Electronic Supplementary Information:

DNA strand-displacement-induced fluorescence enhancement for highly sensitive and selective assay of multiple microRNA in cancer cells

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1. Experiemntal details

Chemicals. 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 1,4-dithiothreitol (DTT), tris(hydroxymethyl)aminomethane (Tris), disodium ethylenediaminetetraacetic acid (EDTA), and bovine serum albumin (BSA) were purchased from Sigma-Aldrich and used as received. The fluorophore/quencher-labeled DNA probes and miRNAs were synthesized by GenePharma Co. (Shanghai, China). The nucleic acids were HPLC-purified and freeze-dried. The sequences of the probes and miRNA were summarized in Table S1. DNA hybridization buffer (HB) was phosphate-buffered saline (137 mM NaCl, 2.5 mM Mg^{2+}, 10 mM Na_2HPO_4, and 2.0 mM KH_2PO_4, pH 7.4). DNA stock solutions were prepared using Tris-HCl buffer (10 mM, pH 8.0) containing 1 mM EDTA. All solutions were prepared with doubly distilled water.

Fluorescent miRNA assays. Fluorescence spectra were collected with a Cary Eclipse fluorescence spectrophotometer (Varian) equipped with a Xenon lamp excitation source. The measurements were performed in HB solution at 37 ºC and pH 7.4. In a typical assay, 1 μL of FAM-labeled capture probe DNA (CP-141, 0.1 μM) were mixed with 2 μL of Dabcyl-labeled output probe DNA (OP-141, 0.1 μM) to form miR-141 molecular beacons, and then diluted to 100 μL using HB solution. After a stable background fluorescence was attained, different concentrations of the target miR-141 (T1) were added to the solution and allowed to hybridize with the molecular beacons for 1 h at 37 °C, which is longer enough to ensure the complete hybridization of T1 with CP-141 and the release of the OP-141 from the molecular beacons. Finally, the fluorescence emission spectra of FAM were recorded under excitation of 494 nm.

For multiple miRNA assay, a solution containing CP-141 (FAM-labeled, 1 μL, 0.1 μM), CP-21 (ROX-labeled, 1 μL, 0.1 μM), and CP-126 (Cy 5-labeled, 1 μL, 0.1 μM) was mixed with 2 μL of OP-141 (Dabcyl-labeled, 0.1 μM), OP-21 (Eclipse-labeled, 0.1 μM), and OP-126 (BHQ2-labeled, 0.1 μM) to form miR-141, miR-21, and miR-126 molecular beacons, and then diluted to 100 μL using HB solution. After 1 h incubation, the sample containing different concentrations of the target miR-141 (T1), miR-21 (T2), and miR-126 (T3) were added to the solution, and allowed to hybridize with the molecular

beacons for 1 h (Figure 1). Then, the enhanced fluorescence signals of FAM, ROX, and Cy 5 at 518, 605, and 663 nm, respectively, were recorded with Synergy 2 microplate reader (Biotek). The excited wavelength for FAM, ROX, and Cy 5 were set at 494, 575, and 646 nm, respectively.

Cell culture. A549 (human lung cancer cell), DU 145 (prostate carcinoma cell), AsPc-1 (pancreatic cancer cell), HeLa (cervical cancer cell), and MDA-MB231 (human breast cancer cell) cell lines were obtained from the cell bank of type culture collection of the Chinese Academy of Sciences (Shanghai, China) and cultured at 37 °C in RPMI 1640 medium supplemented with 10% fetal bovine serum (FEB), 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin in a 5% CO_2 environment. HMVEC cells (human microvascular endothelial cell, PromoCell, Heidelberg, Germany) were cultured in endothelial cell growth medium (EGM2-MV medium) containing 2% FBS at 37 °C with humidified 95% air containing 5% CO_2. MCF-10A cells (human normal breast epithelial cell, ATCC) were cultured in DMEM/F-12 medium containing 5% horse serum, 100 U/mL penicillin, 100 mg/mL streptomycin, 100 ng/mL cholera toxin, 10 ng/mL epidermal growth factor, 0.5 mg/mL hydrocortisone, 10 mg/mL insulin, and 1% L-glutamine at 37 °C under an atmosphere of 5% CO_2. After growing to 90% confluence, the cells were washed with PBS (0.145 M NaCl, 1.9 mM NaH_2PO_4, 8.1 mM K_2HPO_4, pH 7.4) and replaced the culture medium by 1 mL PBS and the cell number was estimated by a hemocytometer.

Preparation of cellular extracts. The preparation of cellular extracts was conducted according to the reported method.[1] Briefly, ~10^7 cells were washed once with phosphate-buffered saline and twice with buffer A (10 mM HEPES, pH 7.9 at 4 °C, 1.5 mM $Mg(NO_3)_2$, 10 mM KNO_3 and 0.5 mM DTT). The cell pellet was suspended in buffer B (20 mM HEPES (pH 7.9), 25% (v/v) glycerol, 0.42 M $NaNO_3$, 1.5 mM $Mg(NO_3)_2$, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride (PMSF) and 0.5 mM DTT)/0.1% Nonidet P-40 (20 μL). After incubating for 15 min on ice, the lysed cellular suspension was briefly mixed on a Vortex and microcentrifuged for 10 min at 4 °C. The supernatant was diluted with 80 μL of buffer C (20 mM HEPES (pH 7.9), 20% (v/v) glycerol, 0.1 M KNO_3, 0.2 mM EDTA, 0.5 mM

PMSF, and 0.5 mM DTT) and stored at –80 °C. The RNA was routinely assessed by gel electrophoresis and UV-vis spectrophotometry following the reported procedures.[2]

References

1 Y.-Q. Liu, M. Zhang, B.-C. Yin and B.-C. Ye, *Anal. Chem.* **2012**, *84*, 5165-5169.

2 J. Sambrook, E. F. Fritsch and T. Maniatis, *Molecular Cloning, A Laboratory Manual*, 2nd ed.; Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY, 1989.

Table S1. Sequences of oligonucleotides used in this work

description	Sequence
22-base 5′-terminus FAM-labeled single-stranded capture probe DNA for miR-141 (CP-141)	5′-FAM-CCA TCT TTA CCA GAC AGT GTT A-3′
22-base 5′-terminus ROX-labeled single-stranded capture probe DNA for miR-21 (CP-21)	5′-ROX-TCA ACA TCA GTC TGA TAA GCT A-3′
22-base 5′-terminus Cy 5 (cyanine dye 5)-labeled single-stranded capture probe DNA for miR-126 (CP-126)	5′-Cy 5-CGC ATT ATT ACT CAC GGT ACG A-3′
12-base 3′-terminus Dabcyl-labeled single-stranded output probe DNA for miR-141 (OP-141)	5′-TGG TAA AGA TGG-Dabcyl-3′
9-base 3′-terminus Dabcyl-labeled single-stranded output probe DNA for miR-141 (OP′-141)	5′-TAA AGA TGG-Dabcyl-3′
15-base 3′-terminus Dabcyl-labeled single-stranded output probe DNA for miR-141 (OP″-141)	5′-GTC TGG TAA AGA TGG-Dabcyl-3′
12-base 3′-terminus Eclipse-labeled single-stranded output probe DNA for miR-21 (OP-21)	5′-GAC TGA TGT TGA-Eclipse-3′
12-base 3′-terminus BHQ2 (black hole quencher 2)-labeled single-stranded output probe DNA for miR-126 (OP-126)	5′-AGT AAT AAT GCG-BHQ2-3′
22-base miR-141 (T1, perfect matched target for CP-141)	5′-UAA CAC UGU CUG GUA AAG AUG G-3′
22-base modified miR-141 (T2, one-base mismatch. The mismatched base is underlined)	5′-UAA CAC UGU CUG GUA AAG GUG G-3′
22-base modified miR-141 (T3, two-base mismatch)	5′-UAA CAC UGU CUA GUA AAG GUG G-3′
22-base modified miR-141 (T4, three-base mismatch)	5′-UAA AAC UGU CUA GUA AAG GUG G-3′
22-base miR-21 (T5, perfect matched target for CP-21)	5′-UAG CUU AUC AGA CUG AUG UUG A-3′
22-base miR-126 (T6, perfect matched target for CP-126)	5′-UCG UAC CGU GAG UAA UAA UGC G-3′
22-base miR-122 (an important component of gene regulatory networks in the liver for normal hepatocyte function, playing a key role in the regulation of hepatitis C virus replication)	5′-UGG AGU GUG ACA AUG GUG UUU G-3′
22-base miR-15b (induces the apoptosis of activated pancreatic stellate cell, which plays a pivotal role in the development of pancreatic diseases, especially chronic pancreatitis and pancreatic cancer)	5′-UAG CAG CAC AUC AUG GUU UAC A-3′
22-base let-7a (conservative among human cells)	5′-UGA GGUAGU AGG UUG UAU AGU U-3′
22-base miR-429 (belongs to the miR-200 family, induces mesenchymal-to-epithelial transition in metastatic ovarian cancer cells)	5′-UAA UAC UGU CUG GUA AAA CCG U-3′
22-base miR-34a* (a transcriptional target of the *p*53 tumor suppressor protein and subject to epigenetic inactivation in colorectal cancer and numerous other tumor types)	5′-CAA UCA GCA AGU AUA CUG CCC U-3′

Figures

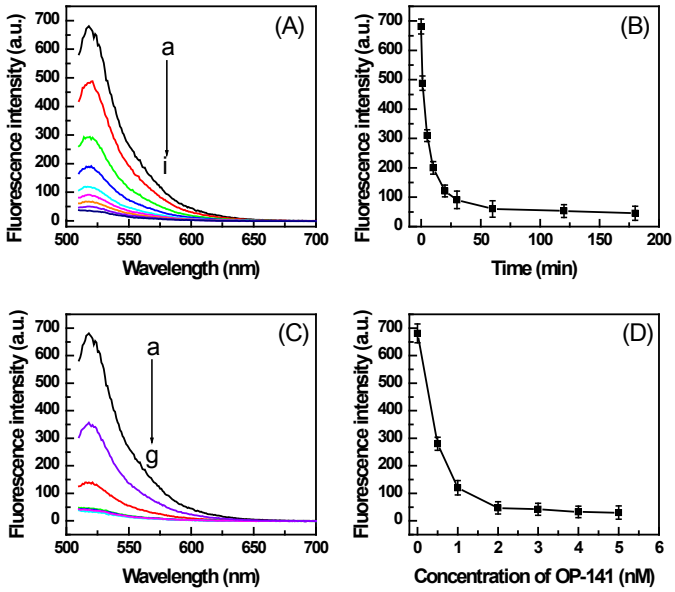

Fig. S1 (A) Fluorescence spectra of FAM-labeled CP-141 (1 nM) after hybridized with 2 nM Dabcyl-labeled OP-141 for (a–i) 0, 1, 5, 10, 20, 30, 60, 120, and 180 min. (B) The hybridization time-dependent fluorescence intensity of FAM (1 nM) at 518 nm. (C) Fluorescence spectra of CP-141 (1 nM) after 1-h hybridization with (a–g) 0, 0.5, 1, 2, 3, 4, and 5 nM of OP-141. (D) Dependence of the fluorescence intensities of FAM (1 nM) on the concentration of OP-141. Error bars were based on five measurements.

The fluorescence quenching efficiency displays an OP-141 concentration-dependent manner (Fig. S1C, D). We fixed the concentration of CP-141 at 1 nM, and then added different concentrations of OP-141 (0.5, 1, 2, 3, 4, and 5 nM, respectively) to the solution. After 1-h hybridization, the fluorescence spectra were recorded (Fig. S1C). The fluorescence intensity of FAM was rapidly quenched with increasing concentrations of the OP-141 in solution (curves a–c, Fig. S1C), and completely quenched when the OP-141 concentration is higher than 2 nM (curves d–g, Fig. S1C). The dependence of the fluorescence intensity on the OP-141 concentration depicted in Fig. S1D also indicated that the maximal quenching efficiency was attained at OP-141 concentration of ~2 nM. Consequently, to maximize the quenching efficiency for the synthesis of molecular beacons, we set the optimal OP-141/CP-141 (quencher/fluorophore) molar ratio at 2 (2 nM OP-141 to 1 nM CP-141) in the following measurements.

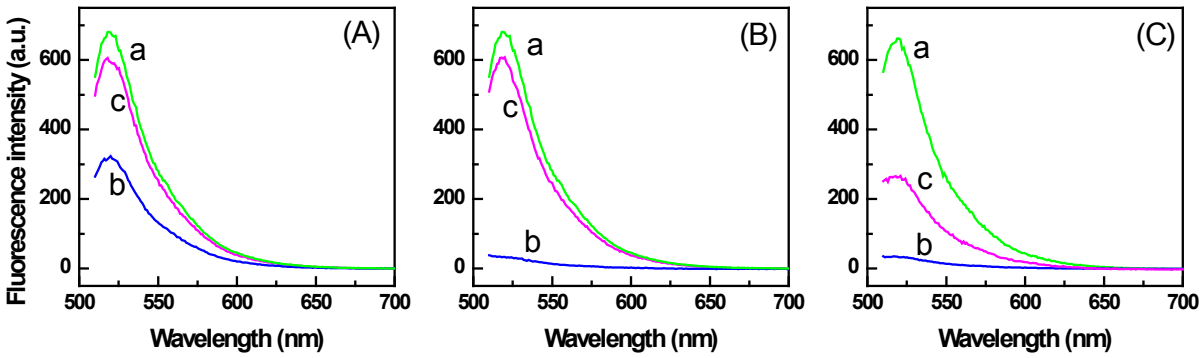

Fig. S2 Effects of the length of the output probe DNA on the quenching of the miR-141 molecular beacons and the fluorescence enhancement of the molecular beacon. Curve (a) in panels (A), (B), and (C) is the fluorescence spectrum of 1 nM FAM-labeled CP-141, (b) in panels (A), (B), and (C) is the fluorescence of the FAM-labeled CP-141 (1 nM) after 1-h hybridization with 2 nM Dabcyl-labeled OP′-141 (9-base) (A), OP-141 (12-base) (B), and OP″-141 (15-base) (C), respectively. Curve (c) in panels (A), (B), and (C) is the fluorescence spectrum of the duplexes of the CP-141/OP′-141 (A), CP-141/OP-141 (B), and CP-141/OP″-141 (C) after hybridized with miR-141 (T1, 50 pM) for 1 h. The CP-141/OP′-141, CP-141/OP-141, and CP-141/OP″-141 duplexes were prepared by hybridizing with 1 nM FAM-labeled CP-141 with 2 nM Dabcyl-labeled OP′-141 (9-base), OP-141 (12-base), and OP″-141, respectively, for 1 h.

We studied the dependence of the fluorescence quenching efficiency of the output probe DNA to the CP-141 on the length of the OP. We hybridized 1 nM of CP-141 with 2 nM Dabcyl-labeled OP′-141 (9-base), OP-141 (12-base), and OP″-141 (15-base), respectively, for 1 h, and then recorded the fluorescence spectra of the CP-141 (Fig. S2). When the length of the OP probe is shorter than 12-base, the fluorescence of the CP-141 cannot be well-quenched. For example, after 1-h hybridization, OP′-141 (9-base) can only quench ~50% of the fluorescence intensity of FAM (curves a and b, Fig. S2A). However, OP-141 (12-base) and OP″-141 (15-base) can almost completely quench the fluorescence of FAM (curves a and b, Fig. S2B, C).

We also studied the effects of the degree of the fluorescence enhancement of the quenched FAM on the OP probe length. Although the longer OP probe has good fluorescence quenching efficiency, it is difficult to be displaced by the target miRNA, leading to that the fluorescence of quenched FAM cannot be well-recovered (Fig. S2). For example, after the molecular beacons interacted with miR-141 (T1) for

1 h, the degree of the fluorescence enhancement for molecular beacons of FAM-labeled CP-141 with DAbcyl-labeled OP′-141 (9-base), OP-141 (12-base), and OP″-141 (15-base) was ~90% (curve c in Fig. S2A), 90% (curve c in Fig. S2B), and 38% (curve c, Fig. S2C), respectively. This is due to that the displacement of the OP probe from the molecular beacons is dynamically balanced between the interaction of the capture probe with output probe and the target miRNA. Longer output probe (such as 15-base) has a stronger interaction with capture probe, resulting to be difficultly displaced by the target miRNA.

Therefore, we will select a 12-base OP probe (OP-141) to synthesize capture/output probe molecular beacons by considering the fluorescence enhancement efficiency, although the longer OP probe processes a good quenching efficiency.

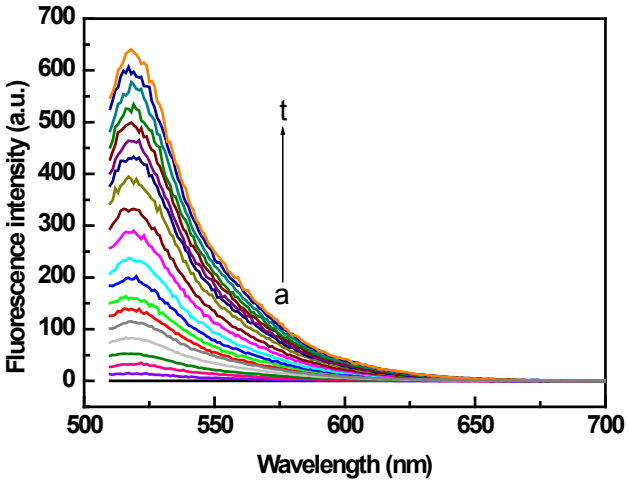

Fig. S3 The background-subtracted fluorescence spectra of miR-141 molecular beacon after 1 h hybridization with (a–t) 0, 0.005, 0.01, 0.02, 0.05, 0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 1.5, 2.0, 2.5, 5.0, 10, 50, 100, 200, and 500 pM of T1, respectively.

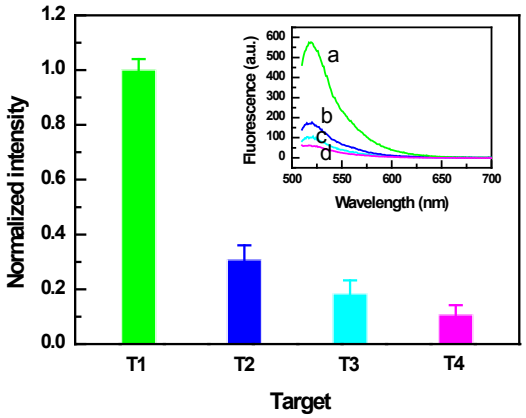

Fig. S4. Dependence of the normalized fluorescence intensity on the number of mismatched bases in the T1 sequence. We obtained the normalized values by comparing the respective fluorescence intensities of T2, T3, and T4 with that of T1. The inset shows the background-subtracted fluorescence spectra of the miR-141 molecular beacon after 1 h hybridization with 50 pM of (a) T1, (b) T2, (c) T3, and (d) T4, respectively. The miR-141 molecular beacon is prepared by hybridizing 1 nM CP-141 with 2 nM OP-141 for 1 h. Error bars were based on five measurements.

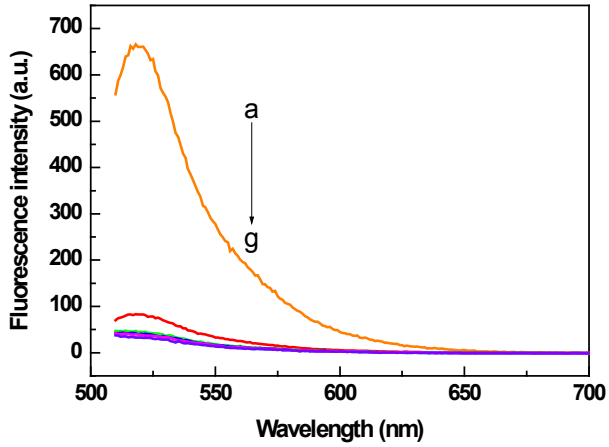

Fig. S5 (a) Fluorescence spectrum of 1 nM FAM-labeled CP-141. (b)-(g) are the fluorescence spectra of miR-141 molecular beacon after hybridizing with 50 pM miR-429 (b), miR-122 (c), miR-15b (d), let-7a (e), miR-34a* (f), and miR-21 (g), respectively, for 1 h. The miR-141 molecular beacon is prepared by hybridizing 1 nM CP-141 with 2 nM OP-141 for 1 h.

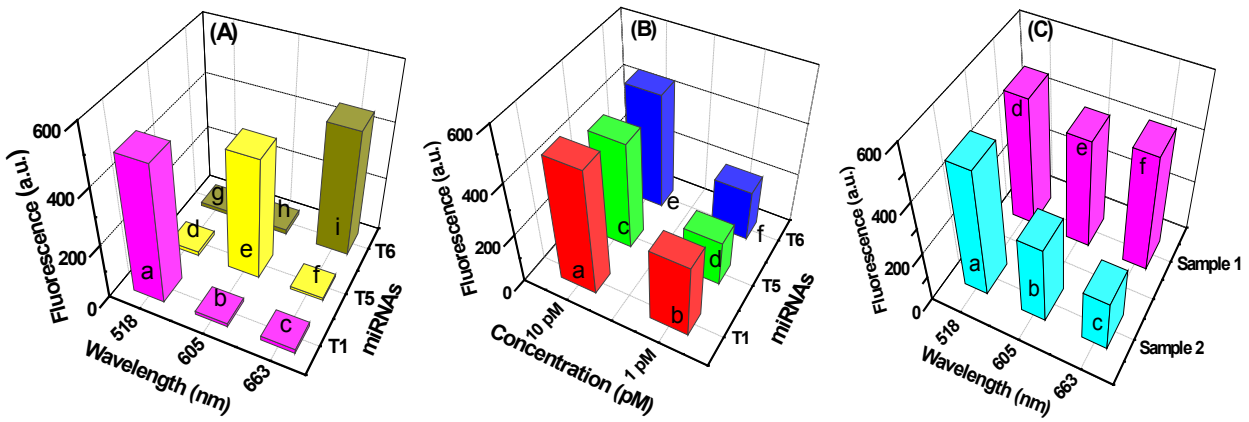

Fig. S6 Multiple miRNAs detection. (A) Enhanced fluorescence signal of miR-141 (a, d, and g), miR-21 (b, e, and h), and miR-126 (c, f, and i) molecular beacons after addition of T1 (10 pM, a, b, and c), T5 (10 pM, d, e, and f), and T6 (10 pM, g, h, and i). The molecular beacons were prepared by hybridizing CP-141 (1 nM), CP-21 (1 nM), and CP-126 (1 nM) with OP-141 (2 nM), OP-21 (2 nM), and OP-126 (2 nM), respectively. (B) Fluorescence signals of miR-141 (a and b), miR-21 (c and d), and miR-126 (e and f) molecular beacons after addition of different concentrations of T1, T5, and T6. The concentrations of target are: 10 pM (a, c, and e) and 1 pM (b, d, and f). (C) Fluorescence signals of the mixtures of molecular beacons (miR-141, miR-21, and miR-126) after addition of the different samples. The sample 1 contains 10 pM T1, 10 pM T5, and 10 pM T6; sample 2 contains 10 pM T1, 5 pM T5, and 1 pM T6.

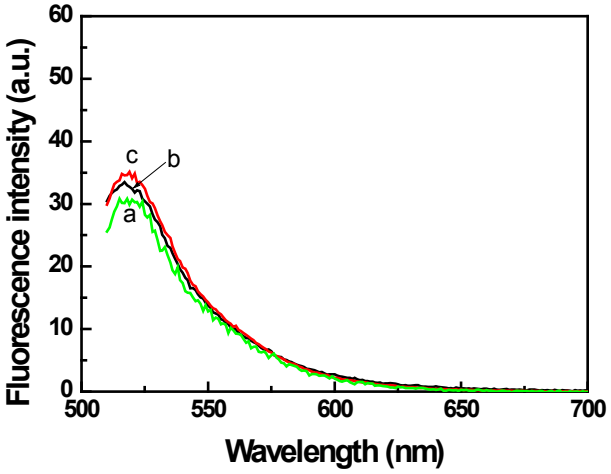

Fig. S7 Fluorescence spectra of 1 nM miR-141 molecular beacon (FAM-labeled) in cell culture medium (RPMI 1640) for 0 (a), 1 (b), and 3 h (c).