Supporting Information

Visualizing the Down-regulation of hTERT mRNA Expression Using Gold-nanoflare Probes and Verifying the Correlation with Cancer Cell Apoptosis

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1. Materials and Reagents.

Chloroauric acid ($HAuCl_4 \cdot 4H_2O$) and trisodium citrate was obtained from Shanghai Chemical Reagent Company (Shanghai, China). 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonic acid (CHAPS), tris(2-carboxyethyl)phosphine hydrochloride (TCEP), epigallocatechin gallate (EGCG), ethylene glycol-bis(β-aminoethyl ether)-N,N,N,N-tetraacetic acid (EGTA), phenylmethyl-sulfonyl fluoride (PMSF) and 3-(4,5-dimethylthiazol-2-yl) -2-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich Inc. (USA). Gel electrophoresis loading buffer and ladder DNA were purchased from Thermoscientific Co. Ltd. (USA). Phosphate buffer saline (PBS, pH 7.4) contained 136.7 mM NaCl, 2.7 mM KCl, 8.72 mM Na_2HPO_4, and 1.41 mM KH_2PO_4. Aqua regia was prepared by mixing HCl and HNO_3 with the volume ratio of 3 : 1. All other reagents were of analytical grade. All aqueous solutions were prepared using ultrapure water (≥ 18 MΩ, Milli-Q, Millipore) and treated with DEPC. Magnesium chloride ($MgCl_2$) solution (1 M), tween-20, potassium chloride (KCl), ethylene glycol tetraacetic acid (EGTA), Ethylenediaminetetraacetic acid (EDTA), Trizma hydrochloride pH 7.5 and pH 8.3 were obtained from Sigma-Aldrich. Human TERT ELISA Kit was from Innovation Beyond Limits (Germany), which contained a bottle of telomerase standard solution (40 $IU \cdot L^{-1}$). DNase I endonuclease were purchased from ThermoFisher Scientific Co. Ltd. (USA).

All the oligonucleotides used here were synthesized and purified by Sangon Biotech Co., Ltd (Shanghai), and all the oligonucleotide sequences are listed in Table 1.

Table S1. DNA and RNA sequences employed in this work.

Name	Sequences
HS-DNA	5’-***TCC A***TG TTC ACA ATC ***GGC C*** A_{10}-SH-3’
Flare-DNA	5’-(Cy5) GCC GAT TGT GA -3’
Target-DNA	5’-GGC CGA TTG TGA ACA TGG A -3’
Target-DNA-m1	5’-GGC CGA TT***C*** TGA ACA TGG A -3’
Target-DNA-m3	5’-GG***T*** CGA TT***C*** TGA ACA ***A***GG A -3’
R-HS-DNA	5’-***GAT G***CA CGT TAG CTA ***ACT G*** A_{10}-SH-3’
R-Flare-DNA	5’-(Cy5) AGT TAG CTA AC-3’
Sense-siRNA	5'-UGAUUUCUUGUUGGUGACAdTdT-3',
Anti-sense-siRNA	5'-UGUCACCAACAAGAAAUCAdTdT-3'

(1) Underlined letters in HS-DNA and R-HS-DNA are LNA. Underlined letters in Target-DNA-m1 and Target-DNA-m3 are the mismatched bases relative to Target-DNA.

(2) The siRNA sequences were designed by a commercial software (Applied Biosystems/Ambion, Austin, TX). The selected sequences have been submitted to BLAST http://www.ncbi.nlm.nih.gov/blast/ to ensure that the selected gene was targeted specifically.

2. Apparatus

The transmission electron microscopic (TEM) images were obtained on a JEM-2100 transmission electron microscope (JEOL Ltd., Japan). The UV-vis absorption spectrum was obtained with a UV-vis spectrophotometer (Nanodrop-2000C, Nanodrop, USA). Dynamic light scattering (DLS) was observed on a 90 Plus/BI-MAS equipment (Brook haven, USA). The fluorescence spectra were obtained on a F-7000 spectrofluorometer (Hitachi, Japan). The cell images were gained on a FV1200 laser scanning confocal microscope (Olympus, Japan) and a LSM880 laser scanning confocal microscope (Zeiss, Germany). MTT assay was performed on a microplate reader (BioTek ELX808, USA). Real-time PCR was performed using the QuantStudio® 5 Real-Time PCR system (Applied Biosystems, USA). Inductively coupled plasma atomic emission spectroscopic (ICP-AES) data were determined on a Perkin Elmer Optima 2000DV ICP-AES system.

3. Synthesis and Characterization of AuNPs and AuNP-probe

Gold Nanoparticles (AuNPs) were prepared according to the previous report.[S1,S2] After heating 100 mL $HAuCl_4$ solution (1 mM) to reflux, 10.0 mL trisodium citrate (38.8 mM) was added quickly to the boiling solution under continuous stirring. The reaction mixture was stirred at 125 °C for 1 h until the color turned deep red and then stored at 4 °C. TEM of the AuNPs showed

an average diameter of 14 ± 1.5 nm with a narrow distribution (Figure S1A), and the UV-vis spectrum showed the characteristic peak of AuNPs at 520 nm (Figure S1B). The concentration of AuNPs was determined by measuring their extinction at 524 nm ($\varepsilon = 2.7\times10^{8}\ L\cdot mol^{-1}\cdot cm^{-1}$).

After the AuNPs were functioned with the hybridized-DNA, the maximum absorption in the UV−vis absorption spectra was red-shifted from 520 to 524 nm (Figure S1B). In addition, the dynamic light scattering (DLS) experiments showed that the average hydrodynamic size increased from 14.7 to 36.80 nm (Figure S1C and S1D).

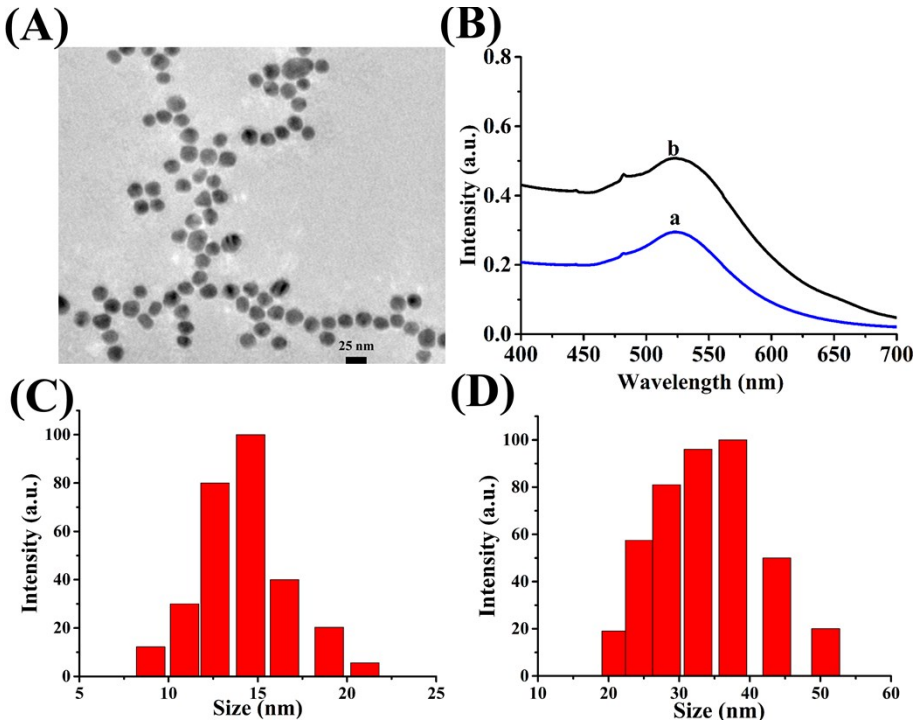

Fig. S1 (A) TEM image of AuNPs. (B) UV−vis spectra of AuNPs (a) and the AuNP probes (b). Average hydrodynamic sizes characterization by dynamic light scattering of the AuNPs (C) and the AuNP probes (D).

4. Evaluation of the amount of the combined DNA assembled on the AuNPs

To measure the amount of the hybridized-DNA system assembled on the probe, a standard curve was obtained with a series of the hybridized-DNA system (Fig. S2). AuNP probe (1.5 nM) incubation 24 h at room temperature with mercaptoethanol for 0, 0.1, 0.5, 1, 5, 10 mM, which can disrupt labelled probes by displacing the alkylthiol-capped oligonucleotides from the AuNPs.

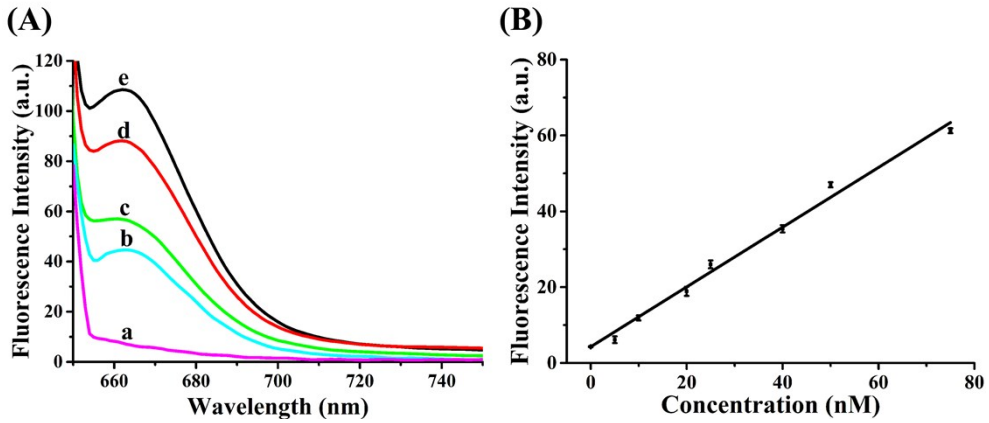

Fig. S2 (A) Fluorescence spectra of the supernatant with probe (1.5 nM) and mercaptoethanol for 0 (a), 0.1 (b), 0.5 (c), 1 (d), 10 mM (e). (B) Plot of fluorescence intensity vs. concentration of the standard hybridized-DNA.

5. Optimization of the Concentration of AuNP Probe

To demonstrate the effect of the concentration of AuNP-probe for the detection of hTERT mRNA target, a series of AuNP-probe (0, 0.5, 1.0, 1.5, 2.0, 3.0, and 4.0 nM) was mixed with the same amount of hTERT mRNA target (200 nM). After incubation at 37 °C for 6 h, fluorescence intensity of the mixture was determined. As shown in Figure S3, a gradual increase of fluorescence intensity with an increasing AuNP concentration was observed which demonstrates more Flare-DNA being released. But the fluorescent signal reached the maximum value when the AuNP-probe concentration was 1.5 nM (curve d). After that, the fluorescent intensity shows an obvious dropping caused by the dynamic fluorescence quenching, and reveal the best AuNP-probe concentration was 1.5 nM.

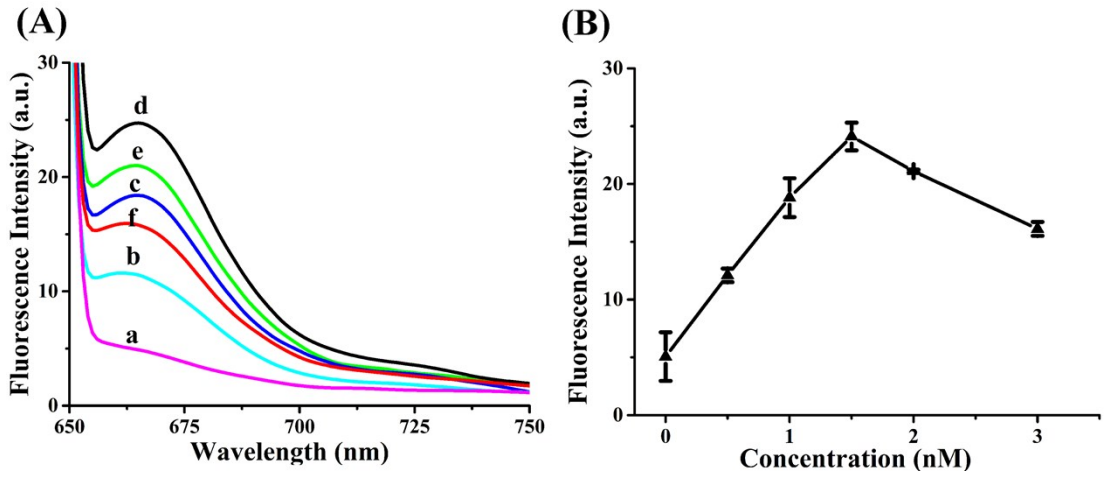

Fig. S3 (A) Fluorescence spectra of hTERT mRNA target (200 nM) and AuNP-probe at 0 (a), 0.5 (b), 1.0 (c), 1.5 (d), 2.0 (e), and 3.0 nM (f); (B) Plot of fluorescence intensity vs. concentration of AuNP-probe. Data are shown as mean ± SD of three independent experiments performed in duplicate.

6. RNA Extraction

HeLa cells were seeded at 1×10^5 mL^{-1} in DMEM with 10% fetal bovine serum. After 24 h, the cells were harvested, and RNA in cells were extracted following a regular Trizol method, according to the manufacturer's instructions.

7. Cellular Uptake AuNP-probe in Different Cells

Four cancer cell lines (HeLa, A549, HepG2 and U2OS) and two normal cell lines (HL-7702 and HBL-100) were respectively seeded at 1×10^5 mL^{-1} in DMEM with 10% fetal bovine serum. After 24 h, cells were incubated with the AuNP-probe (1.5 nM) for 4 h. At the end of incubation period, the cells were washed three times with PBS buffer and trypsinized to remove them from the bottom. Then the cells were counted and collected in centrifuge tubes. Next, the cells were treated with 0.2 mL of aqua regia (3:1 hydrochloric acid/nitric acid). Following the incubation overnight, the sample was diluted to 5 mL using ultrapure water. All samples was then analyzed

for total gold content by ICP-AES and the measurement was repeated three times. As described previously,[S3] the amount of AuNP as determined by ICP-AES analysis was then converted to the number of nanoparticles using the average AuNP diameter as determined via TEM imaging. Based on this protocol, the numbers of Au-NP in a cell can uptake were calculated to be (2.3 ± 0.62) × 10^4 (in HeLa), (2.1 ± 0.45) × 10^4 (in A549), (2.6 ± 0.67) × 10^4 (in HepG2), (2.5 ± 0.92) × 10^4 (in U2OS), (2.3 ± 0.77) × 10^4 (in HBL-100) and (2.8 ± 1.02) × 10^4 (in HL-7702), respectively.

8. Optimization of the Response Time of AuNP-probe to hTERT mRNA Target

To optimize the response time of AuNP-probe to hTERT mRNA target, a series of AuNP-probes (1.5 nM) was incubated with the same amount of hTERT mRNA target (200 nM) for different times at 37 °C. After that, fluorescence intensity of the mixture was determined. As shown in Fig. S4A, a gradual increase of fluorescence intensity with increasing reaction time was observed which demonstrates more Flare-DNA being released. But the fluorescent intensity reached the plateau at about 240 min (Figure S4B).

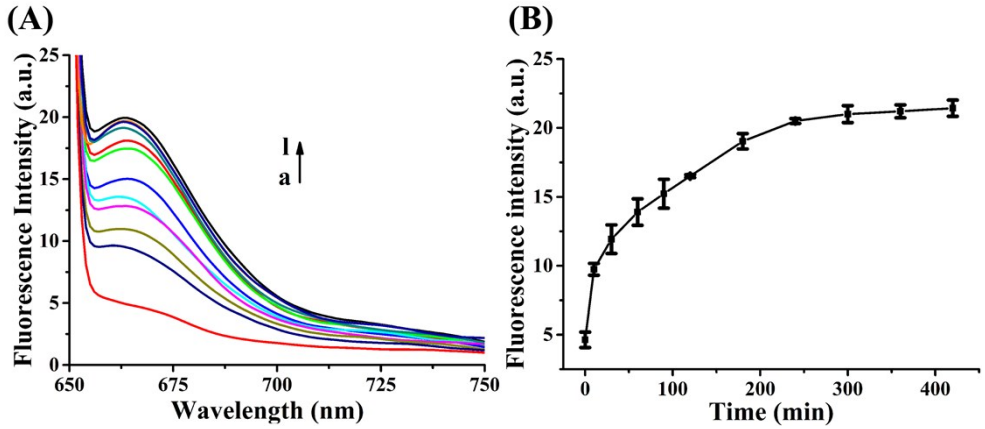

Fig. S4 (A) Fluorescence spectra of the AuNP probe after incubation with hTERT mRNA for different time (from a to l); (B) Plot of fluorescence intensity vs. incubation time. Data are shown as mean ± SD of three independent experiments performed in duplicate.

9. Verification of the specificity of the AuNP-probe

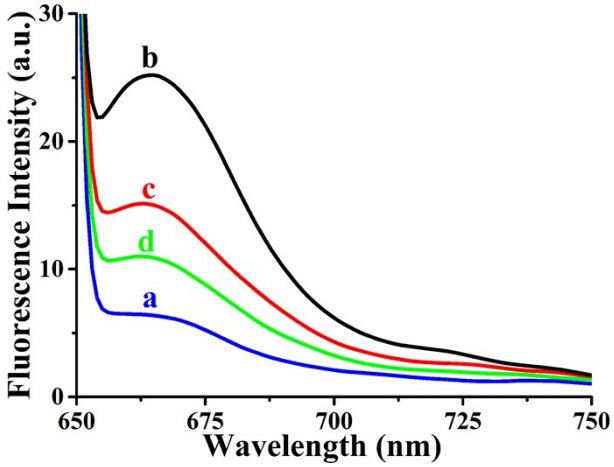

Fig. S5 Fluorescence intensities produced by buffer (no target, a), complementary Target-DNA (b), and those containing 1 (c) or 3 (d) mismatches Target-DNA with AuNP-probes.

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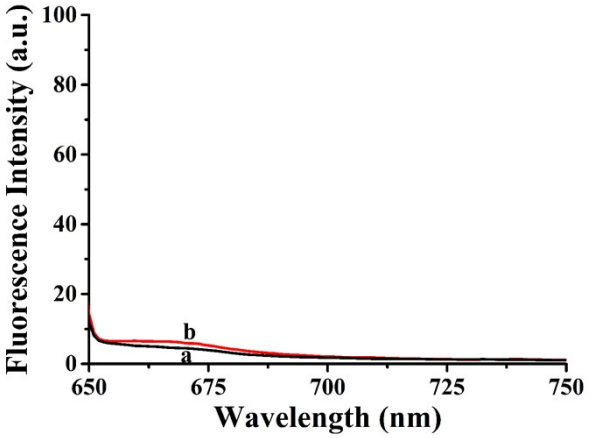

Fig. S6 Fluorescence intensity of AuNP-probe analogues (1.5 nM) incubated without (a) or with RNA extracts of 5000 HeLa cells (b).

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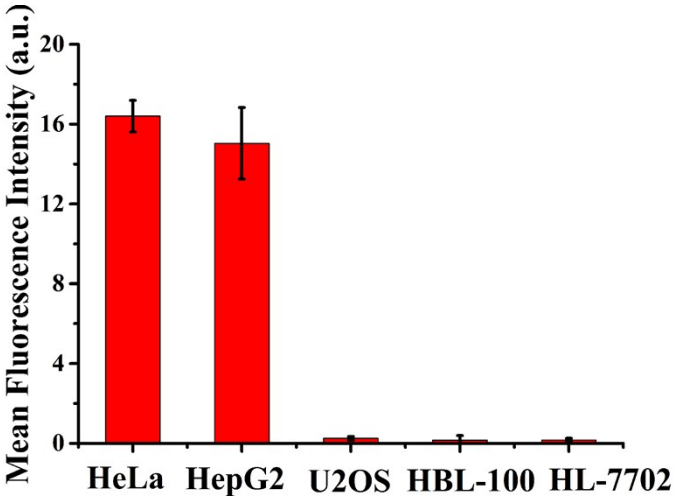

Fig. S7 Mean fluorescence intensity of different cells in CLSM images shown in Fig. 1C.

12. Intracellular Imaging of hTERT mRNA with the AuNP-probe Analogue

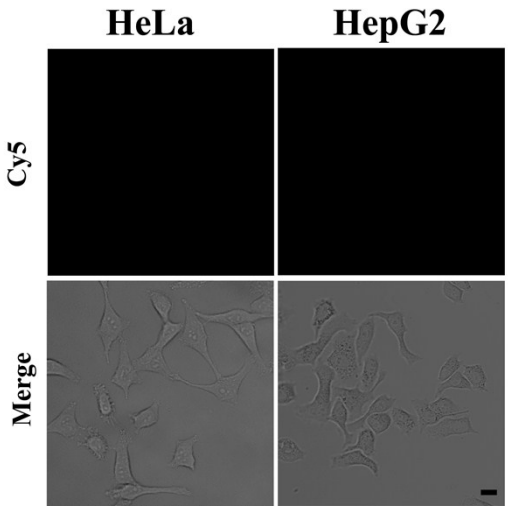

Fig. S8 Confocal images of HeLa, HepG2 cells after incubation with AuNP-probe analogue: fluorescence field images (top line) and overlapping fluorescence and bright-field images (bottom line). Scale bars are 50 μm.

13. Protection Ability of AuNP to Combined DNA Duplexes Against Nuclease And GSH

To investigate the protective properties of AuNP probe against damage by intracellular enzymes, DNase I was added to the AuNP probes in phosphate-buffered saline (PBS), and almost no fluorescence signal enhancement was observed compared with the background, which ensured the intracellular usage of the probe.

200 μL of AuNP probe (1.5 nM) was treated with or without DNase I or GSH (2 mM) in the PBS buffer or DMEM containing serum, the fluorescence intensity of the solution was recorded along with incubation time, which showed negligible fluorescence recovery (Figure S8), indicating the protection ability of AuNP probe and good probe stability.

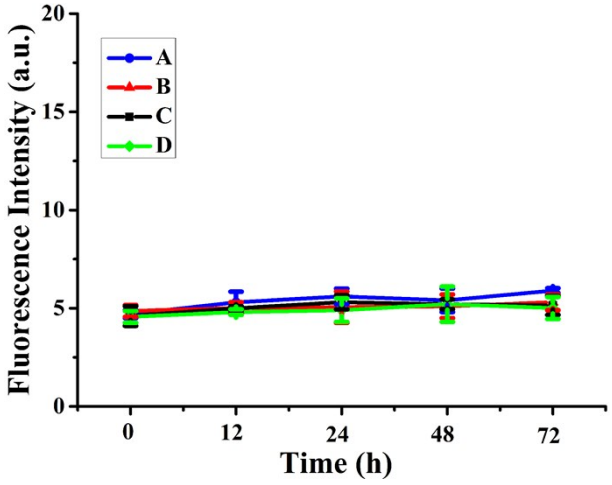

Fig. S9 Plots of fluorescence intensity of the AuNP probe in the absence (A) and presence of DNase I in PBS (B) or in cell culture medium containing serum (C), in PBS containing GSH (D) vs. incubation time. Data are shown as mean ± SD of three independent experiments performed in duplicate.

14. Expression of hTERT of different cells by ELISA kit

Telomerase in different cell samples was extracted according to the following procedure. First, cells (0.4 mL, 1×10^6 mL^{-1}) were seeded a 20-mm confocal dish for 24 h. Then, all cell samples were collected, and 4×10^6 cells were dispensed in a 1.5-mL EP tube, washed twice with 0.1 M ice-cold PBS (pH 7.4), and resuspended in 200 μL of ice-cold CHAPS lysis buffer containing 10 mM Tris-HCl (pH 7.5), 1 mM $MgCl_2$, 1 mM EGTA, 0.1 mM PMSF, 0.5% CHAPS, and 10% glycerol. The mixture was incubated for 30 min on ice and then centrifuged at 16000 rpm at 4 °C for 20 min. The supernatant was collected and diluted to 200 μL for analysis or stored frozen at −105 °C.

To quantify the expression of hTERT in different samples, a standard curve was constructed using a commercial hTERT ELISA Kit with a series of standard samples at a concentration range from 0 to 20 $IU{\cdot}L^{-1}$. The ELISA analysis was performed by adding 50 μL of cell extracts in the wells of the ELISA plate to incubate at 37 °C for 30 min. The plate was washed with washing buffer, still for 30 s then drain, repeat 5 times, dry by pat and added with 50 μL of HRP-Conjugate reagent (from the kit) to incubate at 37 °C for 30 min. Afterward, the medium was removed, and Chromogen Solution A 50 uL and Chromogen Solution B 50 uL (from the kit) were added to vibrate, evaded the light preservation for 10 min at 37 °C. Stop Solution 50 μL (from the kit) was finally added to each well to stop the color reaction, and the absorbance was measured at 450 nm on a microplate reader.

The hTERT protein expression level in different cell extracts was determined by analysis with the ELISA Kit and comparison to the standard curve.

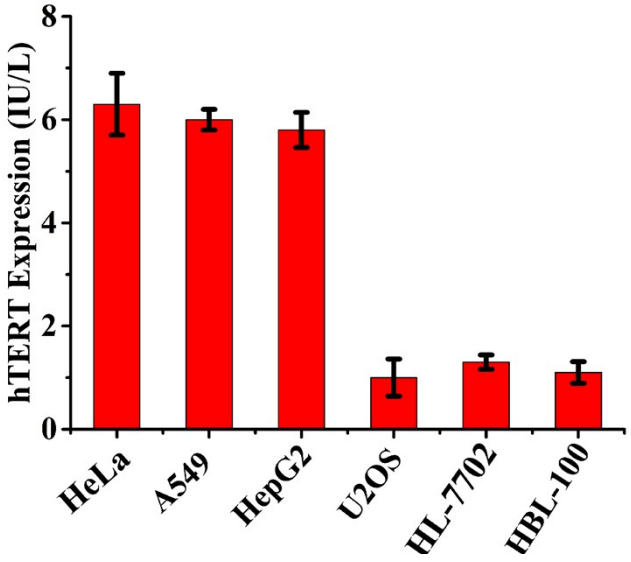

Fig. S10 Expression of hTERT of different cells by ELISA kit. Data are shown as mean ± SD of three independent experiments performed in duplicate.

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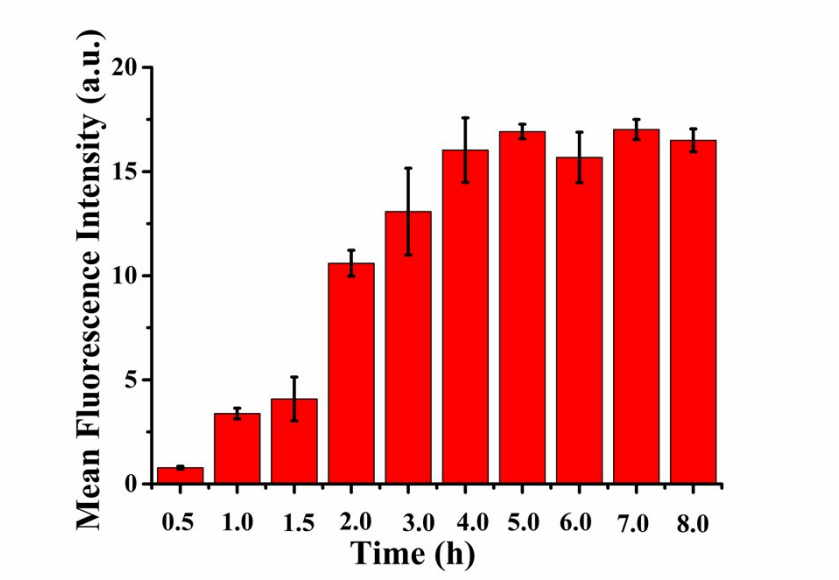

Fig. S11 Mean fluorescence intensity in the time course of CLSM images shown in Fig. 2A.

16. Three-dimensional CLSM images of cells incubated with AuNP-probe

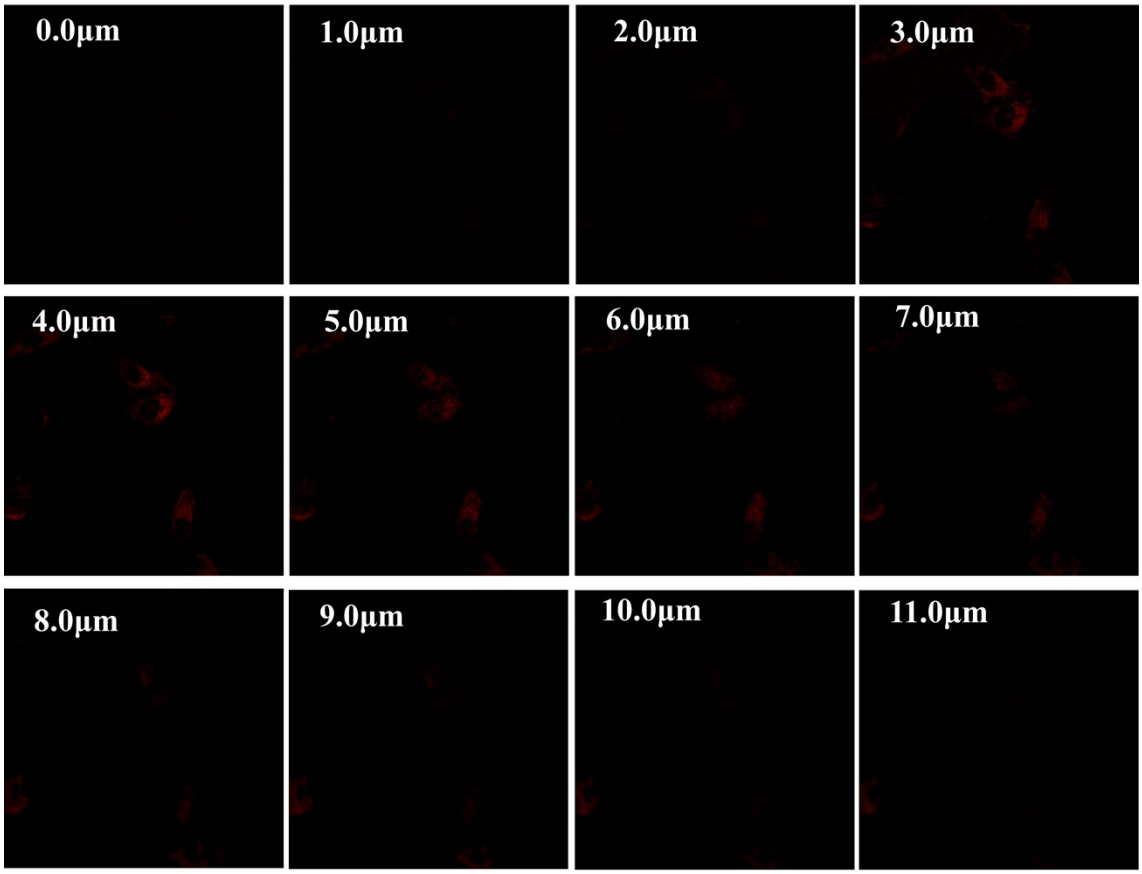

Fig. S12 The three-dimensional fluorescence image obtained by the CLSM tomographic imaging at different depths along the Z-stack of HeLa cells.

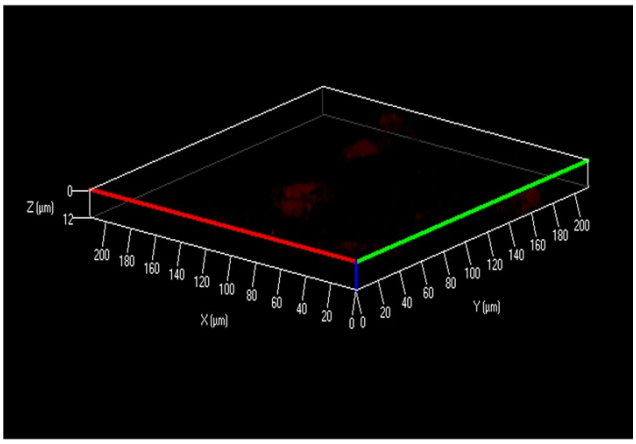

Fig. S13 Z-stack images of the fluorescence signal of Cy5 in the HeLa cells after incubation with the probe for 4 h. The images were taken in a series of 12-step (1.0 μm-step-sizes) measurements.

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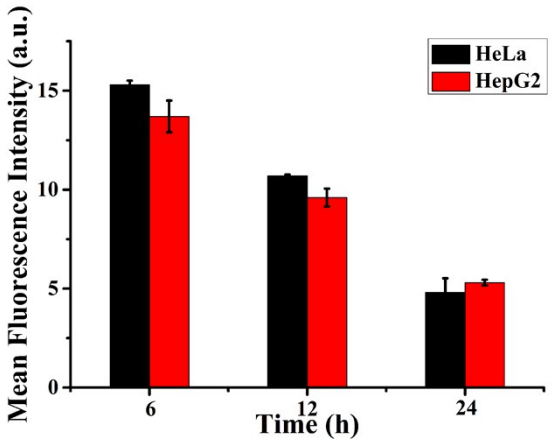

Fig. S14 Mean fluorescence intensity in the time course of CLSM images shown in Fig. 3.

18. Assessment of the Specificity of the AuNP-probe for hTERT mRNA

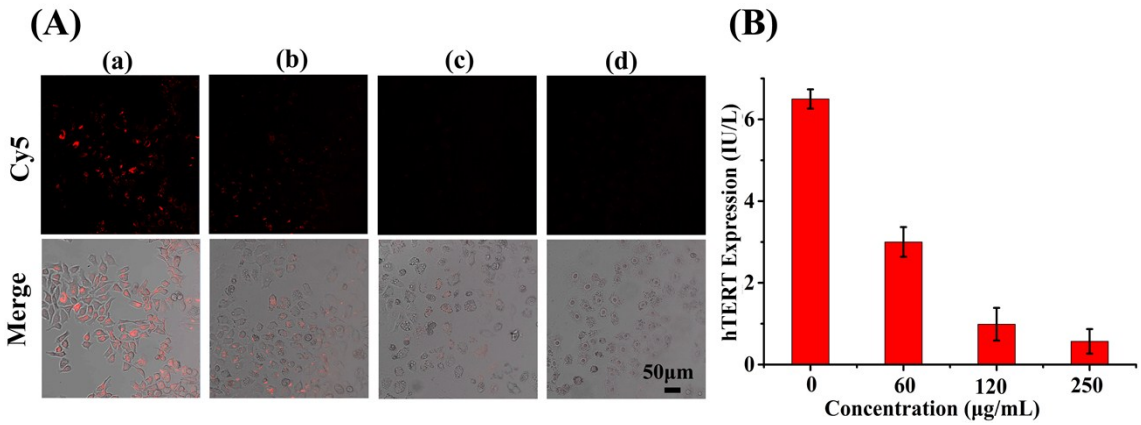

Fig. S15 (A) Confocal images of HeLa cells treated with 0, 60, 120 and 250 μg.mL^{-1} EGCG (from a to d) for 24 h and then AuNP-probe (1.5 nM) for 4 h. (B) Expression of hTERT of cells treated for different concentrations of EGCG determined by ELISA kit. Data are shown as mean ± SD of three independent experiments performed in duplicate.

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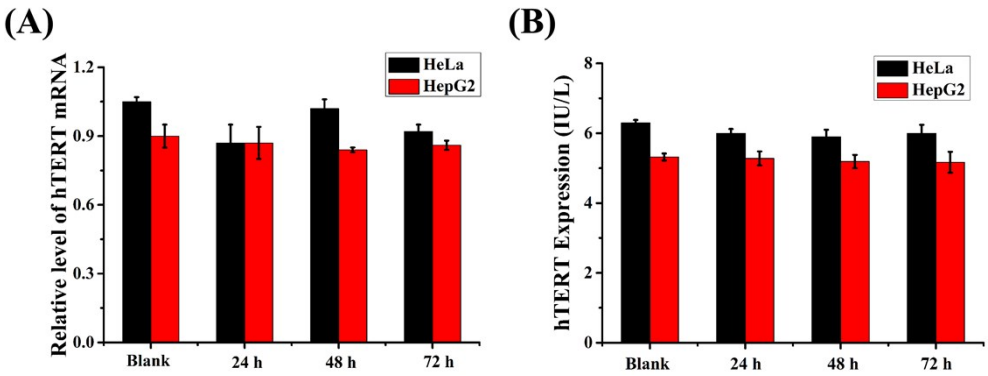

Fig. S16 (A) Expression analysis of hTERT mRNA in HeLa and HepG2 cells treated without (Blank) or with the AuNP-probe analogues for different time by qRT-PCR. The relative mRNA expression was calculated using the $2^{(-\Delta\Delta CT)}$ method, with GAPDH as an internal control. (B) Assessment of the expression of hTERT of HeLa and HepG2 cells treated without (Blank) or with the AuNP-probe analogues for different time using a hTERT ELISA kit.

20. Characterization of HeLa cells morphology after treatment with AuNP-probe

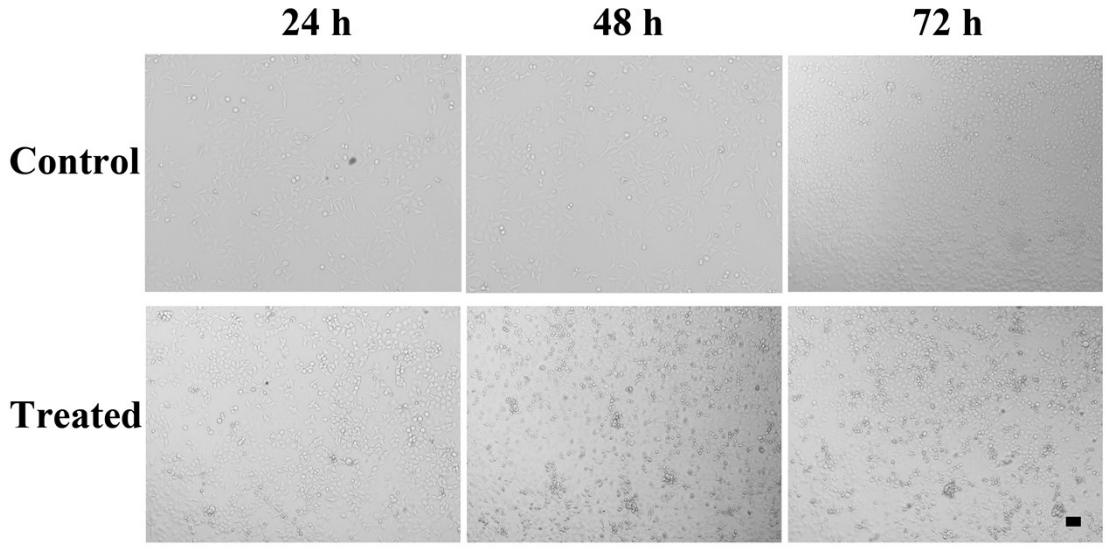

Fig. S17 Morphology of HeLa cells treated without (Control) or with (Treated) the AuNP-probes for 24, 48 and 72 h. Cells were visualized at 10 × magnification. Scale bars are 50 μm.

Reference:

(S1) M. Hong, H. Sun, L. Xu, Q. Yue, G. Shen, M. Li, B. Tang, C.-Z. Li, *Ana. Chim. Acta*, 2018, **1021**, 129-139.

(S2) Y. J. Yang, J. Huang, X. H. Yang, K. Quan, H. Wang, L. Ying, N. l. Xie, M. Ou and K. M. Wang, *J. Am. Chem. Soc.*, 2015, **137**, 8340-8343.

(S3) S. A. Love and C. L.Haynes, *Anal. Bioanal. Chem.,* 2010, **398**, 677-688.