Supporting Information

An mRNA-Initiated, Three-Dimensional DNA Amplifier Able to Function Inside Living Cells

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Experimental Section

Chemicals and Materials

All DNA oligonucleotides (Table S1, Supporting Information) were purchased from Sangon Biotech Co., Ltd. (Shanghai, China). The oligonucleotides were purified by high-performance liquid chromatography (HPLC). Tamoxifen was purchased from Sigma-Aldrich Chemical Co. Ltd. (St. Louis, MO). Unless otherwise specified, all other reagents used in this work were of analytical grade, commercially purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China), and used without further treatment. Human hepatocellular liver carcinoma cell line (HepG2) and human hepatocyte cell line (HL7702) were obtained from our lab and cultured in 1640 (GIBCO) medium with 10% fetal calf serum (FBS) and 1% penicillin-streptomycin (PS, 10000 IU penicillin and 10000 μg/mL streptomycin, Multicell).

Instruments

All solutions used in the experiments were prepared using ultrapure water (resistance > 18 MΩ cm), which was obtained through a Millipore Milli-Q ultrapure water system (Billerica, MA, USA). The hydrodynamic size was measured by the Zetasizer Nano (Malvern). All fluorescence measurements were carried out on a Fluoromax-4 (HORIBA Jobin Yvon Inc., Edison, NJ) spectrofluorometer with a temperature controller. The confocal fluorescence imaging studies were performed on an FV1000 confocal laser scanning microscope.

Preparation and Characterization of EDTD

EDTD consists of two major modules: the ET module and the FT module. The ET and FT modules were self-assembled according to previous reports with slight modifications.[1-2] In brief, for the synthesis of ET, six customized single-stranded oligonucleotide strands (P1, P2, P3, P4, P5, and P6) were mixed in equal molar ratio in TM buffer (20 mM Tris, 50 mM $MgCl_2$, pH = 8.0). The stock solutions, which had final concentrations of 2 μM, were heated to 95 °C for 5 min and then cooled to 4 °C

within 1 min and kept in a buffer for future use. Similarly, for the synthesis of FT, four customized single-stranded oligonucleotide strands (P1, P2, P3, and P7) were mixed in equal molar ratio in TM buffer (20 mM Tris, 50 mM $MgCl_2$, pH = 8.0). The stock solutions, which had final concentrations of 2 μM, were heated to 95 °C for 5 min and then cooled to 4 °C within 1 min and kept in a buffer for future use. Successful formation of ET and FT was verified by use of 12.5% N-PAGE and DLS. The gels were run in ice-cold 1 × TBE (tris-borate-EDTA) buffer at a constant voltage of 110 V for 1h. DLS was employed to measure the hydrodynamic size of ET and FT. Briefly, a 50 μL sample (2 μM) was first centrifuged at high speed at 10000 rpm for 30 min at 4 °C before the supernatant (30 μL) was retrieved, and the hydrodynamic diameter in ultrapure water was determined using the Zetasizer Nano (Malvern).

Fluorescence Measurements

All fluorescence measurements were performed using a Fluoromax-4 (HORIBA Jobin Yvon Inc., Edison, NJ) spectrofluorometer with a temperature controller. For detection of targets in the buffer solution, different concentrations of synthetic DNA targets were added to 200 μL of Tris-HCl buffered solution (25 mM Tris with 5 mM $MgCl_2$, pH = 8.0) containing 50 nM EDTD. The fluorescence spectra of the mixture were collected from 500 to 650 nm with 488 nm excitation in a 200 μL quartz cuvette. All experiments were repeated at least three times. For the nuclease stability experiment, we incubated 10% FBS with EDTD and EBeacon, respectively. FAM signal was monitored and recorded over a period of 6 h at 20 min intervals. Likewise, all experiments were repeated at least three times.

Cytotoxicity

The cytotoxicity of EDTD was assessed by the CCK-8 assay. HepG2 cells (1×10^5 cells/well) were cultured in a 96-well microtiter plate and incubated at 37 °C in 5% CO_2 for 24 h to reach 85% confluency, and then cells were treated with varying concentrations of EDTD (0, 50, and 100 nM). HepG2 cells were incubated with EDTD for 6, 12, 24, and 48 h. Subsequently, the cells were washed with DPBS buffer

three times. Then 10 % (v/v) of CCK-8 cells were added to each well with incubation at 37 °C in 5% CO_2 for 0.5 h. Next, the cytotoxic effects by CCK-8 were determined by recording the OD450 nm using a multimode microplate.

qRT-PCR

Total cellular RNA was extracted from HepG2 cells or HL7702 cells using Trizol reagent S5 (Sangon Co. Ltd., Shanghai, China) according to the indicated protocol. The cDNA samples were prepared by using the reverse transcription (RT) reaction with AMV First Strand cDNA Synthesis Kit (BBI, Toronto, Canada). The qRT-PCR analysis of mRNA was performed with SG Fast qPCR Master Mix (2X) (BBI) on a LightCycler480 Software Setup (Roche). The primers (from 5' to 3') used in this experiment are listed below. We evaluated all the data with respect to the mRNA expression by normalizing to the expression of GAPDH and using the $2^{-\Delta\Delta Ct}$ method.

TK1 forward: CTCCTACCCACTGGTCTGCTTA

TK1 reverse: CAGGGAGAACAGAAACTCAGCA

GAPDH forward: TGGGTGTGAACCATGAGAAGT

GAPDH reverse: TGAGTCCTTCCACGATACCAA

Confocal Fluorescence Imaging

HepG2 and HL 7702 cell lines were grown in 1640 (GIBCO) medium with 10% FBS and 1% penicillin-streptomycin with 5% CO_2 at 37 °C. All cells were plated on a 35-mm confocal laser culture dish for 24 h. Then EDTD (final concentration of 100 nM) was respectively delivered into HepG2 cells and HL 7702 cells in 1640 medium at 37 °C for the appropriate time. After washing with DPBS three times, confocal fluorescence imaging studies were performed on the FV1000 confocal laser scanning microscope. In addition, in the experiments for expression levels of TK1 mRNA, two groups of HepG2 cells were treated with tamoxifen (10^{-6} mol/L) for 24 h and then incubated with EDTD and EBeacon, respectively. Other steps were performed as described above.

Table S1. Sequences of oligonucleotides used in this work.

Oligo	Sequence (5'—3')
P1	ACATTCCTAAGTCTGAAACATTACAGCTTGCTACACGAGAAGAGCCGCCATAGTA
P2	TATCACCAGGCAGTTGACAGTGTAGCAAGCTGTAATAGATGCGAGGGTCCAATAC
P3	TTCAGACTTAGGAATGTGCTTCCCACGTAGTGTCGTTTGTATTGGACCCTCGCAT
P4	TCAACTGCCTGGTGATAAAACGACACTACGTGGGAATCTACTATGGCGGCTCTTCT TTTTTTTTTTTTTTTCCCTCAAGTATGCCAAAGAC
P5	TGGAGACGTAGGGTATTGAATGAGGGCCGTAAGTTAGTTGGAGACGTAGG-Dabcyl
P6	FAM-CCTACGTCTCCAACTAACTTACGG
P7	TCAACTGCCTGGTGATAAAACGACACTACGTGGGAATCTACTATGGCGGCTCTTCT TTTTCCTACGTCTCCAACTAACTTACGGCCCTCAAGTATGCCAAAGAC
P8	TTTTTTTTTTTTTTTTCCCTCAAGTATGCCAAAGAC
P9	CCTACGTCTCCAACTAACTTACGGCCCTCAAGTATGCCAAAGAC
P10	TGGAGACGTAGGGTATTGAATGAGGGCCGTAAGTTAGTTGGAGACGTAGG
P11	Alexa Fluor 488-CCTACGTCTCCAACTAACTTACGG
Target	CAAGTATGCCAAAGACACTCGC
mA	CAAGTAAGCCAAAGACACTCGC
mG	CAAGTAGGCCAAAGACACTCGC
mC	CAAGTACGCCAAAGACACTCGC
iA	CAAGTAATGCCAAAGACACTCGC
iG	CAAGTAGTGCCAAAGACACTCGC
iC	CAAGTACTGCCAAAGACACTCGC
iT	CAAGTATTGCCAAAGACACTCGC
dT	CAAGTAGCCAAAGACACTCGC
a1	AGGCAGTTGAGACGAACATTCCTAAGTCTGAAATTTATCACCCGCCATAGTAGAC GTATCACC
a2	CTTGCTACACGATTCAGACTTAGGAATGTTCGACATGCGAGGGTCCAATACCGAC GATTACAG
a3	GGTGATAAAACGTGTAGCAAGCTGTAATCGACTCTAGCGAGTGTCTTTGGCATACT TCTCGCGGCTCACTACTATGGCG
a4	FAM-TAGAGACGGTATTGGACCCTCGCATGACTCAACTGCCTGGTGATACGAGAGC C-Dabcyl

DNA Tetrahedron-Based Molecular Beacons (DTMB) were self-assembled by a1, a2, a3 and a4.

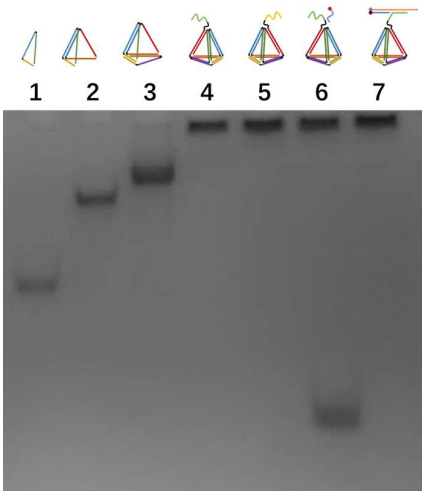

Figure S1. Analysis by 12.5% native PAGE. Lane 1 is strand P1; Lane 2 is strand P1 + strand P2; Lane 3 is strand P1 + strand P2 + strand P3; Lane 4 is strand P1 + strand P2 + strand P3 + strand P4; Lane 5 is strand P1 + strand P2 + strand P3 + strand P7; Lane 6 is strand P1 + strand P2 + strand P3 + strand P4 + strand P6; Lane 7 is strand P1 + strand P2 + strand P3 + strand P4 +strand P5 + strand P6.

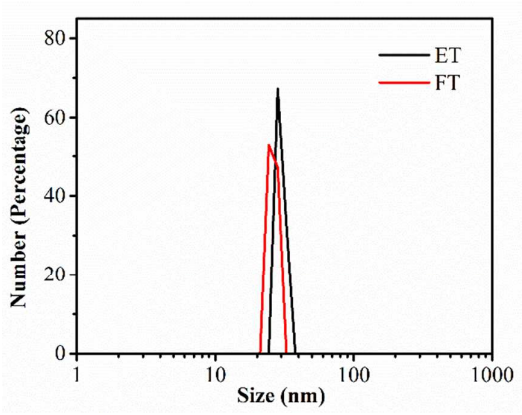

Figure S2. DLS measurement revealed that the mean hydrodynamic diameter of ET was above 29.7 nm and that of FT was above 26.2 nm.

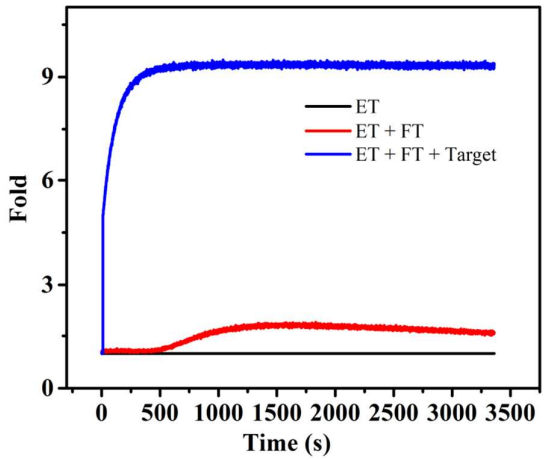

Figure S3. Catalytic feasibility of EDTD, as determined by fluorescence kinetics monitoring. The system showed remarkable response toward the target, while leakage remained insignificant.

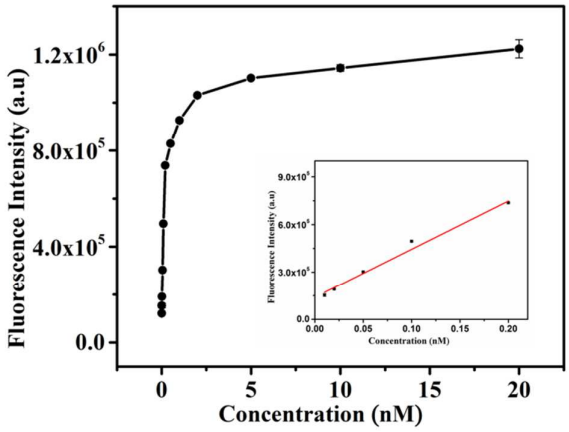

Figure S4. The relationship between fluorescence intensity and target concentration. The inset shows the responses of the sensing system to targets at low concentration.

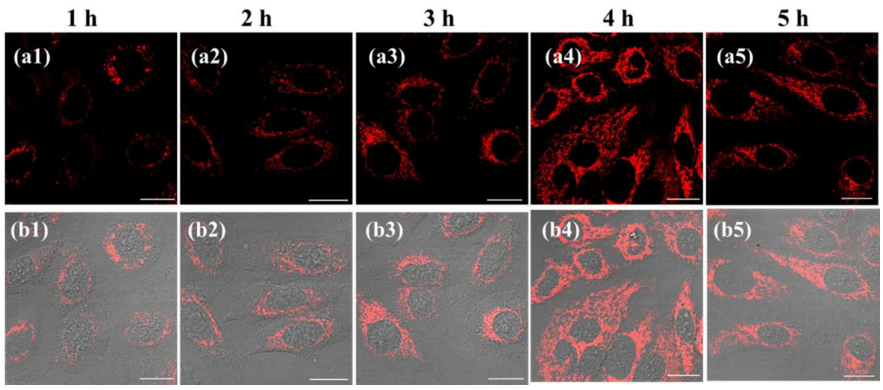

Figure S5. Optimization of incubation time for EDTD in living cells. HepG2 cells were incubated with EDTD for different time points at 37 ° C for confocal microscopy. (a) Fluorescence; (b) merged with DIC. Scale bars are 20 μm.

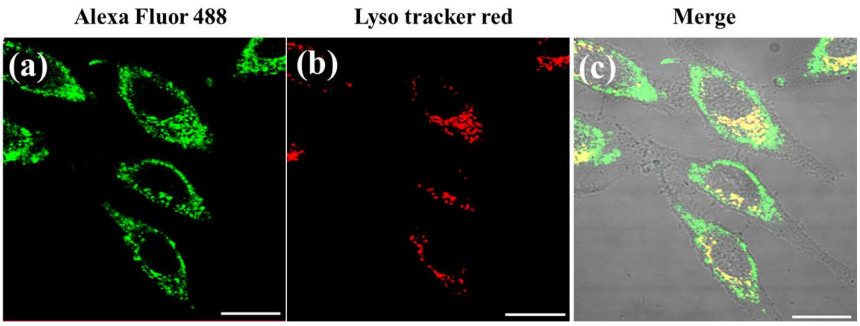

Figure S6. Fluorescence co-localization imaging for determining the distribution of ET in HepG2 cells. The green fluorescence shows the position of ET. The red fluorescence indicates the lysosome. Scar bars are 20 μm. This result shows that the DNA nanostructure can efficiently escape from lysosome.

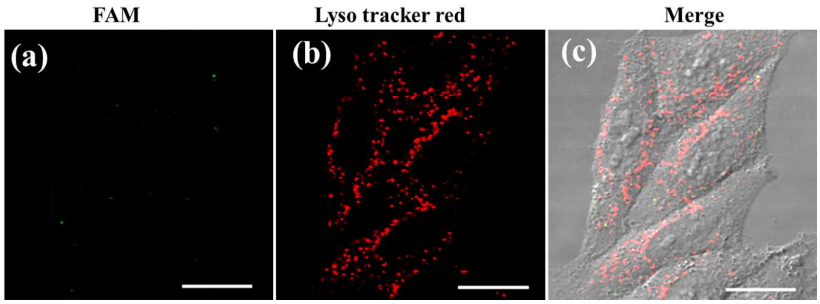

Figure S7. Fluorescence co-localization imaging to show the influence of endosomal acidity on the fluorescence of ET. The green color indicates the fluorescence of FAM and the red color indicates the fluorescence of Lyso tracker red. Scar bars are 20 μm. This result suggests that endosomal acidity shows little influence on the fluorescence of FAM due to the efficient quenching of FAM fluorescence by Dabcyl on ET.

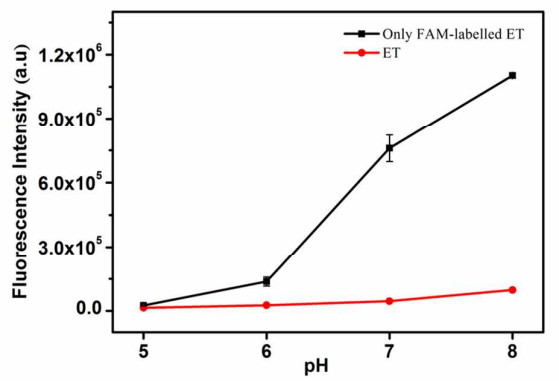

Figure S8. The effect of pH on the fluorescence of ET (red curve) and only FAM-labeled ET (black curve). The fluorescence date showed that pH variation causes insignificant interference to FAM fluorescence of the ET due to the effective quenching of FAM by Dabcyl.

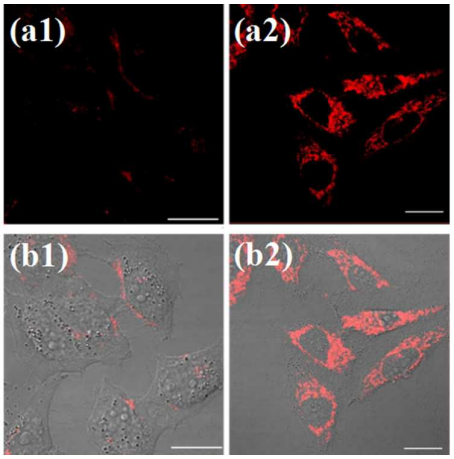

Figure S9. (A) Fluorescence image of TK1 mRNA in HL7702 cells (1) and HepG2 cells (2) by EDTD. (a) Fluorescence; (b) merged with DIC. Scale bars are 20 μm.

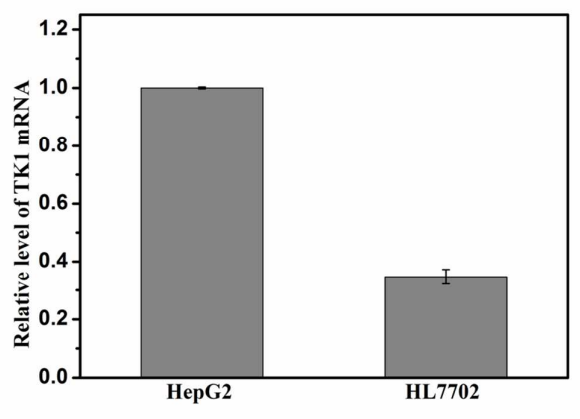

Figure S10. The relative expression levels of TK1 mRNA in HepG2 and HL7702 cell lines.

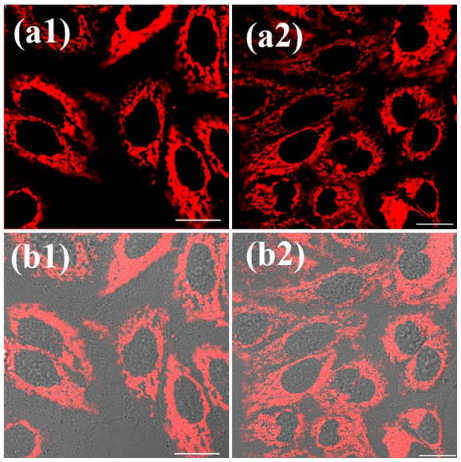

Figure S11. Fluorescence imaging for measuring the cellular uptake of ET in untreated HepG2 cells (a1 and b1) and tamoxifen-treated HepG2 cells (a2 and b2). Scale bars are 20 μm.

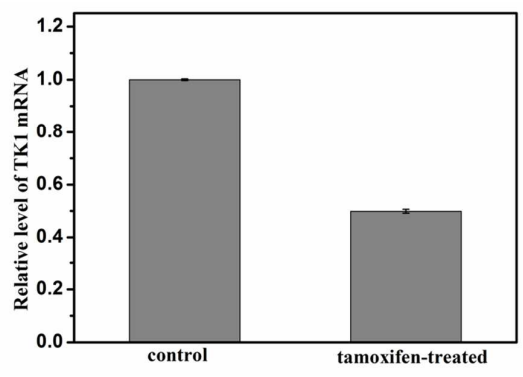

Figure S12. The relative expression levels of TK1 mRNA in untreated HepG2 cells (control) and tamoxifen-treated HepG2 cells.

Supplementary References

(1) He, L.; Lu, D. Q.; Liang, H.; Xie, S.; Luo, C.; Hu, M.; Xu, L.; Zhang, X.; Tan, W. *ACS Nano* **2017**. *11*, 4060-4066

(2) Liang, L.; Li, J.; Li, Q.; Huang, Q.; Shi, J.; Yan, H.; Fan, C. *Angew. Chem., Int. Ed*. **2014**, *53*, 7745-7750.