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Supporting Information For

Crosslinking Catalytic Hairpin Assembly for High Contrast Imaging of Multiple mRNAs in Live Cells

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Materials. Tamoxifen and survivin expression repressor YM155 were purchased from Sigma-Aldrich (St. Louis, USA). Streptavidin (SA) from Streptomyces avidinii was purchased from Sangon Biotechnology Co., Ltd. (Shanghai, China). HeLa cells (human cervical carcinoma cell line) were obtained from the cell bank of Central Laboratory at Xiangya Hospital (Changsha, China). MCF-7 (human breast adenocarcinoma cell line) and MCF-10A cells (immortalized nontumorigenic human mammary epithelial cell line) were purchased from Cell Bank of the Committee on Type Culture Collection of Chinese Academy of Sciences (Beijing, China). Cell culture media was obtained from Thermo Scientific HyClone (MA, USA). Lipofectamine 3000 and Opti-MEM were purchased from Invitrogen (MA, USA). Oligonucleotide used in this study were synthesized and purified through HPLC by Sangon Biotechnology Co., Ltd. (Shanghai, China).

DNA sequences used in this work

1. CCHA design for survivn mRNA:

H1: Biotin-TTTTTTTTTTGAGATGCGGT(Cy3)GGTCCTTGAGAAGCCATGTGTAGA TTCTCAAGGACCACC-BHQ2

H2: Biotin-TTTTTTTTTTCTTGAGAATCTACACATGGCTTCTCAAGGACCAC GCCATGTGTAGA

Survivin mRNA target region: TTCTCAAGGACCACCGCATCTC

2. CCHA design for TK1 mRNA:

H1: Biotin-TTTTTTTTTT GCGAGT GT (Cy5)CTT TGG CAT ACT TG CTGTGTGTAGTC CAAGTATGCCAAAGAC-BHQ2

H2: Biotin-TTTTTTTTTTCTTGAGAATCTACACATGGCTTCTCAAGGACCAC GCCATGTGTAGA

TK1 mRNA target region: AGTATGCCAAAGACACTCGCTAC

3 Selectivity assay of CCHA

survivin single-base mismatch target: TTCTCAAGGACCACCGCAGCTC

survivin two-bases mismatch target: TTCTCAATGACCACCGCAGCTC

TK1 single-base mismatch target: AGTATGCCAAAGACACTCTCTAC

TK1 two-bases mismatch target: AGTATGCTAAAGACACTCTCTAC

Instruments. Agarose gel was visualized via a Tocan 240 gel imaging system (Tanon Science & Technology Co. Ltd., China). The atomic force microscopy (AFM) image was performed by means of Bruker Bioscope system (Bruker, USA). The fluorescence measurements were carried out at room temperature in a quartz cuvette on an FL-7000 spectrometer (Hitachi, Japan). For the Real-time fluorescence intensity study, a Tecan Infinite M-1000 microplate reader was used. All fluorescence images were acquired using an oil dipping objective (60×, 1.4 NA) on Nikon TI-E+A1 SI confocal laser scanning microscope (Japan). The 540 nm laser and 640 nm laser were respectively used as excitation source of Cy3 and Cy5, and emission was collected at two separate channels, green (570-620 nm) and red (665-735 nm). Flow cytometric analysis of cells was performed on a CytoFLEX™ flow cytometer (Beckman Counter, Inc., USA).

Gel electrophoresis analysis. All the reactions were performed in 20 μL aliquot of 1x PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM phosphate, pH 7.4) incubating at 37 °C for 2 h. These products were also analyzed using 3% agarose gel electrophoresis in TBE buffer at room temperature. The gel was stained using 0.5 μg/mL GoldView and 0.5 μg/mL ethidium bromide (EB). Electrophoresis was performed at a constant voltage of 110 V for 1.5 h with a load of 10 μL of sample in each lane.

AFM imaging. The samples were diluted 10 times. 10μL diluted sample were deposited onto freshly cleaved mica (Ted Pella, Inc., USA) and left to absorb for 5 min. Samples were then gently rinsed with deionized water 5 times and dried at room temperature. AFM imaging experiments were performed in air under ScanAsyst mode. The image background was flattened by NanoScope Analysis software.

Fluorescence assay In Vitro. To prepare DNA hairpins for DNA nanotetrads, the all hairpins were dissolved in 1x PBS and then were kept at 95 °C for 5 min, then quickly quenched on ice for 5 min, finally transferred to room temperature for 60 min before further use. Subsequently, appropriate SA was added to form DNA nanotetrads. 100 nM DNA nanotetrads were incubated with RNA target of a varied concentration in 50 μL 1x PBS buffer at 37 °C for 2 h. All fluorescence measurements were performed on

a FL-7000 spectrometer, using a quartz fluorescence cell with an optical path length of 1.0 cm. The excitation wavelength of Cy3 was 540 nm with a recording emission range from 555 to 680 nm and the excitation wavelength of Cy5 was 630 nm with a recording emission range from 650 to 720nm. All the excitation and emission slits were set at 10 nm.

For the real-time fluorescence intensity study, the fluorescence responses of each sample with 100 μL volume were performed with a time interval of 2 min in a 384-well black microplate on a Tecan Infinite M-1000 microplate reader. The excitation wavelength of Cy3 was fixed at 540 nm and the emission wavelength was 562 nm with both excitation and emission bandwidths of 10 nm. Cy5 fluorescence was recorded with excitation wavelength of 640 nm and emission wavelength of 663 nm.

Cell Culture. MCF-7 cells and HeLa cells were cultured in DMEM medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 U/mL streptomycin. MCF-10A cells were grown in Dulbecco's-modified Eagles medium (DMEM) containing 100 ng mL-1 cholera toxin supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 U/mL streptomycin. All cell lines were maintained at 37 °C in a 100% humidified atmosphere containing 5% CO_2.

Fluorescence imaging of cells was performed as follows: In a comparative experiment of cancer cells and normal cells, all Cells were plated on a 35 mm Petri dish with 10 mm well in 2 mL culture medium at 37 °C for 24 h, then incubated with 1 mL culture medium containing 100 nM nanotetrads survivin and nanotetrads TK1 at 37 °C for 2 h. For experiment of conventional CHA, 250 μL of Opti-MEM supplemented with 0.75 μL lipofectamine 3000 are mixed with 250 μL Opti-MEM containing 200 nM H1, 200 nM H2 for 5 min, then cells were incubated with this mixture supplied with 55 μL fetal bovine serum at 37 °C for 2 h. After washing three times with 1x PBS, the cells were incubated with 1 mL fresh medium at 37 °C before imaging.

In the experiments for the expression levels of tumor mRNA, MCF-7 cells was pretreated with tamoxifen to down regulate the expression of TK1 mRNA and YM155 to down regulate the expression of survivin mRNA for 48 h. Then the cells were washed and incubated 500 μL culture medium containing 100 nM DNA nanotetrads survivin

and nanotetrads TK1 at 37 °C for 2 h. Cells were washed by 1x PBS three times before imaging.

Flow cytometry assay. In CCHA system, MCF-7 cells and MCF-10A cells were incubated with 1 mL culture medium containing 100 nM nanotetrads survivin and 100 nM nanotetrads TK1 at 37 °C for 2 h. In CHA system, 200 nM H1 and 200 nM H2 were transfected using lipofectamine 3000 for 2 h. And then washed to remove residual probes. After treatment with trypsin, cells were suspended in 1x PBS buffer and analyzed by a CytoFLEX™ flow cytometer.

Table S1. Comparison of detection limit for intracellular mRNA detection.

Method	Target	Detection limit	Ref

FRET Based DNA Tetrahedron Nanotweezer	mRNA	330 pM	S1
Entropy-driven DNA Nanoamplifier	mRNA	3.3 pM	S2
Hybridization Chain Reaction Based Amplification	mRNA	10.9 pM	S3
Catalytic Hairpin Assembly based Amplification	mRNA	9 pM	S4
Transformable Tetrahedral DNA Probe	mRNA	30 pM	S5
Crosslinking Catalytic Hairpin Assembly	mRNA	0.8/0.9 pM	This work

Reference

1. He, L., Lu, D. Q., Liang, H., Xie, S., Luo, C., Hu, M1., Xu, L., Zhang, X., Tan, W. ACS Nano, 2017, 11, 4060−4066.
2. He, L., Lu, D., Liang, H., Xie, S., Zhang, X., Liu, Q., Yuan, Q., Tan, W. J. Am. Chem. Soc., 2018, 140, 258-263.
3. Ren, K., Xu, Y., Liu, Y., Yang, M., Ju, H. ACS Nano, 2018, 12, 263−271.
4. Li, J. J., Li, W. N., Du, W. F., Lv, M. M., Wu, Z. K., Jiang, J. H. Chem. Commun., 2018, 54, 10626-10629.
5. Wan, Y., Zhu, N., Lu, Y., Wong, P. K. Anal. Chem., 2019, 91, 2626−2633.

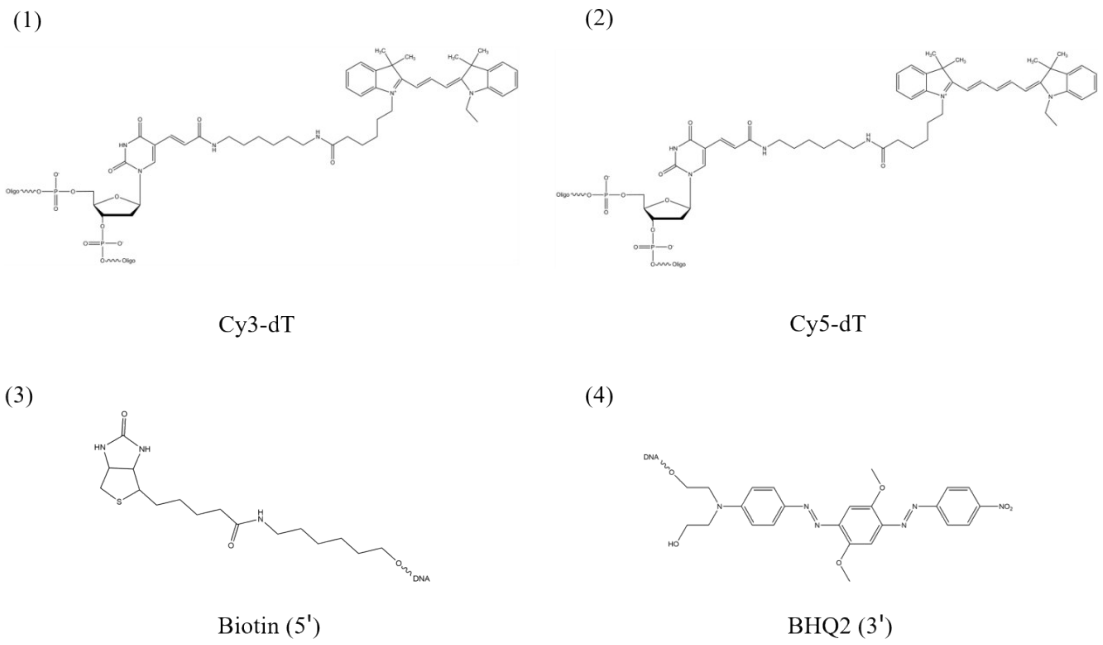

Fig. S1 Chemical Structures of (1) Cy3-dT; (2) Cy5-dT; (3) 5'-Biotin; (4) BHQ2-3'.

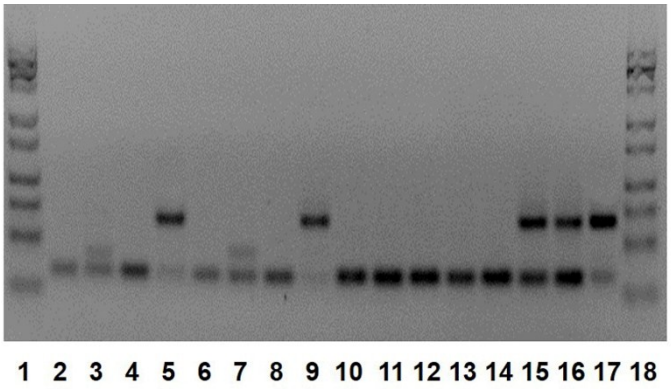

Fig. S2 Agarose gel image of CHA assay. Lane 1 and Lane 18：DNA ladder; Lane 2: survivin H1; Lane 3: survivin H1 + survivin mRNA; Lane 4: survivin H1 + survivin H2; Lane 5: survivin H1 + survivin H2 + survivin mRNA; Lane 6: TK1 H1; Lane 7: TK1 H1 + TK1 mRNA; Lane 8: TK1 H1 + TK1 H2; Lane 9: TK1 H1 + TK1 H2 + TK1 mRNA; Lane 10: survivin H1 + survivin H2 + TK1 H1; Lane 11: survivin H1 + survivin H2 + TK1 H2; Lane 12: survivin H1 + TK1 H1 + TK1 H2; Lane 13: survivin H2 + TK1 H1 + TK1 H2; Lane 14: survivin H1 + survivin H2 + TK1 H1 + TK1 H2 ; Lane 15: survivin H1+ survivin H2 + TK1 H1 + TK1 H2 + survivin mRNA ; Lane 16: survivin H1+ survivin H2 + TK1 H1 + TK1 H2 + TK1 mRNA ; Lane 17: survivin H1+ survivin H2 + TK1 H1 + TK1 H2 + survivin mRNA + TK1 mRNA.

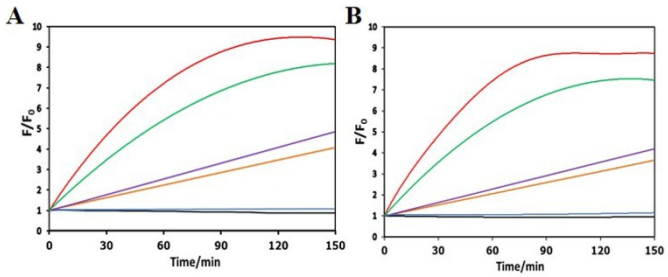

Fig. S3 (A-B) Real-time fluorescence responses of CCHA and CHA to survivin (A) and TK1 (B). From bottom to up, the sample of 200 nM H1 and 200 nM H2 (black), 100 nM nanotetrads (blue), 200 nM H1 and 200 nM H2 with 5 nM target mRNA (orange), 100 nM nanotetrads with 5 nM target mRNA (purple), 200 nM H1 and 200 nM H2 with 50 nM target mRNA (green), 100 nM nanotetrads with 50 nM target mRNA (red), respectively.

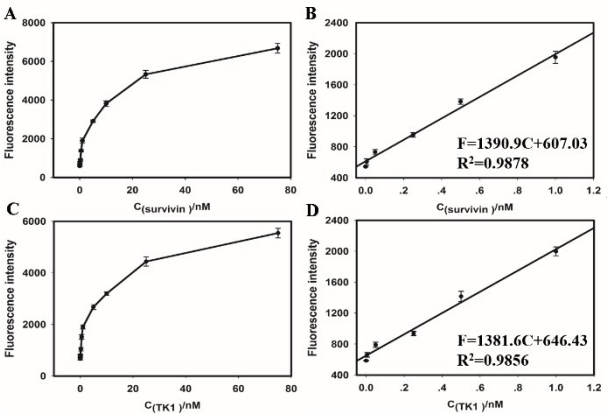

Fig. S4 Calibration curves of fluorescence intensities versus targets concentrations (A) survivin and (B) TK1. The linear relationship between fluorescence intensities and target concentrations in the linear range (C) surviving and (D) TK1. The inset tables are the fitting equations and correlation coefficients.

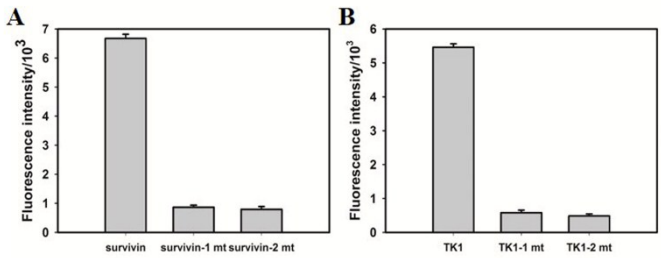

Fig. S5 Specificity of CCHA over full complementary, single-base mismatch and two-bases mismatch of target mRNA.

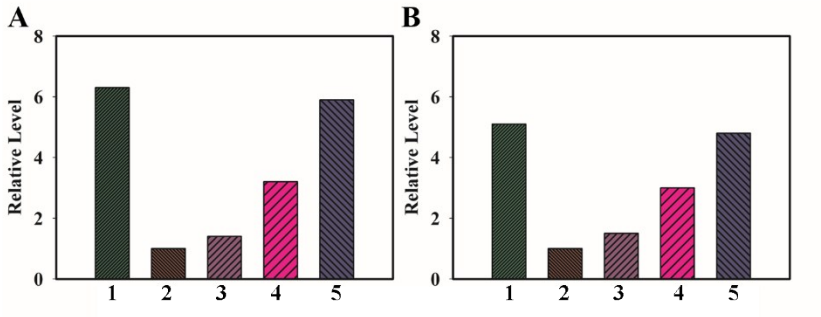

Fig. S6 Mean fluorescence intensity measured from confocal images in Fig. 3, A: survivin mRNA; B: TK1 mRNA. (1) MCF-7 cells incubated with nanotetrads survivin and nanotetrads TK1; (2) MCF-10A cells incubated with nanotetrads survivin and nanotetrads TK1; (3) MCF-7 cells pretreated with YM155 and tamoxifen followed by incubation with nanotetrads survivin and nanotetrads TK1. (4) MCF-7 cells incubated with nanotetrads survivin-H1 and nanotetrads TK1-H1; (5) MCF-7 cells transfected with all H1 and H2 probes in auxiliary of Lipofectamine 3000.

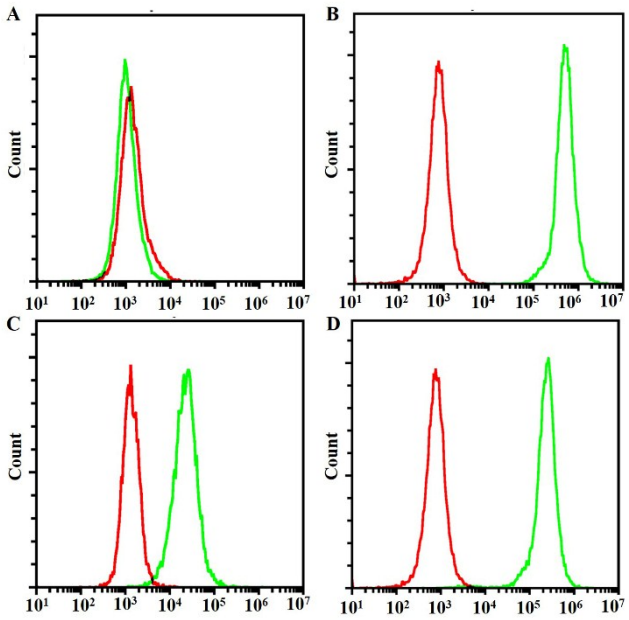

Fig. S7 Flow cytometric assay of CCHA for TK1 mRNA assay. The red curve is obtained for cells incubated without probes as control. The green curve is the response for the cells of interest. (a) MCF-10A cells incubated with nanotetrads TK1, (b) MCF-7 cells incubated with nanotetrads TK1, (c) MCF-7 cells incubated with nanotetrads TK1-H1, (d) MCF-7 cells transfected with TK1 H1 and TK1 H2 using Lipofectamine 3000.

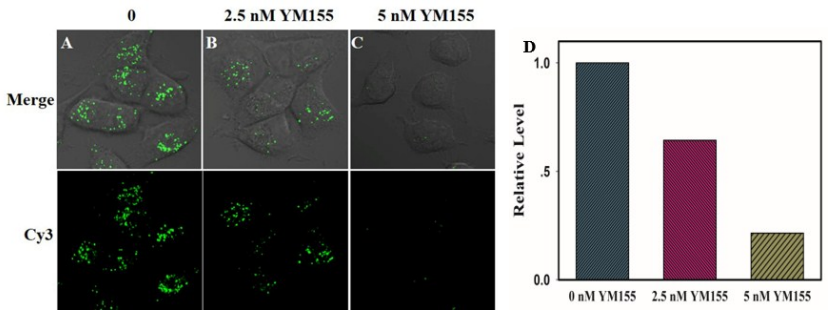

Fig. S8 Fluorescence images for MCF-7 cells pretreated with (A) 0 nM, (B) 2.5 nM, (C) 5 nM survivin expression repressor YM155 followed by incubation with nanotetrads survivin, (D) Mean fluorescence intensity measured from the confocal images.

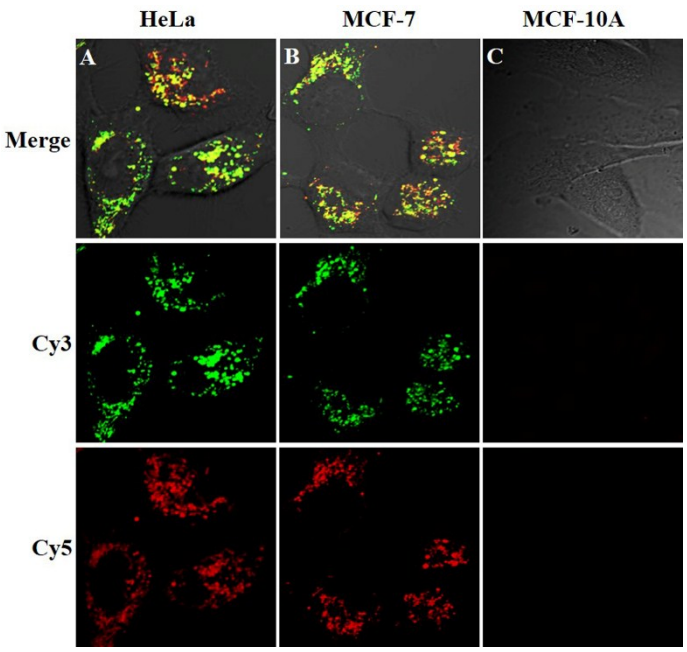

Fig. S9 Fluorescence imaging for (A) HeLa cells, (B) MCF-7 cells and (C) MCF-10A cells incubated with nanotetrads survivin and nanotetrads TK1.

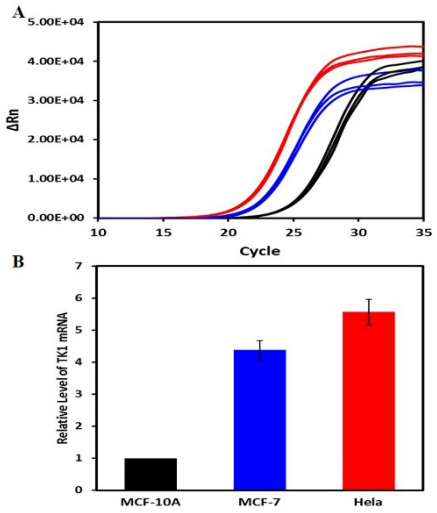

Fig. S10 Expression analysis of survivin mRNA in MCF-10A (black), MCF-7 (blue) and Hela (red) cells. (A) Real-time fluorescence curves in qRT-PCR analysis, (B) Relative expression levels for survivin mRNA.

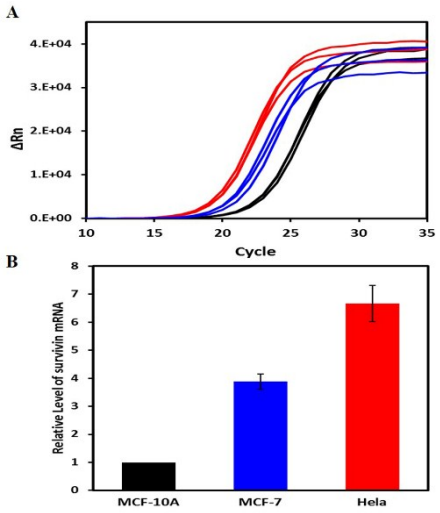

Fig. S11 Expression analysis of TK1 mRNA in MCF-10A (black), MCF-7 (blue) and Hela (red) cells. (A) Real-time fluorescence curves in qRT-PCR analysis, (B) Relative expression levels for TK1 mRNA.