Electronic Supplementary Information

A sequentially lighting-up multicolor DNA tetrahedron nanoprobe for analyzing human telomerase in living cells

Ruiyuan Zhang, Ruixue Zhang, Wei Jiang, and Xiaowen Xu*

School of Chemistry and Chemical Engineering, Shandong University, 250100 Jinan, P. R. China.

E-mail: xuxw@sdu.edu.cn; Fax: +86 531 88363916.

Experimental Section

Materials and reagents. DNA sequences (Table S1) were synthesized and purified by Sangon Biotech Co., Ltd. (Shanghai, China). Diethyl pyrocarbonate (DEPC) treated water, 40% acrylamide/bisacrylamide (19:1) solution, ammonium persulfate (APS), N,N,N',N'-tetramethylethylenediamine (TEMED), Tris base, bovine serum albumin, ethylenediaminetetraacetic acid tetrasodium (EDTA), egtazic acid-glycol ether diamine tetraacetic acid (EGTA), boric acid and deoxynucleotide triphosphates (dNTPs) were obtained from Sangon Biotech Co., Ltd. (Shanghai, China). 3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonic acid (CHAPS) lysis buffer was bought from Millipore (Bedford, MA, USA). SYBR gold nucleic acid gel stain was bought from Thermo Fisher Scientific. Dimethyl sulfoxide (DMSO), epigallocatechin gallate (EGCG) and 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma-Aldrich (Missouri, USA). Fetal bovine serum (FBS), MEM cell medium, RPMI-1640 cell medium and phosphate-buffered saline (PBS) buffer were obtained from Biological Industries (Israel). All other reagents were of analytical grade and used as received. Ultrapure water (18.25 MΩ•cm) obtained from a UP water purification system was used throughout the experiment.

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

Name	Sequence (5' to 3')
DT-1	ACATTCCTAAGTCTGAAACATTACAGCTTGCTACACGAGAAGA GCCGCCATAGTA
DT-2	TCAACTGCCTGGTGATAAAACGACACTACGTGGGAATCTACTA TGGCGGCTCTTC
DT-3	TATCACCAGGCAGTTGACAGTGTAGCAAGCTGTAATAGATGCG AGGGTCCAATAC
DT-4	TTCAGACTTAGGAATGTGCTTCCCACGTAGTGTCGTTTGTATTG GACCCTCGCATCTAACTCTCACCTAC
MB1	FAM-AGGGTTCCATAGCCTAACCC/iDabcyldT/AACTTCCACTTCTACAC TCC
MB2	Cy5-GCGGTTGTACGCACTAACCGC/iBHQ2dT/ACCTCTGCACTACACT ACG
MB3	HEX-CAGGTTCCAATCACTAACC/iDabcyldT/GCGATATACCCTCACCCT AAC
LD	GTAGGTGAGAGTTAGGTTAGGGTGAGGCGTAGTGTAGTGGGAG TGTAGAAG*AATCCGTCGAGCAGAGTT*
cDNA for MB1	AAGTTAGGGTTAGGCTA
cDNA for MB2	AGGTAGCGGTTAGTGCG
cDNA for MB3	TATCGCAGGTTAGTGAT
MB1'	AGGGTTCCAAAAACTAACCCTAACTTCCACTTCTACACTCC
TS	AATCCGTCGAGCAGAGTT
CX	CCCTTACCCTTACCCTTACCCTAA
LcDNA1	GGAGTGTAGAAGAATCCGTCGAGCAGAGTT**AAGTTAGGGTTA GGCTA**
LcDNA2	CGTAGTGTAGTGAATCCGTCGAGCAGAGTT**AGGTAGCGGTTA GTGCG**
LcDNA3	GTTAGGGTGAGGAATCCGTCGAGCAGAGTT**TATCGCAGGTTA GTGAT**

[a] The complementary bases between DT-4 and LD are marked in blue, which are utilized to link the DNA carrier and MBs-loaded LD. The complementary bases between MB1, MB2, MB3 and LD are respectively marked in green, red and yellow, which are utilized to set MB onto LD. The

stem region of MB is marked in purple. The binding regions of telomeric repeats on MB are underlined. Telomerase substrate domain is marked in italic in LD. For the connected longer cDNA (termed as LcDNA) that can accommodate MB, the complementary bases to MB1, MB2, MB3 are respectively marked in green, red and yellow in LcDNA1, LcDNA2 and LcDNA3, and the same sequences to cDNA are in bold.

Instrumentation. UV–vis absorption spectra were recorded on a U-2910 spectrometer (Hitachi, Japan). Fluorescence emission spectra were recorded on a F-7000 fluorospectrophotometer (Hitachi, Japan). Absorbance measurements in MTT experiments and fluorescence intensity measurements were conducted on a multimode microplate reader (Tecan, Switzerland). Stained polyacrylamide gel was imaged on GelDocTM XR^+ imaging system (Bio-Rad Laboratories Inc., USA). Confocal fluorescence images were recorded on a SP8 confocal laser scanning microscope (Leica, Germany) with an oil dipping objective (63×).

Preparation of SLMN. The DNA tetrahedron was prepared according to the previous method.[1] Four types of single-stranded oligonucleotides (DT-1, DT-2, DT-3, DT-4) were mixed at an equivalent molar ratio in TM buffer (20 mM Tris, 50 mM $MgCl_2$, pH 8.0). The reaction solution was heated and holds at 95 °C for 3 min, and then cooled down rapidly to 4 °C in ice-water bath. The LD strand and three molecular beacon strands (MB1, MB2, MB3) were mixed at a ratio of 2:3:3:3 in PBS buffer (2 mM KH_2PO_4, 8 mM Na_2HPO_4, 136 mM NaCl, 2.6 mM KCl, pH 7.4). The mixture was heated to 90 °C for 10 min and cooled down gradually to room temperature. Finally, equimolar of DNA tetrahedron and MBs-loaded LD strand were incubated at 4 °C for 24 h to assemble the SLMN. SLMN were purified by centrifugation at 12000 rpm for 10 min by ultrafiltration devices (Millipore, 1.5 mL, 30 kD) to remove free MBs in solution. The probes were re-suspended in the TM buffer with a final concentration of 1 μM.

Polyacrylamide gel electrophoresis. 8% polyacrylamide gel was prepared by mixing 5 mL of 40% acrylamide/bisacrylamide solution (19:1), 5 mL of 5× Tris-borate-EDTA (TBE) buffer, 15 mL of deionized water, 180 μL of 0.1 g/mL APS and 18 μL of TEMED. 10 μL of DNA samples were mixed with 2 μL of 6× loading buffer, and 5

μL of the mixture was loaded into the well of the gel. Electrophoresis was run at 100 V for 4 h in 1× TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.0). The gel was then stained with 1× SYBR gold for 40 min and photographed with the fluorescence imaging system. Gel characterization of SLMN was performed by characterizing the samples during each assembling step. Gel characterization of telomerase activity by telomeric repeat amplification protocol was conducted according to our previous work.[2]

Cell culture. Cervical cancer (HeLa) cells, normal liver (HL-7702) cells were grown in DMEM medium supplemented with 10% fetal calf serum and 100 U/mL of penicillin-streptomycin. Breast cancer (MCF-7) cells, liver cancer (HepG2) cells were grown in RPMI-1640 supplemented with 10% fetal calf serum and 100 U/mL of penicillin-streptomycin. The cells were cultured in incubator at 37 °C in humidified atmosphere containing 5% CO_2.

Detection of telomerase activity in cell extracts. Telomerase was extracted by the CHAPS method.[3] To detect telomerase activity, 5 μL of 1 μM SLMN, 10 μL of cell extracts, 10 μL of 10 mM dNTPs, 10 μL of 1 mg/mL BSA, 20 μL of 5×TRAP buffer and 45 μL of DEPC treated water were mixed and incubated at 37 °C. 50 μL of above solution was transferred to the cuvette for fluorescence measurements. For the control experiment in the absence of telomerase, 10 μL of CHAPS lysis buffer was used instead of cell extracts. For the sensitivity study, telomerase extracts from different number of HeLa cells were reacted with SLMN at 37 °C for 4 h, and fluorescence spectra were measured. For the specificity study, different types of cell extracts including HeLa, MCF-7, HepG2 and HL-7702 from 10000 cells were tested. To measure the fluorescence of FAM, HEX and Cy5, the excitation wavelengths were set at 480 nm, 520 nm and 600 nm, and emission wavelengths were recorded from 500 to 550 nm, 540 to 600 nm and 630 to 720 nm, respectively. To establish the calibration curve utilized for calculating the opened MB when SLMN is reacted with telomerase extracted from cell lysates, different concentrations of annealed MB/cDNA duplex were mixed with the reaction buffer that contains cell extracts, dNTPs, BSA in 1×TRAP buffer, and fluorescence is measured in the cuvette on

fluorospectrophotometer.

Cell viability assay. HeLa cells were seeded in a 96 well cell culture plate at a density of 4000 cells per well at 37 °C in 5% CO_2 in 100 μL of culture medium. After 24 h, the cells were treated with different concentrations of SLMN within the range from 25 to 200 nM for 24 h. After abandoning the medium, each well was washed with PBS buffer, followed by addition of 100 μL of 5 mg/mL MTT. After an incubation time of 4 h, MTT solution was discarded, and then 100 μL of DMSO was injected to dissolve purple formazan. The absorbance at 490 nm was measured by the microplate reader and the cell viability was calculated.

In situ imaging of telomerase activity. HeLa cells (or MCF-7, HepG2, HL-7702 cells) were seeded in each confocal dish for 24 h. After removing the medium, 100 μL of fresh medium containing 150 nM SLMN was added to the confocal dish. After incubation at 37 °C for a period of time, the cells were washed with PBS buffer and incubated with 100 μL fresh medium for confocal imaging. To study the variation of intracellular telomerase activity, cells were pre-incubated with different concentrations of EGCG for 24 h before the addition of the SLMN. The confocal fluorescence images were processed by "channel dye separation" tool in LAS AF Lites software (Leica, Germany) to remove overlapping fluorescence from each channel. The mean value of fluorescence intensity for confocal fluorescence images are qualitatively analyzed and provided by LAS AF Lites software.

Determination of intracellular telomerase-elongated product length distribution. HeLa cells were seeded in a 96 well cell culture plate at a density of 4000 cells per well and 100 μL of cell culture medium containing 150 nM SLMN was added to each well. After 2.5 h, the wells were washed with PBS buffer, added with fresh medium and then the fluorescence intensity was measured by the microplate reader. For fluorescence measurements, the excitation wavelengths were set at 475 nm, 510 nm and 620 nm, and the emission wavelengths were set at 520 nm, 560 nm and 667 nm, respectively. To establish the calibration curve utilized for calculating the opened MB when SLMN is incubated with cells seeded in the microplate supported by culture medium, different concentrations of annealed MB/cDNA duplex were mixed with the

cell culture medium, and fluorescence is measured in the microplate on microplate reader.

Additional figures and discussion

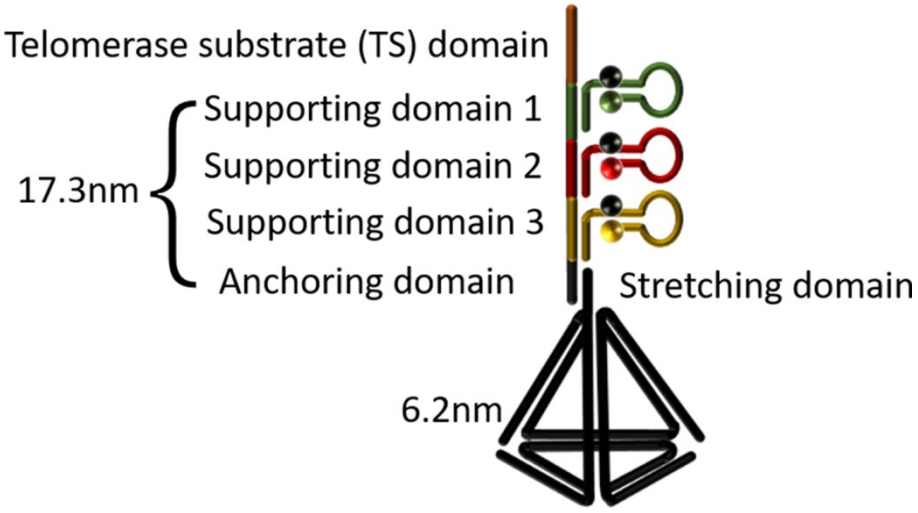

Fig. S1 Schematic illustration of the theoretical size and the corresponding functional DNA domains in SLMN.

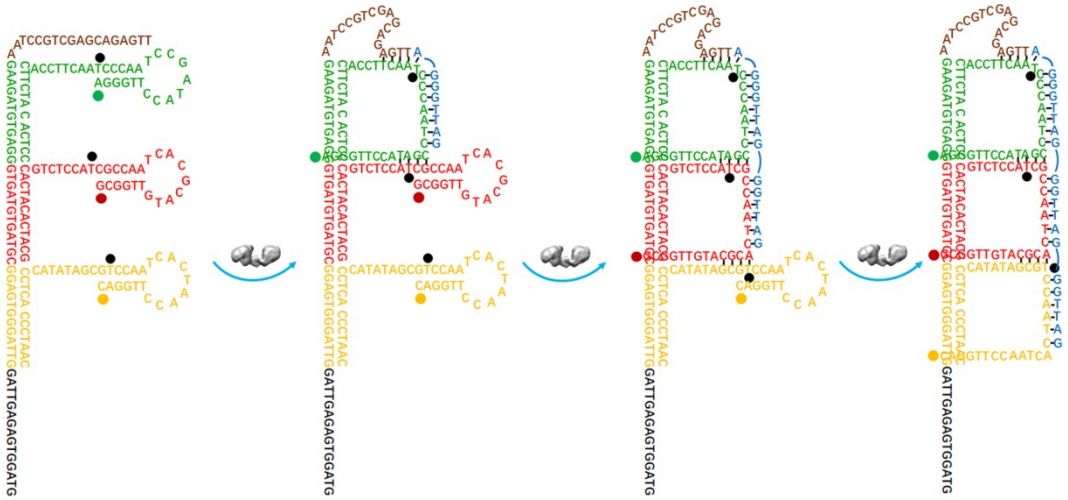

Fig. S2 Schematic illustration of the sequential open of MBs along with the elongation of telomeric repeats with base identifications. When the synthetic non-telomeric oligonucleotide (TS) is used as the substrate, for the processive utilization of human telomerase template, initially 8 dNTPs of "AGGGTTAG" is added onto TS. Then, the elongated TS (TS-1R) realigns for the next round of synthesis to produce

the expected 6-base repeat of "GGTTAG".[4]

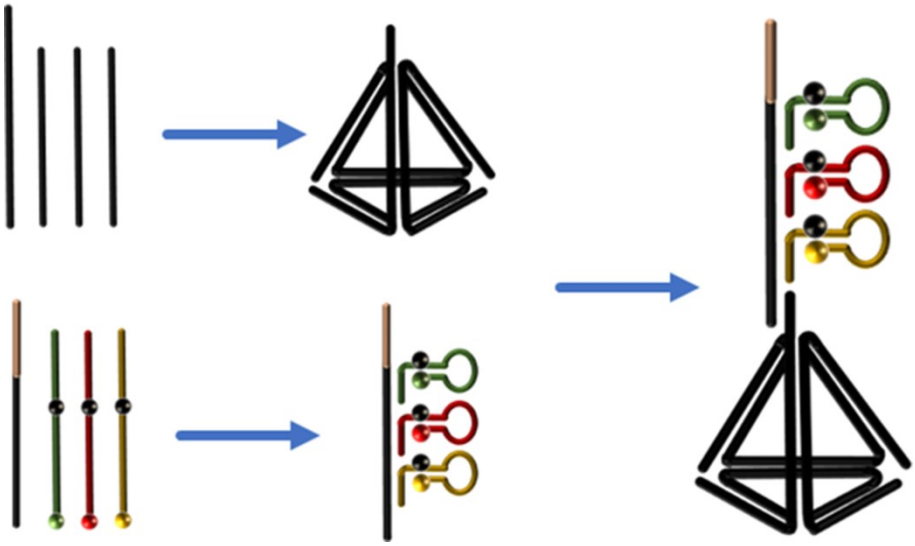

Fig. S3 Schematic illustration of the assembling process of the SLMN.

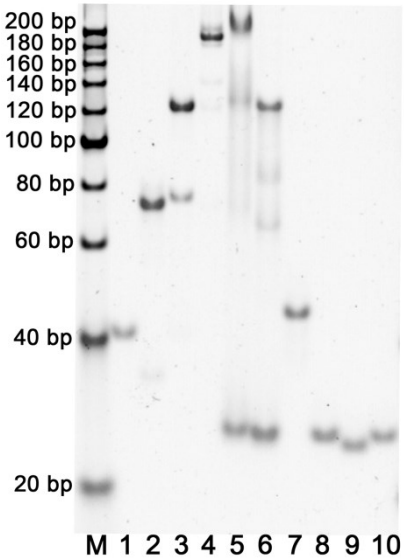

Fig. S4 Native polyacrylamide electrophoresis gel characterization of the SLMN assembling process. Lane M: DNA marker; lane 1: DT-1; lane 2: DT-1 + DT-2; lane 3: DT-1 + DT-2 + DT-3; lane 4: DT-1 + DT-2 + DT-3 + DT-4; lane 5: DNA tetrahedron carrier + MBs loaded LD; lane 6: LD + MB1 + MB2 + MB3; lane 7: LD; lane 8: MB1; lane 9: MB2; lane 10: MB3.

Fig. S4 shows the construction process characterized by native polyacrylamide gel electrophoresis (PAGE). On the one hand, the DNA tetrahedron carrier is gradually assembled as its component strands are introduced one by one (lane 1 to lane 4), and shows a high assembling yield. One the other hand, the free LD (lane 7), and the three types of MBs (lane 8 to lane 10), are integrated together to form MBs-loaded LD (lane 6). It can be observed that only one major band, with the slowest migration rate, appears in lane 6, suggesting that all of the three MBs are hybridized on the LD. The bands corresponding to the LD bearing one or two MBs are faint and negligible, indicating a very low yield of those deficient assemblies. This results from the exclusive complementarity between the designated supporting domain in LD and specific MB, making each MB set in the correct position. Finally, when the DNA tetrahedron carrier is mixed with the MBs-loaded LD, a new band with retarded migration rate is generated (lane 5), which indicates the formation of the SLMN. Note that the bands corresponding to MBs in lane 5 and lane 6 are due to their excess amount in annealing, which are then separated by ultrafiltration.

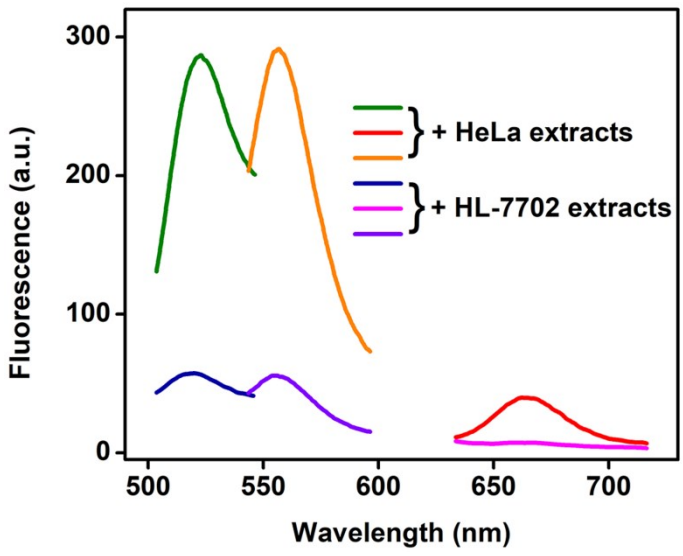

Fig. S5 Fluorescence spectra of SLMN in the presence of telomerase extracts from 10000 HeLa cells or 10000 HL-7702 cells.

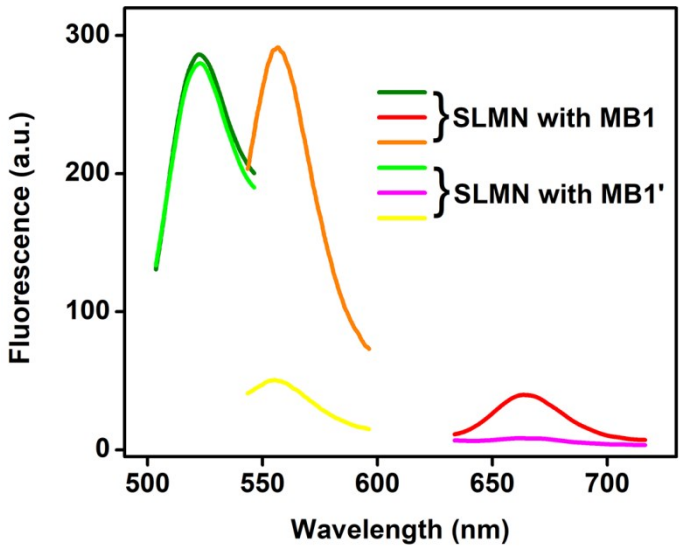

Fig. S6 Fluorescence spectra of MB1-constituted SLMN and MB1'-constituted SLMN in the presence of telomerase extracts from 10000 HeLa cells.

As shown in Fig. S6, it can be seen that when MB1' is substituted for MB1 in SLMN, only the first MB is opened to obtain the fluorescence enhancement of FAM. At this time, MB2 does not get opened as indicated by the fluorescence of Cy5, due to the alternation of auxiliary bases in MB1 that cannot assist the open of MB2. MB3 does not get opened, either, as indicated by the fluorescence of HEX, due to the folding of MB2 that does not expose auxiliary bases for opening MB3.

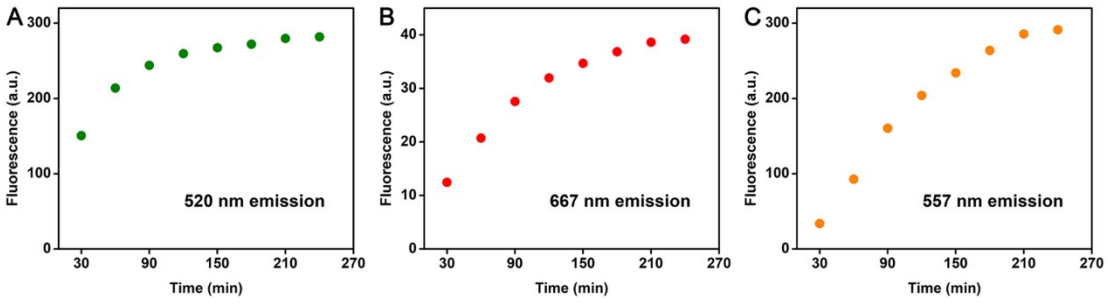

Fig. S7 Time-dependent fluorescence changes of SLMN at 520 nm (A), 667 nm (B) and 557 nm (C) in the presence of telomerase extracts from 10000 HeLa cells.

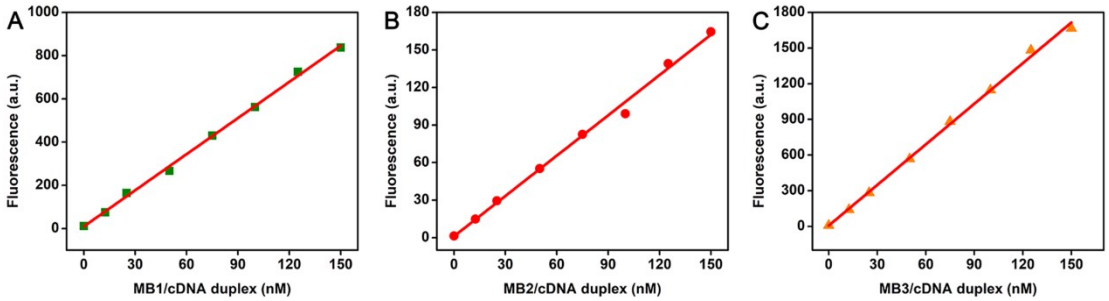

Fig. S8 Calibration curves between fluorescence intensity and concentration of MB/cDNA duplex: fluorescence intensity at 520 nm versus MB1/cDNA duplex (A), fluorescence intensity at 667 nm versus MB2/cDNA duplex (B), and fluorescence intensity at 557 nm versus MB3/cDNA duplex (C).

To test whether the proximity between the complementary DNA and MBs affects the state of opened MB after annealing, longer cDNA that can connect and accommodate MB (termed as LcDNA) was tested, for comparison with the case of the free cDNA. After annealing the corresponding MBs with cDNA or LcDNA in the reaction buffer that contains cell extracts, dNTPs, BSA in 1×TRAP buffer, the similar fluorescence is obtained (Fig. S9). It indicates that regardless whether MB is kept in the proximity with cDNA or not, they can reach the same thermodynamic equilibrium after annealing. However, the hybridization kinetics recorded by measuring time-dependent fluorescence changes after mixing MBs with cDNA or LcDNA at room temperature, suggests a faster reaction rate for the hybridization between MB and LcDNA (Fig. S10), as a result of their proximity. It can be obtained that the proximity between MB and its cDNA mainly influences the hybridization kinetics instead of hybridization thermodynamic equilibrium. Thus, under the annealing experimental condition, the hybridization of MB by cDNA can afford an opened state of MB similar to that in SLMN.

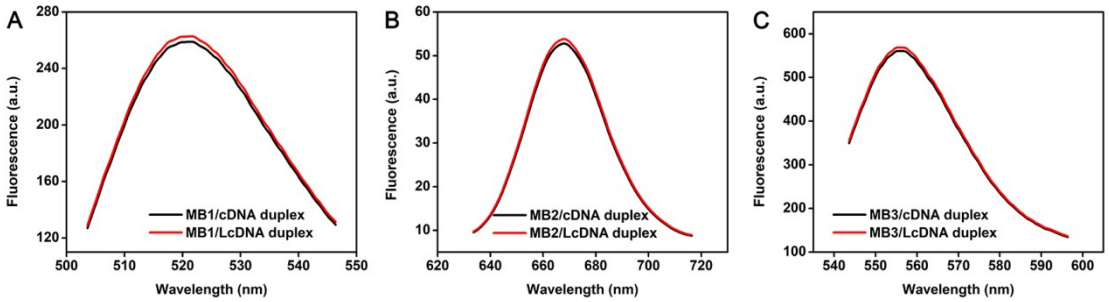

Fig. S9 Comparison of fluorescence spectra by annealing 50 nM MB with 50 nM cDNA or LcDNA.

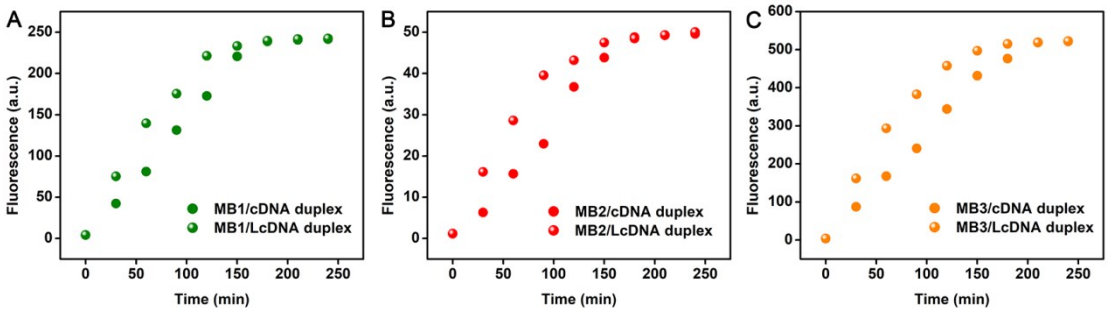

Fig. S10 Time-dependent fluorescence change by mixing 50 nM MB with 50 nM cDNA or LcDNA at room temperature at 520 nm (A), 667 nm (B) and 557 nm.

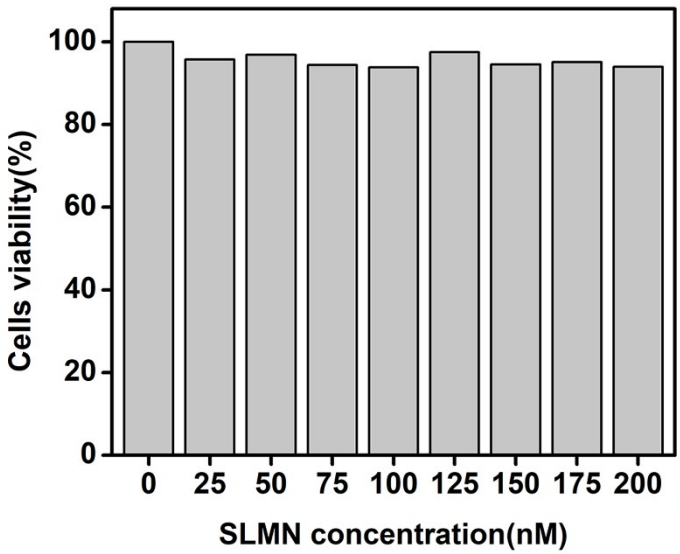

Fig. S11 The cell viabilities of HeLa cells after incubation with different concentrations of SLMN within the range from 25 to 200 nM for 48 h.

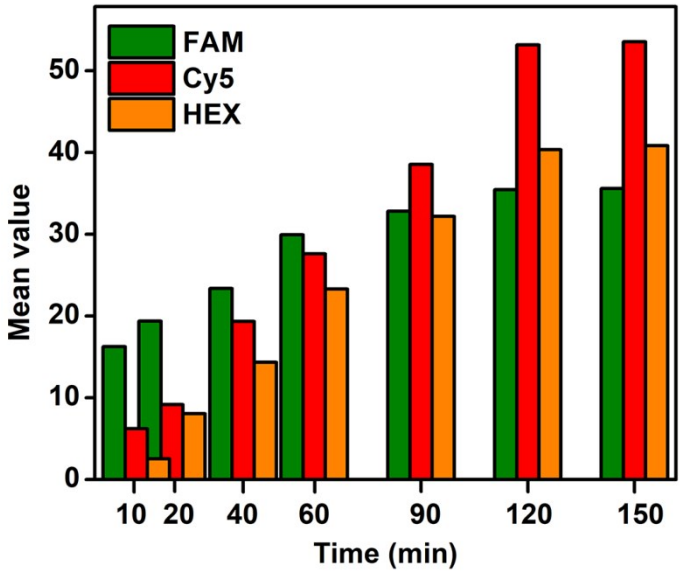

Fig. S12 Mean value of the fluorescence intensity for confocal fluorescence images collected at different time intervals after the incubation of SLMN with identical HeLa cells.

For time-dependent confocal fluorescence images (Fig. 2 in the main text), the mean values of FAM, Cy5 and HEX all gradually increase along with time, indicating the action of intracellular telomerase on TS to generate products that opens MBs. Specially, the fluorescence of FAM is relatively higher at the beginning, while the fluorescence of Cy5 and FAM shows rapid increase along with time and finally exceeds the fluorescence of FAM (Fig. S12), suggesting the sequentially opening of MBs by extended telomeric repeats.

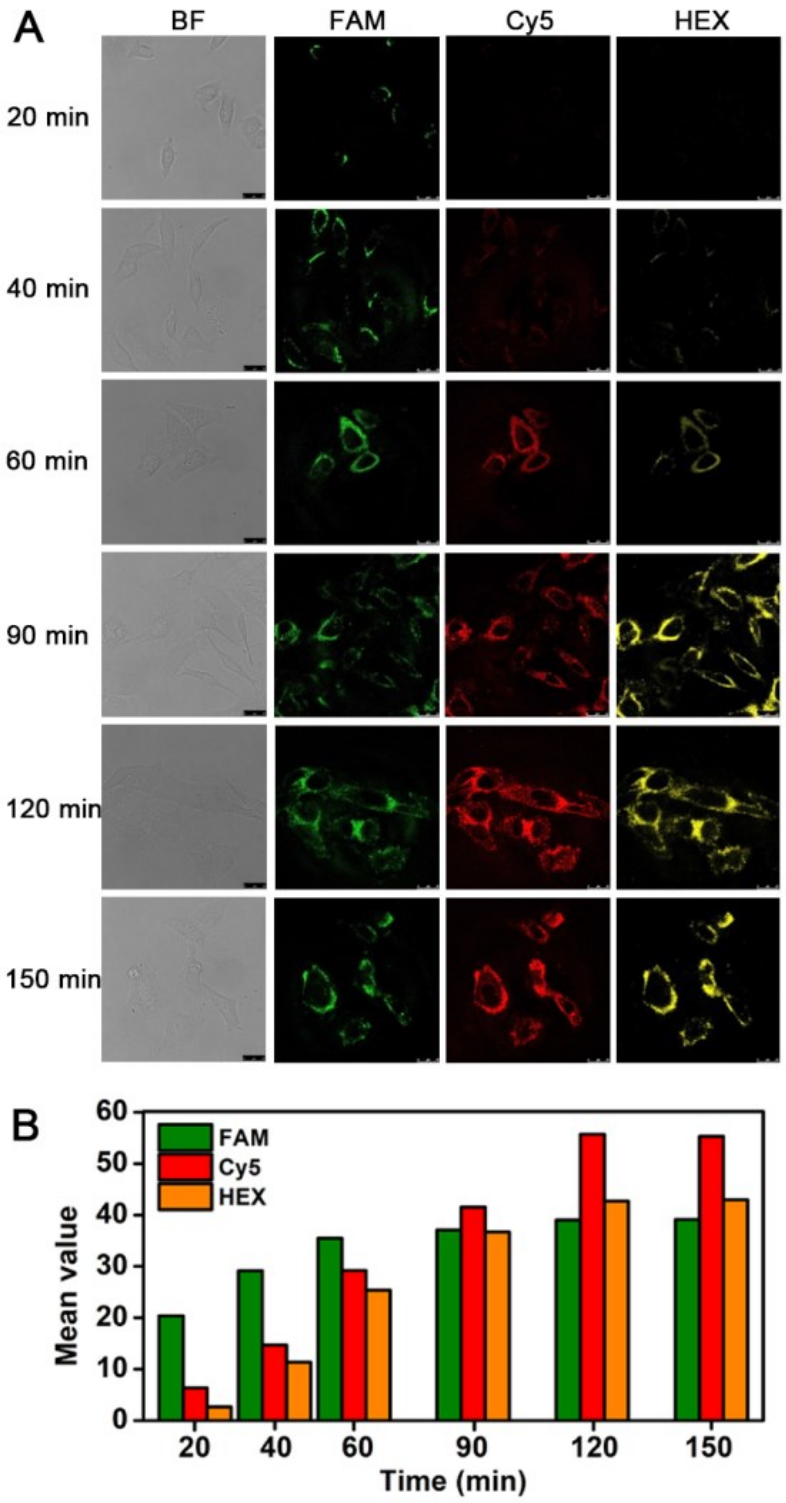

Fig. S13 (A) Time-dependent sequential fluorescence emission for different HeLa cells after the incubation with SLMN. Scale bar: 25 μm. (B) Derived mean value of the fluorescence intensity from confocal fluorescence images.

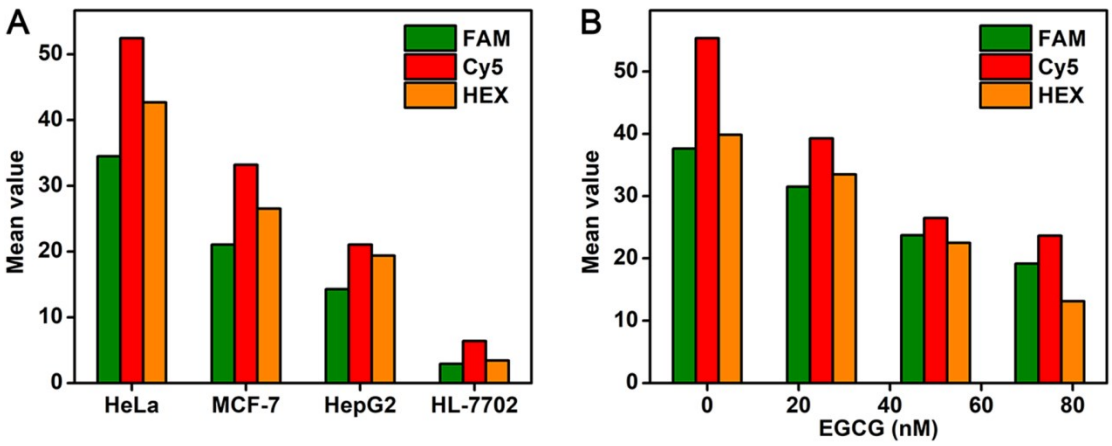

Fig. S14 Mean value of the fluorescence intensity for confocal fluorescence images of telomerase activity in different types of living cells (A) and telomerase variation in HeLa cells treated with different concentrations of EGCG (B).

For confocal fluorescence images of telomerase activity in different types of living cells (Fig. 3A in the main text), the mean values of fluorescence intensity for HeLa, MCF-7, HepG2 and HL-7702 cells indicate that telomerase activity is relatively high in HeLa cells compared with other two tumor cells and SLMN can distinguish the variation of telomerase activity in different cells lines (Fig. S14A). For confocal fluorescence images of telomerase variation in HeLa cells treated with different concentrations of EGCG (Fig. 3B in the main text), the mean values of fluorescence intensity for all types of three channel decrease as the concentration of EGCG increases, suggesting that SLMN can sensitively respond to intracellular telomerase activity change (Fig. S14B).

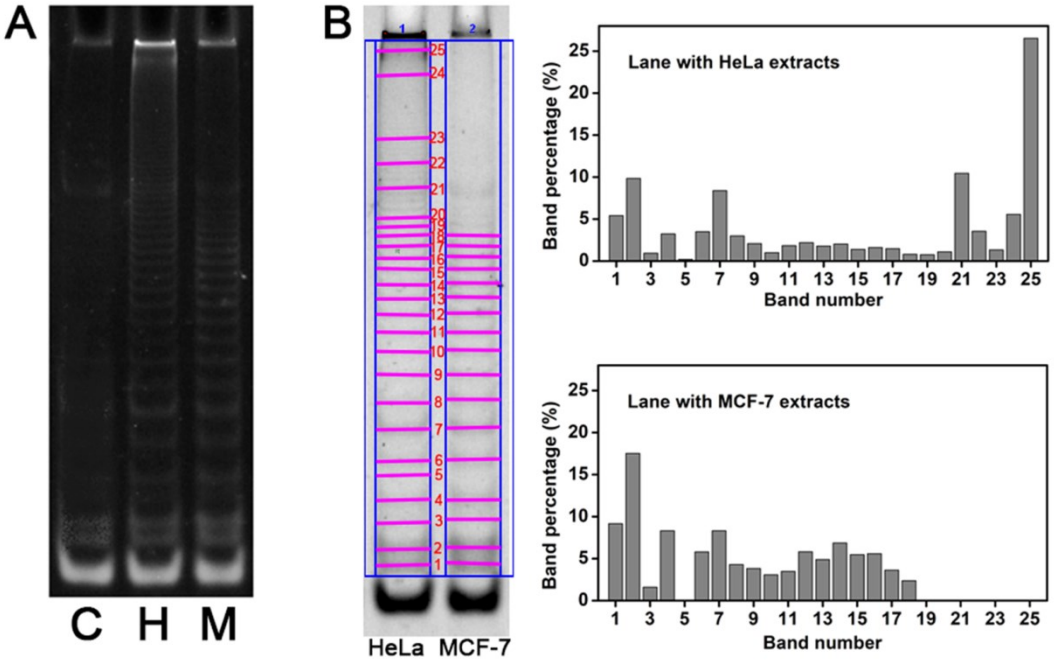

Fig. S15 (A) Gel characterization of telomerase activity by telomeric repeat amplification protocol. lane C: CHAPS lysis buffer, lane H: HeLa cell extracts, lane M: MCF-7 cell extracts. (B) Quantitative analysis of gel result when TS is reacted with telomerase from HeLa extracts or MCF-7 extracts.

For the gel, it can be observed the formation of DNA complex in lane C, the case of lysis buffer. Since TS and CX are used in the negative control of the lysis buffer, the formation of primer dimer complex during PCR is demonstrated as a bright band.[5] Meanwhile, few faint bands that migrate a bit slower can be observed in lane C, and is assigned to PCR-related artifacts that originate from the primer dimer.[5] The gel result in lane H and lane M is quantified by ImageLab software (Bio-Rad Laboratories Inc. USA). First, the gel image is color inverted, and the analysis region is defined. The intense bands with the slowest migration rate are excluded in both lanes for eliminating the influence from possible formation of DNA-protein complex. The bands that are obvious and eligible are then automatically detected and given by the software. It can be seen that 25 bands are detected in the lane of HeLa extracts while 17 bands are detected in the lane of MCF-7 extracts. Specially, when TS is reacted with telomerase from HeLa extracts, it obtains additional bands of products with retarded migration in comparison to MCF-7 extracts. It indicates that longer products are generated under the action of telomerase from HeLa extracts. The band 8 is

selected as a reference band, since it has the similar intensity in both lanes. The volume intensity and band percentage are then be given by the software. For telomerase from HeLa extracts, the longer products that are not detected in MCF-7 extracts (band 19 to band 25), account for 49.3% in the totally elongated products. In comparison, such longer products account for 0% in the totally elongated products for telomerase from MCF-7 extracts. It suggests that telomerase extracted from HeLa cells catalyzes TS to generate longer products, and telomerase in HeLa cells is more processive.

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