Supporting Information

Simultaneous enzyme-free detection of multiple long non-coding RNAs in cancer cells at single-molecule/particle level

Yan Zhang,[‡,†] Chen Wang,[‡,†] Xiaoran Zou,[‡,†] Xiaorui Tian,[†] Juan Hu,[,§] and Chun-yang Zhang[*,†]*

[†] College of Chemistry, Chemical Engineering and Materials Science, Collaborative Innovation Center of Functionalized Probes for Chemical Imaging in Universities of Shandong, Key Laboratory of Molecular and Nano Probes, Ministry of Education, Shandong Provincial Key Laboratory of Clean Production of Fine Chemicals, Shandong Normal University, Jinan 250014, China. E-mail: cyzhang@sdnu.edu.cn; Fax: +86 531-82615258; Tel.: +86 531-86186033

[§] School of Chemistry and Chemical Engineering, Southeast University, Nanjing 211189, China. E-mail: hujuan@seu.edu.cn

[‡] These authors contributed equally.

Table of Contents

EXPERIMENTAI SECTION

Materials and reagents

lncRNA MALAT1, lncRNA HOTAIR, RNase inhibitor and RNase-free water were purchased from TaKaRa Bio. Inc. (Dalian, China). All DNA oligonucleotides (Table S1) were synthesized by Sangon Biotechnology Co. Ltd. (Shanghai, China). The streptavidin-coated magnetic beads were obtained from Invitrogen (California, CA, USA). Human lung adenocarcinoma cell line (A549 cells), human cervical carcinoma cell line (HeLa cells), human colon cancer cells (SW480 cells), human breast cancer cell line (MCF-7 cells) and human bronchial epithelial cell line (HBE cells) were bought from Cell Bank, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China). All other reagents were of analytical grade and used as received without further purification.

Table S1. Sequences of the Oligonucleotides

note	sequence (5'-3')
lncRNA MALAT1	UAA GAU UUC CCA AGC AGA CAG CCC GUG CUG CUC CG
lncRNA HOTAIR	GCA ACU CUA UAA UAU GCU UAU AUU AGG UCU AGA AG
helper probe 1	CCA AGC AGA CAG CCC GTG CTA GGC TGA CGG TAG ATA
helper probe 2	TAA TAT GCT TAT ATT AGG GAT CGT ACC GTG AGC GCG
capture probe 1	CG GAG CAG CAC GGG CTG TCT GCT TGG GAA ATC TTA TTT TT - biotin
capture probe 2	CT TCT AGA CCT AAT ATA AGC ATA TTA TAG AGT TGC TTT TT - biotin
signal probe 1	ACA ACA ACC CAC AAT CCC TAT CTA CCG TCA GCC TAG - Cy3
signal probe 2	GGG ATT GTG GGT TGT TGT CTA GGC TGA CGG TAG ATA - Cy3
signal probe 3	CTC ATA TAC CCA TCC ACG CGC GCT CAC GGT ACG ATC - Cy5
signal probe 4	CGT GGA TGG GTA TAT GAG GAT CGT ACC GTG AGC GCG - Cy5
let-7a	UGA GGU AGU AGG UUG UAU AGU U
miR-486-5p	UCC UGU ACU GAG CUG CCC CGA G
MALAT1-forward primer	GCTCTGTGGTGTGGGATTGA
MALAT1- reverse primer	GTGGCAAAATGGCGGACTTT
HOTAIR-forward primer	GGGACAGAAGGAAAGCCCTC
HOTAIR- reverse primer	TTGAGAGCACCTCCGGGATA

GAPDH-forward primer	ATCTCTGCCCCCTCTGCTGA
GAPDH-reverse primer	GATGACCTTGCCCACAGCCT

Preparation of the reporter units

For the preparation of the reporter unit 1 for lncRNA MALAT1, 2 μL of 100 μM helper probe 1, 4 μL of 100 μM signal probe 1, 4 μL of 100 μM signal probe 2 were added to a buffer containing 100 nM Tris-HCl (pH 8.0), 500 nM NaCl with a final volume of 20 μL. The mixture was heated at 95 °C for 5 min, followed by slowly cooling to the room temperature. For the preparation of the reporter unit 2 for lncRNA HOTAIR, 2 μL of 100 μM helper probe 2, 4 μL of 100 μM signal probe 3, 4 μL of 100 μM signal probe 4 were added to a buffer containing 100 nM Tris-HCl (pH 8.0), 500 nM NaCl with a final volume of 20 μL. The mixture was heated at 95 °C for 5 min, followed by slowly cooling to the room temperature.

Conjugation of capture probe with the streptavidin-coated magnetic bead

Prior to the reaction, 27 μL of 10 mg/mL streptavidin-coated MBs was transferred to a centrifuge tube and washed twice to remove the preservative. The supernatant was removed by magnetic separation, and then the MBs were resuspended in the buffer (10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 2 M NaCl) with a final concentration of 5 μg/μL. For the preparation of the MB-capture probe conjugates, 45 μL of 1 μM biotinylated capture probe 1 and capture probe 2 were mixed with the MBs solution, followed by incubation at room temperature for 15 min in a roller mixer. Subsequently, the mixture was washed five times to remove the excess capture probes. The MB-capture probe conjugates were resuspended in 15 μL of 1× TDT buffer (10 mM $Mg(Ac)_2$, 20 mM Tris-Ac, 50 mM KAc, pH 7.9).

Preparation of the MB-capture probe-reporter unit complexes

For the preparation of the MB-capture probe-reporter unit complexes, the above reporter unit 1 for lncRNA MALAT1 and the reporter unit 2 for lncRNA HOTAIR were mixed with the MB-capture probe conjugate solution. Then the mixture was placed in a roller mixer and incubated for 30 min in the dark to form the complexes. Subsequently, the mixture was washed five times to remove the free reporter units. The obtained complexes were dispersed in 15 μL of 1× TDT buffer for subsequent use.

Gel Electrophoresis

To verify the formation of reporter units, the HCR products were analyzed by 2.5% agarose gel electrophoresis in 1× TAE (40 mM Tris-acetic acid, 2 mM EDTA, pH 8.0) at 110 V constant voltages for 70 min at room temperature.

To analyze the target lncRNA-catalyzed strand displacement reaction, the released reporter units in the presence of target lncRNA were analyzed by 10% nondenaturating polyacrylamide gel electrophoresis (PAGE) analysis in 1× TBE (9 mM Tris-HCl, 9 mM boric acid, 0.2 mM EDTA, pH 7.9) at a 110 V constant voltage for 50 min at room temperature. After electrophoresis, the gel was analyzed by a ChemiDoc™ MP Imaging System (Bio-Rad, Hercules, CA, USA).

Cell culture and the extraction of total RNA

The MCF-7 cells, HeLa cells, SW480 cells, A549 cells and HBE cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, USA) with 10% fetal bovine serum and 1% penicillin-streptomycin at 37 °C with 5% CO_2. The total RNA was obtained by miRNeasy mini kit (Qiagen, German) according to the manufacturer's procedure, and its concentration was determined by the NanoDrop 2000c spectrophotometer (Thermo Scientific, Wilmington, U.S.A.).

LncRNA assay

For target-catalyzed strand displacement reaction, different concentrations of synthetic lncRNA / total RNA samples were heated at 95 °C for 5 min, followed by adding to a hybridization solution which contains 3 μL of the complexes, 20 U of RNase inhibitor and 1× TDT buffer with a final volume of 20 μL. The mixture was incubated at room temperature for 80 min in the dark to release the fluorescent reporter units. After magnetic separation, the supernatant was incubated with 30 μL of 1× NEBuffer1 containing 10 U of Exo III at 37 °C for 30 min to release Cy3 and Cy5 molecules. The obtained solution was subjected to fluorescence spectra measurement and single-molecule detection.

Measurement of fluorescence spectra

Fluorescence spectra of Cy3 and Cy5 were measured by using a fluorescence spectrophotometer (Hitachi F-7000, Tokyo, Japan) with an excitation wavelength of 535 nm and 635 nm, respectively. The Cy3 emission spectrum was scanned from 550 to 700 nm, and the Cy5 emission spectrum was scanned from 650 nm to 750 nm. The fluorescence intensity at 570 nm was used for quantitative analysis of lncRNA MALAT1, and the fluorescence intensity at 670 nm was used for quantitative analysis of lncRNA HOTAIR, respectively.

Single-molecule detection and data analysis

In the single-molecule measurement, the reaction products were diluted 2500-fold for imaging the Cy3 fluorescence signal and 350-fold for imaging the Cy5 fluorescence signal, respectively, with a buffer (10 mM Tris-HCl, 50 mM KCl, 5 mM $MgCl_2$, 1 mM Trolox, pH 8.0). The 10 μL of sample was placed on a coverslip for

single-molecule fluorescence imaging with a total internal reflection fluorescence microscopy (Ti-E TIRF, Nikon, Japan). The Cy3 and Cy5 fluorescent molecules were simultaneously excited by using 561-nm laser and 640-nm laser. Fluorescence signals were acquired through the Cy3 detection channel (573 – 613 nm filter) and the Cy5 detection channel (661.5 – 690.5 nm filter). For data analysis, Image J software was used to select regions of interest of 600 × 600 pixels. The average Cy3 counts and the average Cy5 counts were obtained by calculating ten frames.

The qRT-PCR assay

Total RNA obtained from different cell lines was reverse transcribed to cDNA using the PrimeScript™ RT reagent Kit with gDNA Eraser (TaKaRa, Dalian, China) according to the manufacturer's instructions. Then the lncRNA levels were quantified by RT-PCR using TB Green Premix Ex Taq II (Tli RNaseH Plus) (TaKaRa, Dalian, China) in a BIO-RAD CFX connect Real-Time system. The results were analyzed using the ΔΔCT method with the HBE cells as a control.

Real-time imaging of the MB-capture probe-reporter unit complexes in living cells

MCF-7 cells were plated in 20-mm glass-bottom cell culture dish and incubated overnight in DMEM medium containing 10% fetal bovine serum. The cells were washed twice with 1× PBS. Transfection assays were performed using Lipofectamine 3000 reagent according to the manufacturer's instructions (Invitrogen). Briefly, 7.5 μL of Lipofectamine 3000 was diluted in 250 μL of Opti-MEM. The 10 μL of MB-capture probe-reporter unit complexes (3 μM) and 10 μL of P3000 were diluted in 250 μL of Opti-MEM. The Opti-MEM transfection mixtures were prepared by mixing the above solutions and incubated at room temperature for 15 min. The cells were incubated with the Opti-MEM transfection mixtures in a humidified incubator containing 5% CO_2 for 1 h at 37 °C. After the transfection, the cells were washed five times with 1× PBS, and the dishes were filled with fresh DMEM medium containing 10% fetal bovine serum. The cell images were obtained on inverted microscope with 10× objective. Fluorescence spots of Cy3 and Cy5 were simultaneously excited by 561-nm laser and 640-nm laser.

In order to discriminate cancer cells from normal cells by their different lncRNA expression levels, MCF-7 cells and HBE cells were plated in 20-mm glass-bottom cell culture dish, respectively, and incubated overnight in DMEM medium containing 10% fetal bovine serum. The 7.5 μL of Lipofectamine 3000 was diluted in 250 μL of Opti-MEM. The 2 μL of MB-capture probe-reporter unit complexes (3 μM) and 10 μL of P3000 were diluted in 250 μL of Opti-MEM. After the transfection, the cell images were obtained on inverted microscope with 60× objective. Fluorescence spots of Cy3 and Cy5 were simultaneously excited by 561-nm laser and 640-nm laser.

Optimization of reporter units-to-capture probe ratio

We optimized the experimental conditions including the ratio of reporter units to capture probe, the reaction buffer, and the reaction time of target-catalyzed strand displacement. As shown in Figure S1, when the ratio of reporter units to capture probe increases from 1:1 to 3:1, the F/F_0 value enhances (F and F_0 are the fluorescence intensity values in the presence and absence of lncRNA, respectively). When the reporter units-to-capture probe ratio exceeds 3:1, the fluorescence signal begins to decrease. Thus, the optimal reporter units-to-capture probe ratio of 3:1 is used in the subsequent experiments.

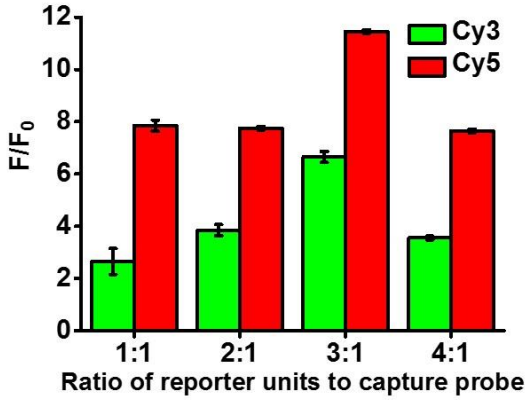

Figure S1. Variance of the F/F_0 value in response to different ratios of reporter units to capture probe in the range from 1:1 to 4:1. F and F_0 are the fluorescence intensity values in the presence and absence of lncRNA, respectively. The concentration of lncRNA MALAT1 is 1 μM, and the concentration of lncRNA HOTAIR is 1 μM. Error bars show the standard deviation of the three experiments.

Optimization of reaction buffer

In order to obtain the ideal buffer for this assay, we compared four buffers including 1× TDT buffer (10 mM $Mg(Ac)_2$, 20 mM Tris-Ac, 50 mM KAc, pH 7.9), 1× Cutsmart buffer (50 mM KAc, 20 mM Tris-Ac, 10 mM $Mg(Ac)_2$, 0.1 mg/ml BSA, pH 7.9), 1× NEB buffer 2 (50 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM DTT, pH 7.9), and 1× Tris-EDTA buffer solution (10 mM Tris-HCl, 1 mM EDTA, pH 7.4). As shown in Figure S2, the F/F_0 value in the presence of TDT buffer is much higher than those in the presence of other three buffers. Thus, TDT buffer was used as the optimal reaction buffer.

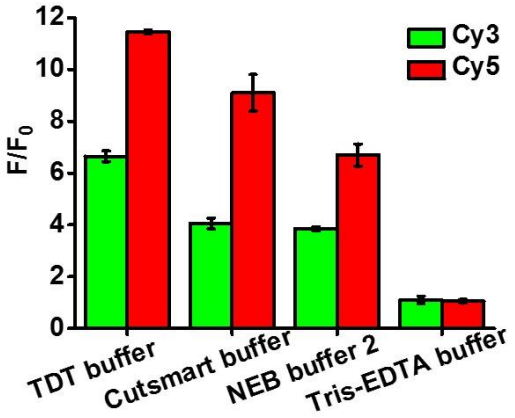

Figure S2. Variance of the F/F_0 value in the presence of different reaction buffers. F and F_0 are the fluorescence intensity in the presence and absence of lncRNA, respectively. The concentration of lncRNA MALAT1 is 1 μM, and the concentration of lncRNA HOTAIR is 1 μM. Error bars show the standard deviation of the three experiments.

Optimization of reaction time of target-catalyzed strand displacement

We further optimized the reaction time of target-catalyzed strand displacement reaction. As shown in Figure S3, in the presence of lncRNA MALAT1, the F/F_0 value increases with reaction time, and reaches the maximum value at 80 min. In the presence of lncRNA HOTAIR, the F/F_0 value reaches the highest value at 80 min. Thus, the reaction time of 80 min is used in the subsequent experiments.

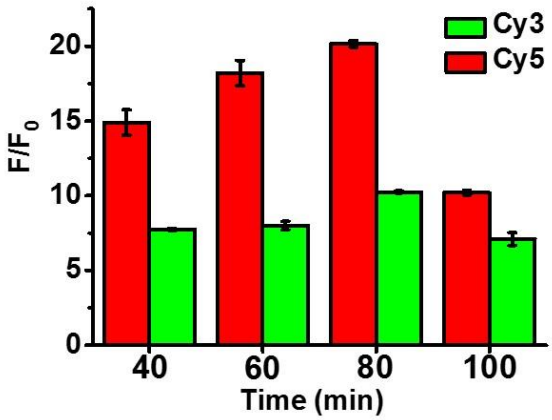

Figure S3. Variance of the F/F_0 value with the reaction times of target-catalyzed strand displacement reaction. F and F_0 are the fluorescence intensity in the presence and absence of lncRNA, respectively. The concentration of lncRNA MALAT1 is 1 μM, and the concentration of lncRNA HOTAIR is 1 μM. Error bars show the standard deviation of the three experiments.

Measurement of lncRNA MALAT1 and lncRNA HOTAIR in different cells by the single-particle detection method

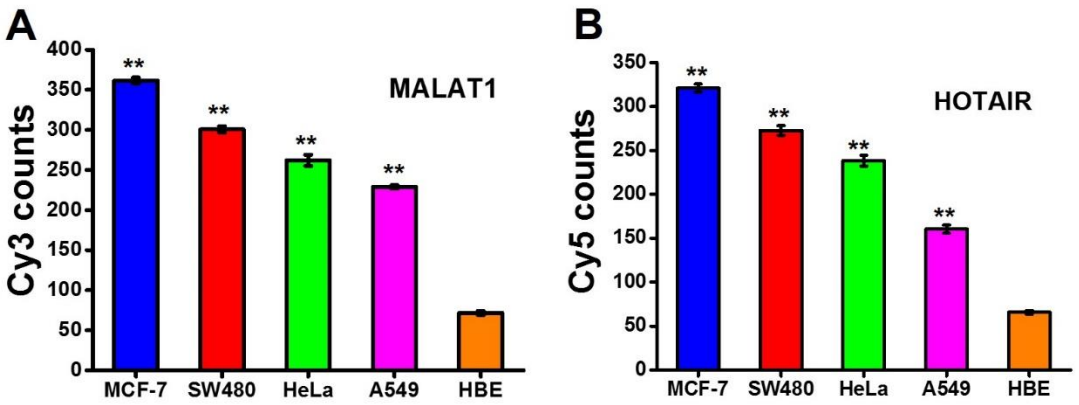

Figure S4. Measurement of lncRNA MALAT1 (A) and lncRNA HOTAIR (B) in the cell extracts obtained from MCF-7 cells, SW480 cells, HeLa cells, A549 cells and HBE cells, respectively. Total RNA extracted from 5×10^5 different cell lines was used in this experiment. $^{**}p < 0.01$ compared with the normal HBE cells. Error bars show the standard deviation of the three experiments.

The qRT-PCR measurement

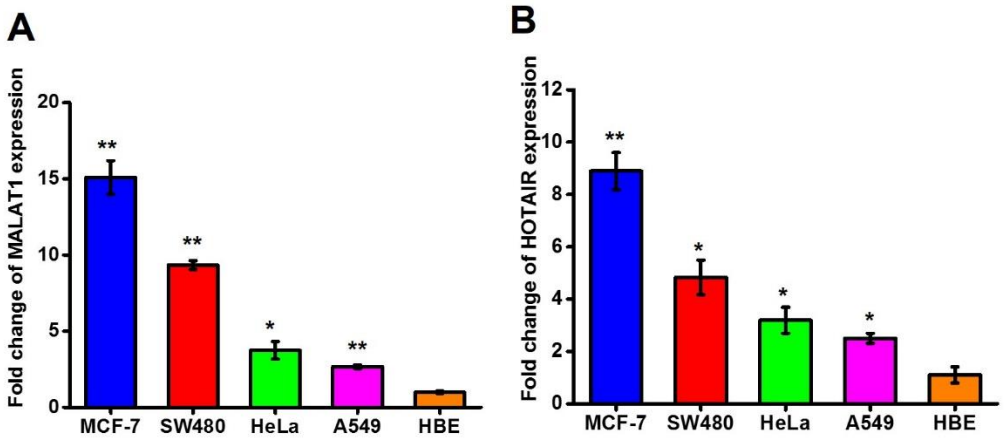

Figure S5. Fold change of lncRNA MALAT1 level (A) and lncRNA HOTAIR level (B) in MCF-7 cells, SW480 cells, HeLa cells, A549 cells and HBE cells quantified by RT-PCR. The results were analyzed using the ΔΔCT method with HBE cells as a control. $^{*}p < 0.05$ and $^{**}p < 0.01$ compared with normal HBE cells. Error bars show the standard deviation of the three experiments.