

## Electronic Supplementary Information

### **Single-molecule counting of oxidative DNA damage in telomeres from cancer cells**

Yan Zhang,<sup>‡</sup> Ruo-nan Hua, <sup>‡</sup> Dongxue Xiang, <sup>‡</sup> and Chun-yang Zhang\*

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.

\* Corresponding author. Tel.: +86 0531-86186033; Fax: +86 0531-82615258. E-mail: cyzhang@sdnu.edu.cn.

#### **EXPERIMENTAL SECTION**

**Materials.** All the oligonucleotides (Table S1) were synthesized by Takara Biotechnology Co. Ltd. (Dalian, China). Deoxyadenosine triphosphate (dATP) was purchased from Takara Biotechnology (Dalian, China). Formamidopyrimidine glycosylase (Fpg), T4 polynucleotide kinase (PNK), terminal deoxynucleotidyl transferase (TdT), 10× terminal transferase reaction buffer (500 mM potassium acetate, 200 mM Tris-acetate, 100 mM magnesium acetate, pH 7.9), 10× cobalt (II) chloride (CoCl<sub>2</sub>) (2.5 mM), Endonuclease IV, NEBNext dsDNA Fragmentase, and 10× NEBNext dsDNA Fragmentase reaction buffer (200 mM Tris-HCl, 150 mM MgCl<sub>2</sub>, 500 mM NaCl, 1 mg/mL BSA, 0.15% TritonX-100, pH 7.5) were obtained from New England BioLabs (Beverly,

MA, USA). The 2',3'-Dideoxycytidine 5'-triphosphate sodium salt solution (ddCTP) was obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). The streptavidin-coated magnetic beads (Dynabeads™ M-280 Streptavidin) were obtained from Invitrogen (California, CA, USA). The SYBR Gold was purchased from Life Technologies (Carlsbad, CA, USA). QIAamp DNA mini kit was obtained from Qiagen (Redwood, CA, USA). Human cervical carcinoma cell line (HeLa cells), human lung adenocarcinoma cell line (A549 cells), human colon cancer cells (SW480 cells) and normal human liver cell line (HL-7702 cells) were obtained from Cell Bank, Shanghai Institutes of Biological Sciences, Chinese Academy of Sciences (Shanghai, China). Fetal bovine serum (FBS) was purchased from Thermo Fisher Scientific (Waltham, MA, USA). All other reagents were of analytical grade and used as received without further purification. Ultrapure water obtained from a Millipore filtration system (Millipore, Milford, MA, USA) was used throughout all experiments.

**Table S1. Sequence of Oligonucleotides<sup>a</sup>**

note	sequence (5'-3')
Telo-0	TTA GGG TTA GGG TTA GGG TTA GGG
Telo-1	TTA GGG TTA <u>GGG</u> TTA GGG TTA ddC
Telo-2	TTA GGG TTA <u>GGG</u> TTA <u>GGG</u> TTA GGG
Telo-Cy5	TTA GGG TTA <u>GGG</u> TTA GGG TTA-Cy5
helper probe	CCT AAC CCT AAC CCT AAddC
capture probe	CCT AAC CCT AAC CCT AAC CCT TTT-Biotin

signal probe	Biotin- TTT TTT TTT XTT TTT TTT-AF488
KRAS	GTA GTT <u>GGA</u> GCT <u>GGT</u> GGC GTA GGC
Random DNA	ATG CAA <u>CGG</u> ACA <u>AGA</u> AGA TCA GTA

---

“The underlined G bases in Telo-1, Telo-2, KRAS and random DNA indicate the 8-oxoG. The ddC indicates 2', 3'-dideoxycytidine. The X in the signal probe indicates a tetrahydrofuranyl analog as the apurinic/apyrimidinic (AP) sites mimic.

**Conjugation of signal probe with the streptavidin-coated magnetic beads.** The coupling of signal probes to the magnetic beads (MBs) was carried out according to the protocol of Invitrogen Corporation. After the 100  $\mu$ L of streptavidin-coated MBs solution (10 mg/mL) was transferred into a centrifuge vial (600  $\mu$ L) and washed twice with 1 $\times$  B&W buffer (5 mM Tris-HCl (pH 7.5), 0.5 mM EDTA, 1 M NaCl), the supernatant was removed by magnetic separation, and the MBs were re-suspended in 2 $\times$  B&W buffer (10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 2 M NaCl) with a final concentration of 5  $\mu$ g/ $\mu$ L. Then 200  $\mu$ L of 1  $\mu$ M biotinylated signal probes was mixed with 200  $\mu$ L of 5  $\mu$ g/ $\mu$ L MBs solution and incubated in the dark for 10 min on a roller mixer at room temperature. The mixture was then washed three times to remove the uncoupled signal probes, and the remaining signal probe-MB conjugates were resuspended in 50  $\mu$ L of TE buffer.

**The 8-oxoG base excision repair and TdT-mediated extension reaction-activated Endo IV-assisted cleavage of signal probes.** The telomere-specific 3'-biotinylated capture probes were used to hybridize with the synthetic telomeres to form the capture probes / telomere duplexes which are subsequently separated by the streptavidin-coated magnetic beads. For the excision of

oxidative damage, 1 nM helper probes, the separated capture probes / synthetic telomere, 1× terminal transferase reaction buffer, 1.6 U of Fpg, and 2 U of PNK were incubated in 10 μL of reaction mixture at 37°C for 1 h. Then 4 U of terminal transferase (TdT), 4 U of endonuclease IV, 1× terminal transferase reaction buffer, 0.25 mM CoCl<sub>2</sub>, 250 μM dATPs, and 5 μL of magnetic bead-coupled signal probe were added to the reaction mixture with a total volume of 50 μL, followed by incubation at 37°C for 60 min. Finally, the streptavidin-coated MBs were separated by magnetic separation for 3 min, and the supernatant solution was subject to single-molecule detection.

**Single-molecule detection.** The images of single molecules were obtained by total internal reflection fluorescence microscopy (TIRF) (Nikon, Ti-E, Japan). After 1000-fold dilution, 10 μL of reaction product samples was used for TIRF imaging. The 488-nm laser was used to excite the AF488. The photons were collected by using an oil immersion 100× objective. The photons from AF488 were imaged onto a EMCCD camera (Photometrics, Evolve 512). For data analysis, an imaging region of 600 × 600 pixels was selected for AF488 molecule counting using image J software. The average AF488 counts were obtained by calculating ten frames.

**Measurement of Fluorescence Spectra.** The 50 μL of reaction products were measured by a Hitachi F-7000 fluorescence spectrophotometer (Tokyo, Japan). The AF488 fluorescence was measured at the excitation wavelengths of 488 nm, and the fluorescence intensity at 532 nm was used for data analysis.

**Gel electrophoresis analysis.** To verify whether the Fpg can remove the 8-oxoG base, 0.5 μM Telo-Cy5/helper probe duplexes were treated with 1.6 U of Fpg in the reaction mixture with a total

volume of 10  $\mu$ L at 37 °C for 1 h. To verify the removal of 8-oxoG base-induced TdT-mediated polymerization reaction, 0.5  $\mu$ M Telo-1/helper probe duplexes were treated with 1.6 U of Fpg and 3 U of PNK at 37 °C for 1 h, and then 4 U of TdT was added into the reaction mixture with a total volume of 15  $\mu$ L, followed by incubation at 37 °C for 1 h. The products stained with SYBR Gold were analyzed by non-denaturing polyacrylamide gel electrophoresis in 1 $\times$  Tris-borate-EDTA (TBE) buffer (9 mM Tris-HCl, 9 mM boric acid, 0.2 mM EDTA, pH 7.9) at a 110 V constant voltage for 40 min at room temperature. The images of gel electrophoresis were visualized by a Bio-Rad ChemiDoc MP Imaging system (Hercules, CA, USA).

**Cell Culture and Preparation of Genomic DNA.** Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, USA) medium with 10% fetal bovine serum (FBS, Invitrogen, USA) at 37 °C under 5% CO<sub>2</sub>. To induce oxidative base damage in vivo, the cells were washed with PBS buffer and treated with different concentrations of H<sub>2</sub>O<sub>2</sub> at 37 °C in a humidified chamber with 5% CO<sub>2</sub> for 1 h. The genomic DNA in cells was extracted using QIAamp® DNA Mini kit (Redwood, CA, USA). Genomic DNA was digested with dsDNA Fragmentase (NEB) to 50-200 bp according to the manufacturer's protocol. To reduce the spontaneous 8-oxoG formation during the preparation of DNA, antioxidants (100 mM deferoxamine, 100 mM butylated hydroxytoluene) were used in all reactions until the oxidative damage excision step. The concentration of genomic DNA was determined by the NanoDrop 2000c spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

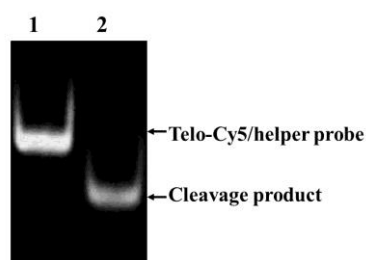
**Detection of oxidative damage in telomere from cancer cells.** To enrich the telomere from the genomic DNA, the telomere-specific biotinylated capture probes and the digested genomic DNA

were incubated in 1× terminal transferase reaction buffer at 95 °C for 5 min, followed by slowly cooling to room temperature. Then the biotinylated capture probes were separated by magnetic beads (MBs). After the magnetic separation and washing for three times, 4 U of TdT, 200 μM ddCTP, 1× terminal transferase reaction buffer, and 0.25 mM CoCl<sub>2</sub> were added to the reaction mixture with a total volume of 10 μL, and incubated at 37 °C for 30 min to cap the 3' ends of DNA strands. The excess TdT and ddCTPs were removed through magnetic separation. Then 100 nM helper probes, 1× terminal transferase reaction buffer, 4 U of Fpg, and 4 U of PNK were added to the reaction system with a final volume of 10 μL, followed by incubation at 37 °C for 1 h. Then 6 U of terminal transferase (TdT), 4 U of endonuclease IV, 1× terminal transferase reaction buffer, 0.25 mM CoCl<sub>2</sub>, 250 μM dATPs, and magnetic bead-coupled signal probes were added to the reaction mixture with a final volume of 50 μL, followed by incubation at 37°C for 60 min. After magnetic separation, the reaction solution was subjected to single-molecule measurement.

## **RESULTS AND DISCUSSION**

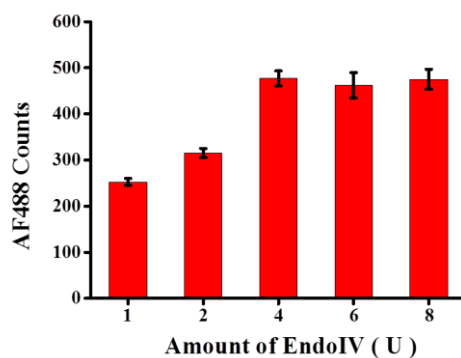
**Verification of the Fpg-mediated removal of oxidative damage.** The Cy5-labeled synthetic telomere containing one 8-oxoG base (Telo-Cy5) was used to verify whether the Fpg can remove the oxidative DNA damage. The helper probe can hybridize with the Telo-Cy5 to form a Telo-Cy5 / helper probe duplex. The non-denaturing polyacrylamide gel electrophoresis (PAGE) analysis shows that only the band of Telo-Cy5/helper probe duplex is observed in the absence of Fpg (Fig. S1, lane 1), suggesting that no 8-oxoG is cleaved. In the presence of Fpg, a new band corresponding to the size of the cleavage product is observed (Fig. S1, lane 2), indicating that Fpg

can remove the damage 8-oxoG base in Telo-Cy5 and incise the DNA backbone to produce a new cleavage product.



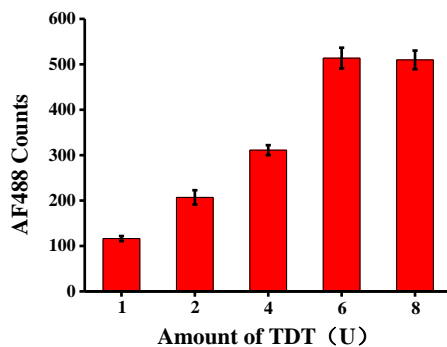
**Fig. S1** PAGE monitoring of the Fpg-induced removal of the oxidative damage base. Lane 1, 0.5  $\mu$ M Telo-Cy5/helper probe; lane 2, 0.5  $\mu$ M Telo-Cy5/helper probe + 1.6 U of Fpg.

**Optimization of Experimental Conditions.** To achieve the best assay performance, we optimized the amounts of Endo IV and TdT, respectively. As shown in Fig. S2, when the amount of TdT is fixed at 8 U, the AF488 counts enhance with the increasing amount of Endo IV, and reach the maximum value at the amount of 4 U. Thus, 4 U of Endo IV is used in the subsequent research. As shown in Fig. S3, when the amount of Endo IV is fixed at 4 U, the AF488 counts enhance with the increasing amount of TdT, and reach the maximum value at the amount of 6 U. Thus, 6 U of TdT is used in the subsequent research.



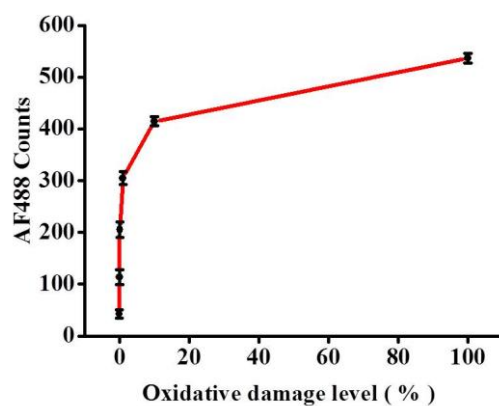
**Fig. S2** Variance of AF488 counts with the amount of Endo IV. The Telo-2 concentration is 10 pM.

Error bars represent the standard deviation of three independent experiments.



**Fig. S3** Variance of AF488 counts with the amount of TdT. The Telo-2 concentration is 10 pM.

Error bars represent the standard deviation of three independent experiments.

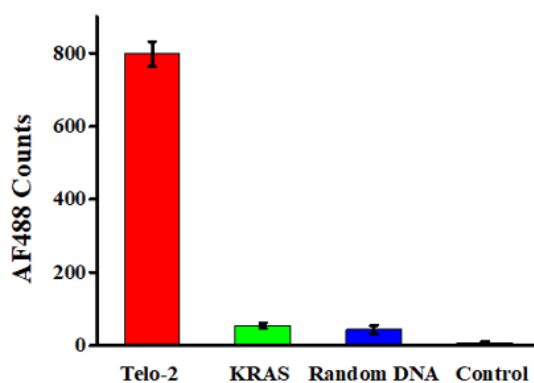


**Fig. S4** Variance of AF488 counts as a function of input oxidative base damage level in the mixture of telomere containing two 8-oxoG bases (Telo-2) and normal telomere without oxidative base damage (Telo-0). Error bars represent the standard deviation of three independent experiments.

**Selectivity of the proposed method.** To investigate the selectivity of the proposed method, we used a synthetic KRAS sequence containing two 8-oxoG bases (KRAS) and a random DNA sequence containing two 8-oxoG bases (random DNA) as the controls. The telomere-specific



capture probes in combination with the magnetic beads were used to separate Telo-2, KRAS, and random DNA, respectively, and then the oxidative damages were detected by the proposed method. Under the identical conditions, a high fluorescence signal is observed in response to Telo-2, while no distinct fluorescence signal is observed in response to KRAS, random DNA, and the control group with only reaction buffer (Fig. S5). This may be explained by the fact that only the telomere can be captured by the telomere-specific capture probe. These results clearly demonstrate that the proposed method can be used for selective detection of oxidative base damage in telomere.



**Fig. S5** Measurement of AF488 counts in response to 1 nM telomere containing two 8-oxoG bases (Telo-2), 1 nM synthetic KRAS containing two 8-oxoG bases (KRAS), 1 nM synthetic random DNA containing two 8-oxoG bases (Random DNA), and the reaction buffer (control), respectively. Error bars show the standard deviation of three experiments.

**Separation of telomere content from genomic DNA.** To enrich the telomere from the genomic DNA, the telomere-specific 3'-biotinylated capture probes and the digested genomic DNA fragments were denatured at 95 °C for 5 min, followed by slowly cooling to room temperature. Then the biotinylated capture probes were separated by magnetic beads. The magnetic beads were

then incubated with 200 mM NaOH for 30 min at room temperature to release the separated telomeres strands. The supernatant with the enriched telomeres was then separated from the magnetic beads, and the amount of telomere is measured by the NanoDrop 2000c spectrophotometer.

**Table S2.** The separated telomere content from genomic DNA

amount of genomic DNA	20 µg	30 µg	50 µg	60 µg
the separated telomere content (%)	0.018%	0.028%	0.032%	0.027%

**Calculation of the number of oxidative damage base in telomeres.** In this research, all the free 3'hydroxyl groups (3'-OH) result only from the excised oxidative damage sites, and they can induce the TdT-mediated polymerization extension-initiated Endo IV-assisted cyclic cleavage of signal probes to release abundant AF488 fluorophores. The measured AF488 counts are proportional to the oxidative damage level. Oxidative base damage, especially 8-oxoG and FapyG, are among the most common oxidative DNA damage. In this research, a standard curve was established by diluting the known concentration of a synthetic telomere containing two 8-oxoG bases (Telo-2). The obtained log-linear regression equation is  $N = 1632.6 + 101.16 \log_{10} C$ , where  $N$  is the measured AF488 counts and  $C$  is the concentration of Telo-2 (Fig. 2A). We further measured the oxidative damage in telomeres which were separated from different amounts of genomic DNA (Fig. 3B). The AF488 counts enhance with the increasing amount of genomic DNA in the range from 0.1 to 100 ng. The standard curve was used to calculate the absolute oxidative

damage level in human telomere. The number of Fpg-sensitive oxidative damage sites in human telomeres in each cell was calculated as follows:

(1) The obtained AF488 counts in human telomeres separated from different amounts of genomic DNA (Fig. 3B) are substituted into the standard curve  $N = 1632.6 + 101.16 \log_{10} C$ , respectively. Thus, the regression equation is: the obtained AF488 counts =  $1632.6 + 101.16 \log_{10} C$ , and the concentration  $C$  can be calculated.

(2) The number of Fpg-sensitive oxidative damage sites (N-telo) in human telomeres separated from different amounts of genomic DNA is: N-telo = the calculated concentration  $C \times 2$  (Telo-2 in standard curve containing two 8-oxoG bases)  $\times$  the reaction volume (10  $\mu$ L)  $\times$  Avogadro's number = the calculated concentration  $C$  (mol/L)  $\times 2 \times 10 \times 10^{-6}$  L  $\times 6.02 \times 10^{23}$  molecules of oxidative damage sites in human telomeres.

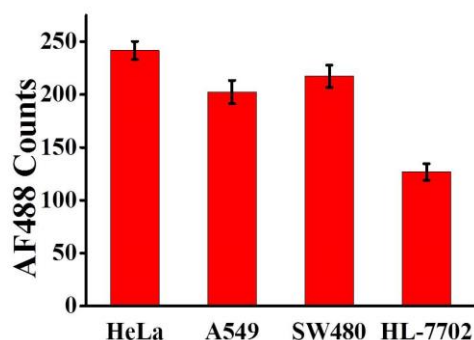
(3) The number of Fpg-sensitive oxidative damage sites in human telomeres in each cell is calculated as: the calculated N-telo / the number of cells corresponds to genomic DNA amount.

Therefore, the number of oxidative damages in telomeres in each cell is estimated to be 34 – 44 each HeLa cell (Table S3).

**Table S3.** The calculated number of oxidative damages in telomeres in each cell

amount of genomic DNA	100 ng	50 ng	20 ng	10 ng	5 ng
number of oxidative damages	40	34	41	43	44
in telomeres in each cell					

**Detection of oxidative damage in human telomeres from different cell lines.** We measured the oxidative damage level in telomeres in different human cell lines including human lung adenocarcinoma cell line (A549 cells), human colon cancer cell line (SW480 cells), and normal human liver cell line (HL-7702 cells). As shown in Fig. S6, different oxidative DNA damage levels in telomeres are observed, with the oxidative DNA damage levels in cancer cells (i.e., HeLa cells, A549 cells, and SW480 cells) being much higher than the normal cells (i.e., HL-7702 cells), consistent with accumulation of oxidative damage in cancer patients. These results suggest that the accurate quantification of oxidative damage level in telomeres may benefit the etiology of oxidative damage-related diseases and early clinic diagnosis.



**Fig. S6** Different oxidative damage level in telomeres from different human cell lines (i.e., HeLa cells, A549 cells, SW480 cells, and HL-7702 cells) treated with 1000  $\mu$ M  $H_2O_2$ . The amount of genomic DNA is 50 ng. Error bars show the standard deviation of three independent experiments.