Supporting Information

A Non-enzymatic Hairpin DNA Cascade Reaction Provides High Signal Gain of mRNA Imaging inside Live Cells

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Experimental Section

DNA Synthesis and Purification

All oligonucleotides were synthesized based on solid-phase phosphoramidite chemistry at a 1 μmol scale using the ABI3400 DNA/RNA synthesizer (Applied Biosystems, Foster City, CA). The specific sequences are presented in Table S1. The synthesis and deprotection procedures were performed using the instructions provided by the reagents' manufacturers. Subsequently, the deprotected DNA was precipitated by adding 1/10 volume of 3 M NaCl and 2.5-times volume of cold ethanol. After placing in a freezer at -20 °C for 30 min, the DNA products were collected by centrifugation at 4,000 rpm for 30 min. The DNAs were then dissolved in 400 μL of 0.1 M triethylamine/acetate (TEAA, Glen Research Corp) and purified by a ProStar HPLC (Varian, Walnut Creek, CA) with a C18 column (Econosil, 5 μm, 250 × 4.6 mm, Alltech, Deerfield, IL) using acetonitrile and 0.1 M TEAA aqueous solution as the mobile phases. Finally, the collected sequences were vacuum-dried and quantified at 260 nm using a Cary Bio-300 UV spectrometer (Varian, Walnut Creek, CA).

Hairpin DNA pairs were dissolved at 100 μM in 200 μL of Tris-acetate/EDTA/Mg^{2+} (TAE/Mg^{2+}) buffer (40 mM Tris-acetate, 1 mM ethylenediaminetetraacetic acid (EDTA), and 12.5 mM magnesium acetate, pH 8.0) and annealed over a temperature gradient from 95 to 25 °C in 2 h. They were then purified with a 1 mm 12% native polyacrylamide gel electrophoresis (PAGE, 120 V, 1 h), followed by staining with Stains-All (Sigma-Aldrich, St. Louis, MO). The sharp bands were excised from the gels, chopped into small pieces and soaked in TAE/Mg^{2+} buffer for 24 h. The extracted solution was concentrated using centrifugal filter devices

(Millipore, Billerica, MA). Finally, the purified hairpin DNAs were quantified by UV absorption and kept in buffer for future usage.

Fluorescence Assays in Buffer

All fluorescence measurements were performed using a Fluorolog spectrometer (Jobin Yvon Horiba) with a 100 µL microcuvette. A Reporter stock solution (10 µM) was prepared by annealing 10 µM Reporter-FAM and 15 µM Reporter-Dabcyl in TAE/Mg^{2+} buffer. A mixture of 100 nM H_1, 400 nM H_2, and 150 nM Reporter was prepared, followed by addition of different amounts of DNA target. The fluorescence excitation and emission were monitored at 488nm and 518 nm at 37 °C, respectively. For comparison of the amplification effect, an assay of two other common molecular probes, linear duplex DNA probe and molecular beacon, was performed under the same conditions.

Cell Culture and Transfection

Human breast cancer cells (MDA-MB-231, ATCC) were grown in relevant media (Leibovitz's L-15 medium) containing 10% FBS (fetal bovine serum, Invitrogen, Carlsbad, CA) and 0.5% mg/mL penicillin-streptomycin (Sigma, St. Louis, MO) at 37 °C in 5% CO_2/air. Cells were plated in 35 mm glass bottomed culture dishes (MatTek Corp., Ashland, MA) and grown to 80% confluency for 48 h before transfection with DNA probes.

Transfection assays were performed according to the manufacturer's protocol. Briefly, transfection was carried out using 3 µL lipofectamine 3000 in 300 µL of Opti-Mem (Invitrogen) at 37 °C for 2~4 h. The hairpin DNA cascade amplifier was transfected using 200 nM H_1, 800 nM H_2, 300 nM Reporter, or linear hybridization probe at 300 nM. Subsequently, the Opti-Mem

transfection mixtures were removed from the cells and replaced with Leibovitz's L-15 medium for imaging.

Confocal Fluorescence Microscopy Imaging

All cellular fluorescent images were collected with a confocal microscope setup consisting of an Olympus IX-81 inverted microscope with an Olympus FluoView 500 confocal scanning system. Hairpin DNA cascade amplifier and linear hybridization probe with Cy3 dye were excited at 543 nm, and the fluorescence was collected at 570 nm. MDA-MB-231 cells were transfected and incubated with DNA cascade amplifier and linear hybridization probe for 2~4 h, followed by washing twice with phosphate buffered saline (PBS) to remove untransfected probes. To avoid unnecessary dye photobleaching and any damage to the cells, the microscope shutter was opened only long enough to allow the laser to illuminate the bound cells while a fluorescence image was collected. MDA-MB-231 cells were treated with 100 μM cordycepin, followed by transfection and incubation with DNA cascade probes or linear duplex probe as described above for 2~4 h.

Flow Cytometric Assay

MDA-MB-231 cells were transfected with hairpin DNA cascade amplifier (200 nM H_1, 800 nM H_2, and 300 nM reporter) or linear hybridization probe (300 nM) for 2~4 h, respectively, and then washed to remove untransfected probes. After treatment with trypsin, cells (10,000) were suspended in PBS buffer and analyzed by a FACScan cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA). The cells transfected with LHP and HDCA were gated according to the parameters of forward scatter (FSC) and side scatter (SSC). The debris,

and dead cells were eliminated and only single cell events were retained for the analysis (86.3% for LHP treated cells and 84.0% for HDCA treated cells, respectively).

Cell Lysate Preparation

MDA-MB-231 cells were plated in a 35 mm cell culture dish (Corning Incorporated, Corning, NY, USA) and grown to about 80% confluency before the experiments. Cells were washed twice with 1 mL PBS, and 0.5 mL of Tris-HCl buffer with proteinase inhibitor was then added to the cell culture dish. Finally, cells were scraped from the cell culture dish and ruptured by mechanical shearing using a Dounce homogenizer.

1. Table of DNA sequences

Name	Detailed sequence information
Target DNA	AAT CAA CTG GGA GAA TGT AAC TG
Hairpin H_1 (1-2-3-4-3*-2*-5*-6*)	CAGTTACA_TTCTCCC_AGTTGATT_CCATGTGTAGA_AATCAACT_GGGAGAA_GTCGCATA_TAGGAACC
Hairpin H_2 (3-4*-3*-2*-4)	AGTTGATT_TCTACACATGG_AATCAACT_GGGAGAA_CCATGTGTAGA
Reporter-F (Cy3 or FAM) (6-5-2)	FAM (or Cy3)-G***G***T***T***CC***T***A_***T***ATGCGAC_TTCTCCC
Reporter-Q (BHQ_2 or Dabcyl) (5*-6*)	GTCGCATA TAGGAACC-Dabcyl (or BHQ_2)
Control Hairpin H_1 (1-2-3-4-3*-2*-5*-6*)	TCCCGCC_TGTGACA_TGCATT_CCATGTGTAGA_AATGCA_TGTCACA_CCTTGTCA_TAGAGCAC
Control Hairpin H_2 (3-4*-3*-2*-4)	TGCATT_TCTACACATGG_AATGCA_TGTCACA_CCATGTGTAGA
Control Reporter-F (6-5-2)	Cy3-G***T***GC***T***C***T***A_T***G***ACAAGG_TGTGACA
Control Reporter-Q (5*-6*)	CCTTGTCA_TAGAGCAC-BHQ_2
MnSOD MB	FAM-GC TAG CCA GTT ACA TTC TCC CAG TTG ATT GCT AGC-Dabcyl
Linear hybridization probe-F (FAM or Cy3) (a-b-c)	FAM (or Cy3)-CA***G***T***T***ACA_***T***TC***T***CCC_AGTTGATT
Linear hybridization probe-Q (Dabcyl or BHQ_2) (b*-a*)	GGGAGAA_TGTAACTG-Dabcyl (or BHQ_2)
Control linear hybridization probe-F (a-b-c)	Cy3-***T***C***T***AAA***T***C_***G***CTATGTG_TCGCTTA
Control linear hybridization probe-Q (b*-a*)	CACATAGC_GATTTAGA- BHQ_2

Italic bold letters denote locked nucleic acid bases.

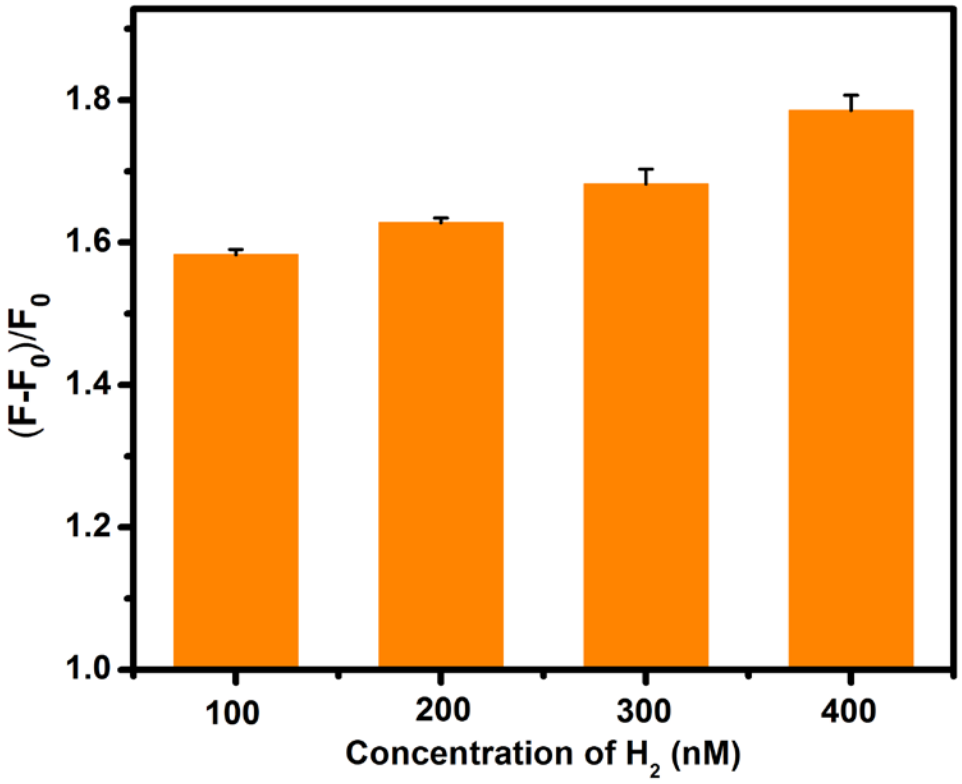

Figure S1. Optimized concentration of metastable hairpin DNA substrate H_2 (100 ~ 400 nM) relative to constant H_1 concentration (100 nM).

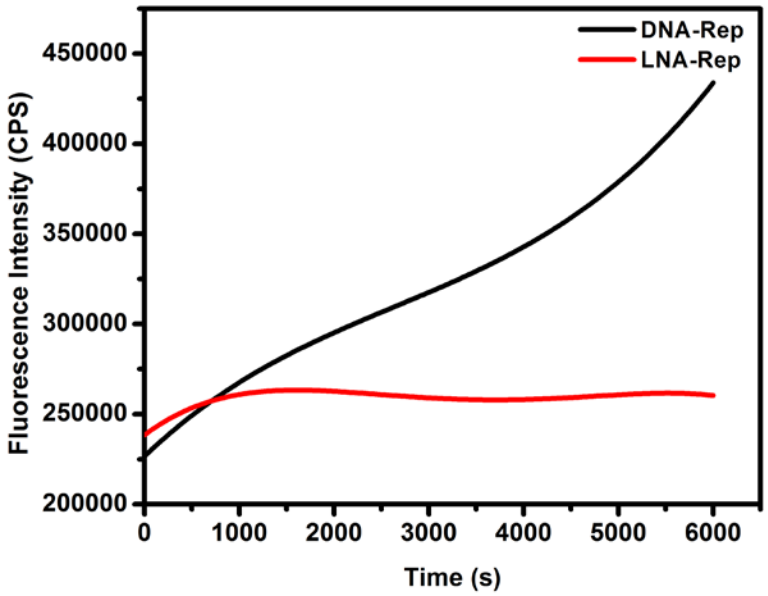

Figure S2. Fluorescence kinetics of locked nucleic acid-modified reporter and regular DNA reporter in MDA-MB-231 cell lysate.

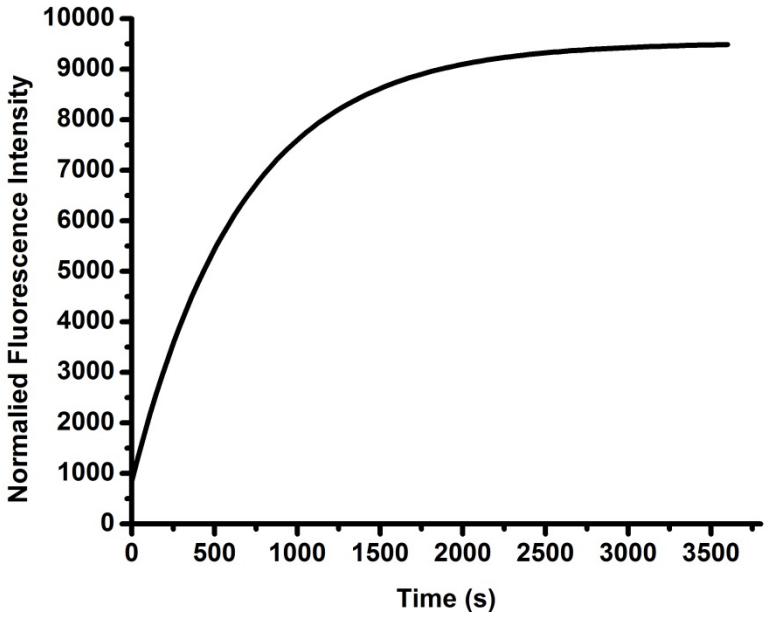

Figure S3. The normalized fluorescence response of HDCA to 1x concentration of target (100 nM).

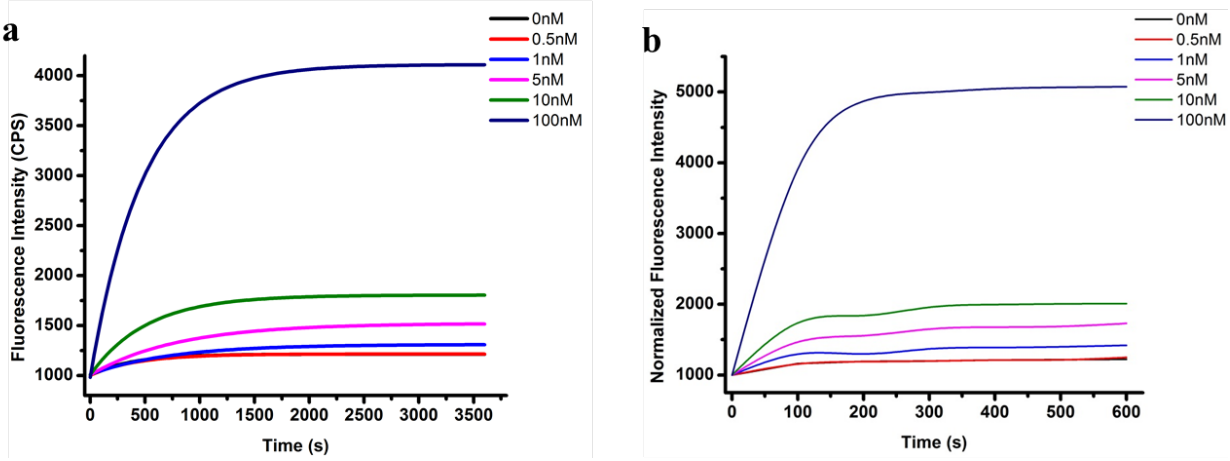

Figure S4. Fluorescence responses of linear hybridization probe (a) and molecular beacon (b) with different concentrations of target DNA (0, 0.5, 1, 5, 10 and 100 nM).

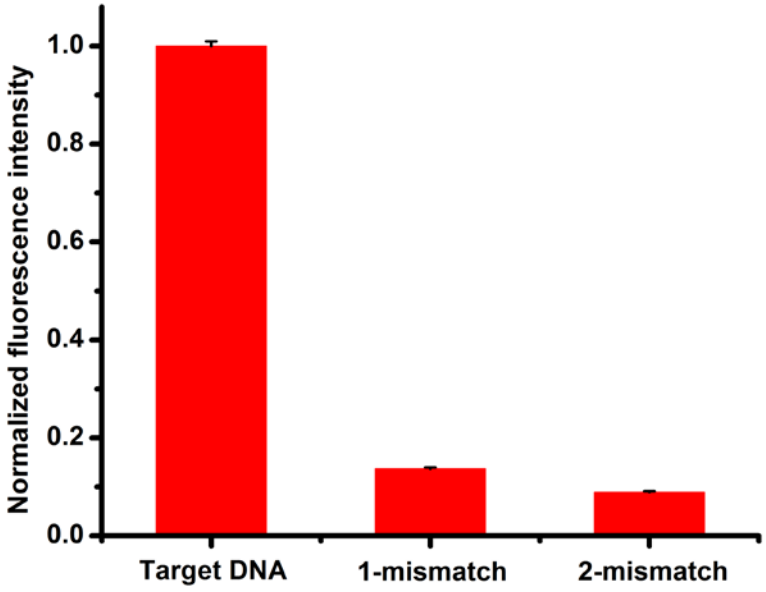

Figure S5. Single nucleotide polymorphism test of hairpin DNA cascade reaction with fully complementary target DNA, single- and double-mismatched DNA sequence.

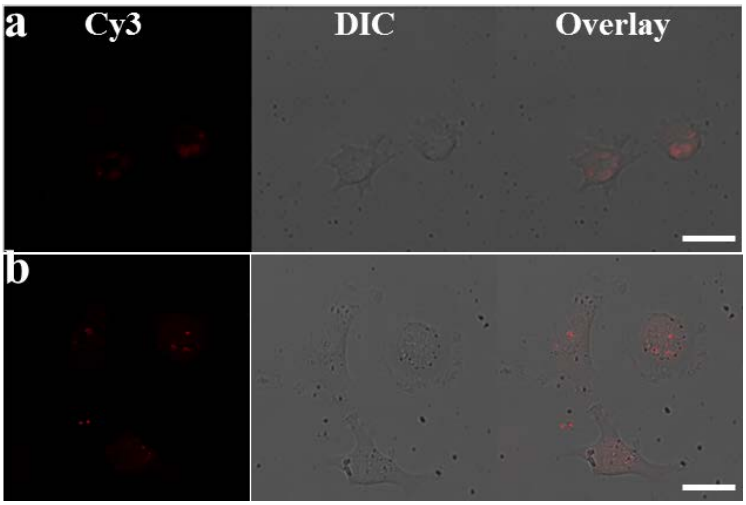

Figure S6. Confocal microscopy images of MDA-MB-231 cells transfected with (a) cHDCA and (b) cLHP, and incubated at 37°C for 2 h.

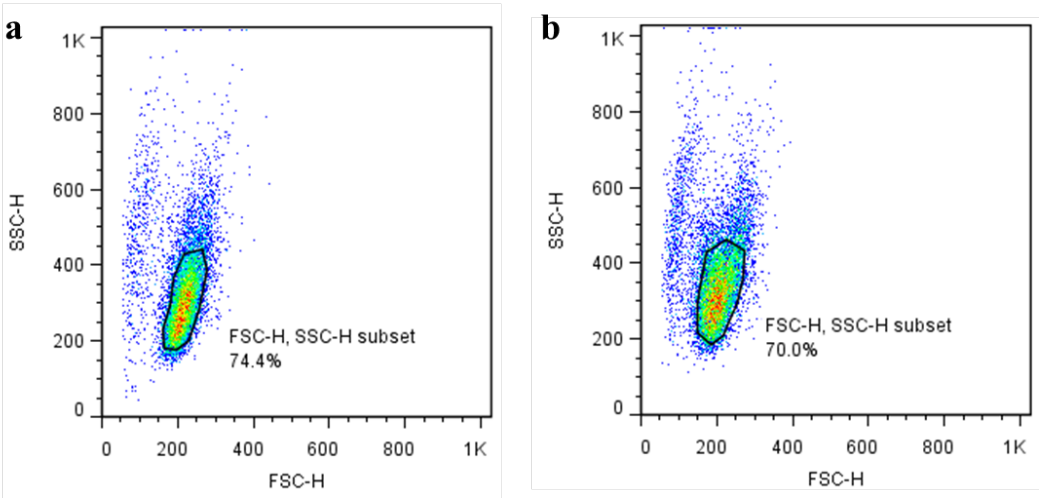

Figure S7. Flow cytometry gating of MDA-MB-231 cells transfected with (a) LHP and (b) HDCA.

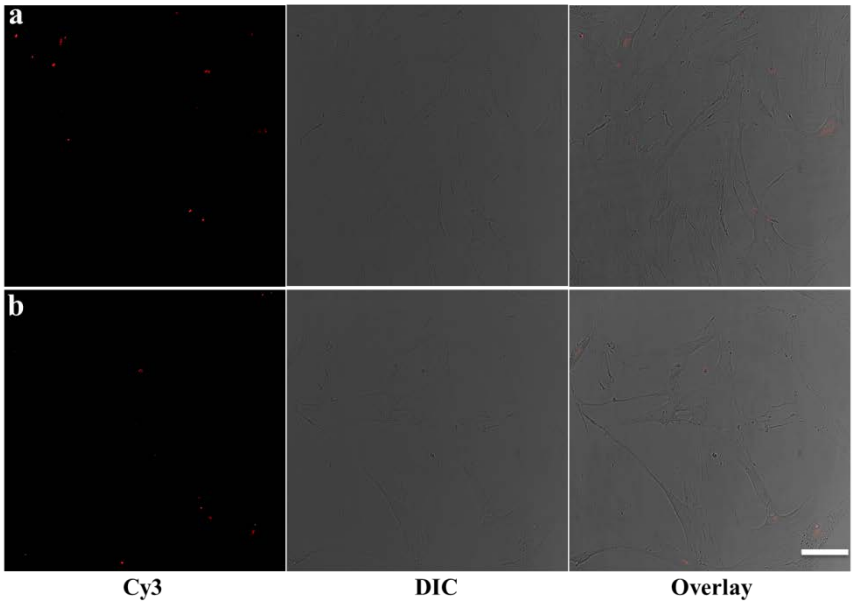

Figure S8. Confocal fluorescence images of IMR-90 cells transfected with (a) HDCA and (b) LHP at 37°C for 2 h. Scale bar: 50 μm.

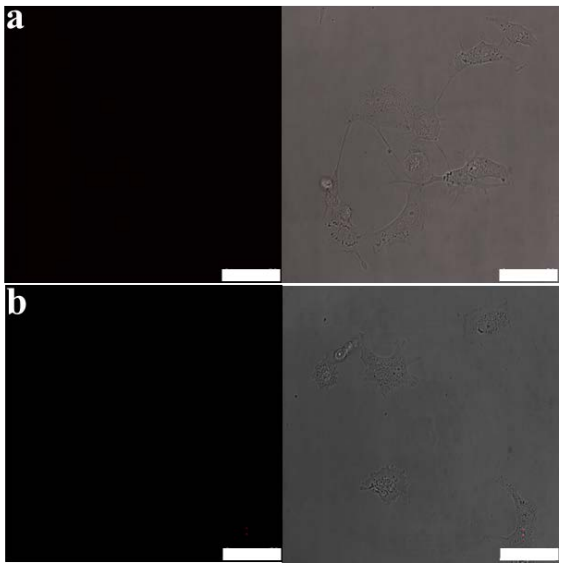

Figure S9. Confocal fluorescence images of MDA-MB-231 cells treated with 100 μM cordycepin, followed by transfection and incubation with (a) cHDCA and (b) cLHP at 37°C for 2 h. Scale bar: 50 μm.

References:

[1] D. Han, G. Zhu, C. Wu, Z. Zhu, T. Chen, X. Zhang, W. Tan. ACS *Nano*, **2013**, 7, 2312-2319.

[2] J. Hemphill, A. Deiters. *J.Am. Chem. Soc.*, **2013**, 135, 10512-10518.