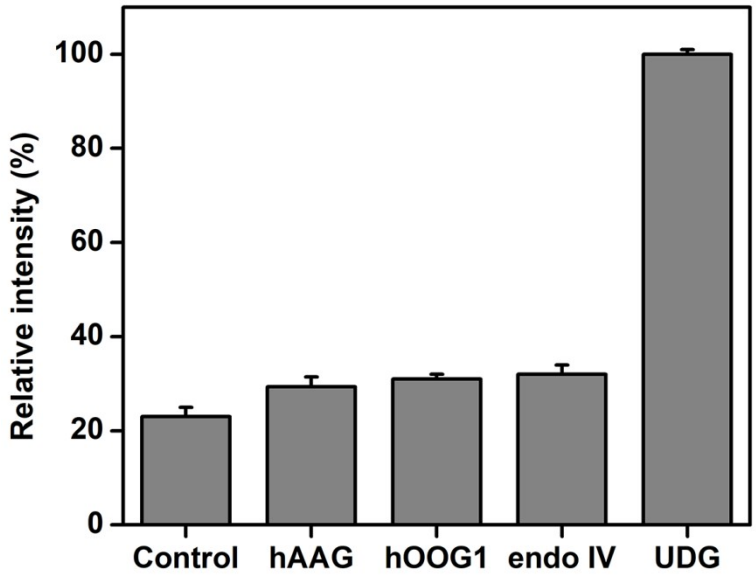

Fig. S7 The specificity of the DNA walker toward the stimuli of reaction buffer (control), 1 U/mL hAAG, 1 U/mL hOGG1, 1 U/mL endo IV and 1 U/mL UDG in the presence of 30 U/mL APE1, respectively.

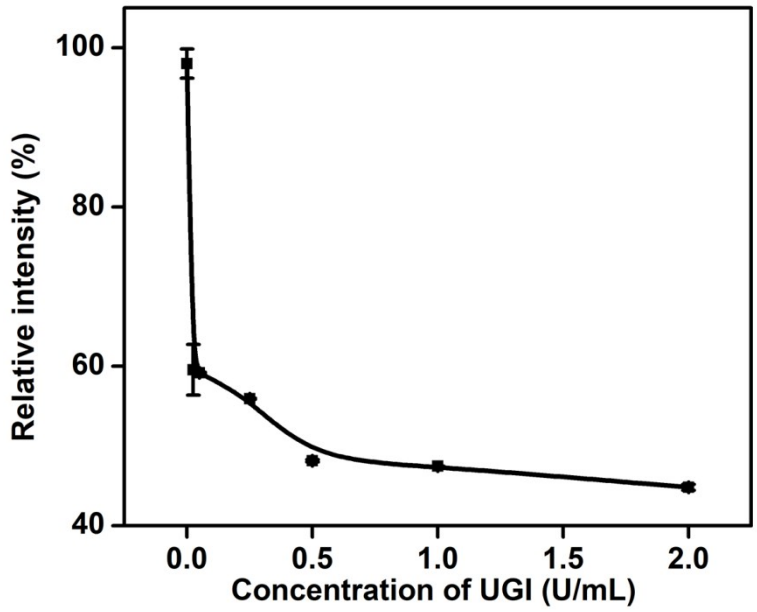

Fig. S8 The inhibition effect of UGI toward the operation of the DNA walker.

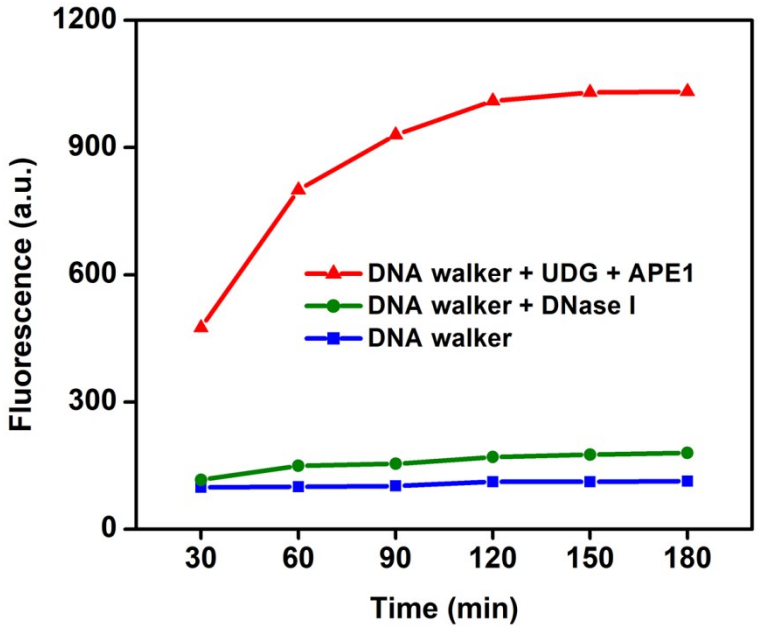

Fig. S9 Time-dependent fluorescence changes of the DNA walker in the presence of UDG and APE1 (red line), DNase I (green line) and reaction buffer (blue line).

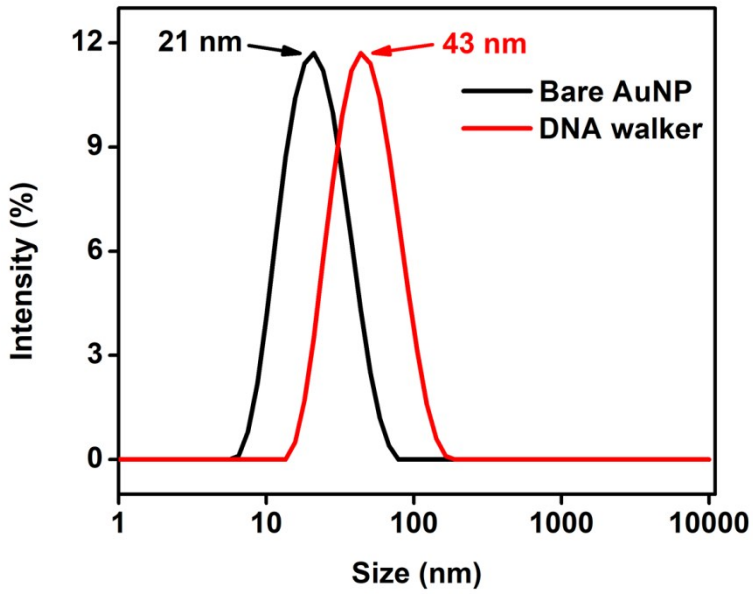

Fig. S10 Dynamic light scattering (DLS) characterization of synthesized AuNPs and constructed DNA walker.

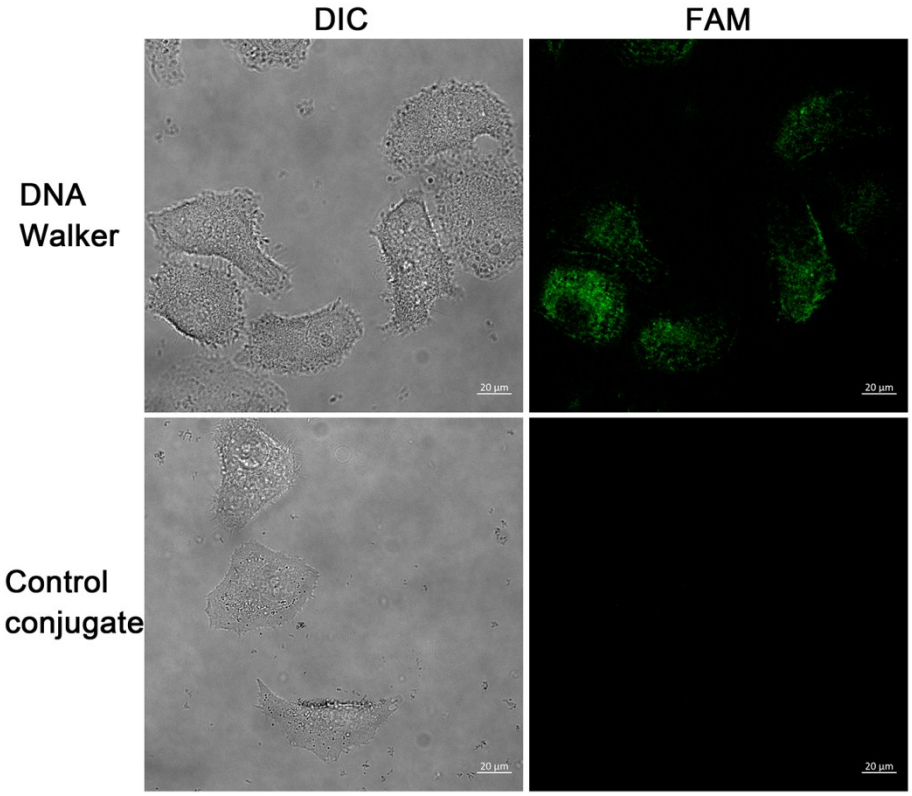

Fig. S11 Fluorescence imaging of UDG activity in HepG2 cells by the DNA walker (top row) and its control DNA-AuNP conjugate that substitutes thymine for uracil (bottom row).

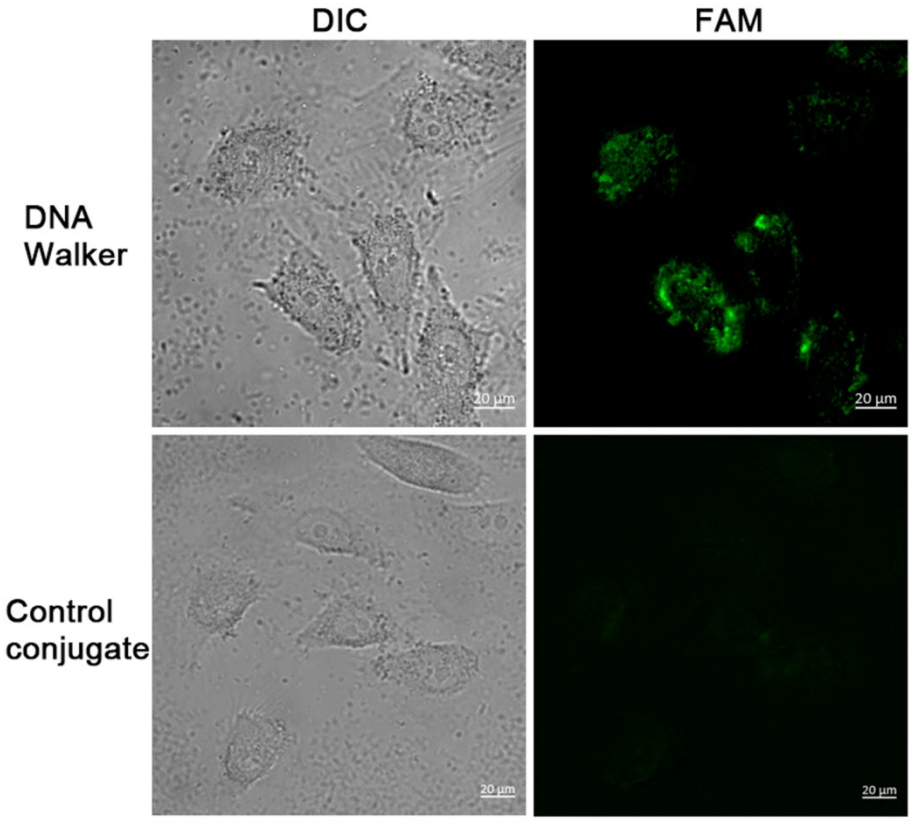

Fig. S12 Fluorescence imaging of UDG activity in MCF-7 cells by the DNA walker (top row) and its control DNA-AuNP conjugate that substitutes thymine for uracil (bottom row).

References:

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