Supporting Information

Cruciate DNA Probes for Amplified Multiplexed Imaging of MicroRNAs in Living Cells

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EXPERIMENTAL SECTION

Reagents and materials. Magnesium chloride hexahydrate ($MgCl_2 \cdot 6H_2O$, AR) was purchased from Aladdin Industrial Co. (Shanghai, China). MDA-MB-231 cells (human breast cancer cell line), A549 cells (human pulmonary carcinoma cell line), HeLa cells (human cervical carcinoma cell line), and L-02 cells (human normal liver cell line) were obtained from the cell bank of Central Laboratory at Xiangya Hospital (Changsha, China). RPMI 1640 and DMEM high glucose mediums, penicillin, streptomycin, heat-inactivated fetal bovine serum (FBS), and lipofectamine 3000 transfection reagent were purchased from Thermo Fisher (MA, USA). Sodium azide (NaN_3) and nuclear stain reagent Hoechst 33342 were purchased from Sigma-Aldrich (MO, USA). 4S Red Plus nucleic acid stain, 5× glycerol gel loading buffer, DNA ladders, and synthesized oligonucleotides were all HPLC-purified and lyophilized by Sangon Biotech Co. Ltd. (Shanghai, China). Sequences of the synthesized oligonucleotides are shown in Supporting Information Table S1. All other chemicals were of analytical grade and purchased from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). Ultrapure water was obtained through a Millipore Milli-Q water purification system (MA, USA) and had an electric resistance >18.25 MΩ.

Quantitative reverse transcription-PCR (qRT-PCR) analysis. Total cellular RNAs were extracted from different cell lines (MDA-MB-231, A549, HeLa, and L-02 cells) with Trizol Total RNA Isolation Kit (Sangon Biotech, China). The cDNA samples were prepared by reverse transcription reaction with an Evo M-MLV RT Kit for qPCR (Accurate Biology, China). The SYBR® Green Premix Pro Taq HS qPCR Kit (Accurate Biology, China) was used for the qPCR analysis of miRNA-21 and miRNA-155 on the ABI StepOnePlus qPCR instrument (Thermo Fisher, USA). The PCR protocol was as follows: an initial 95 °C for 30 s followed by 40 cycles of 95 °C for 5 s and 60 °C for 30 s. The relative levels of miRNA-21 and miRNA-155 were calculated by normalizing U6 RNA expression and using the $2^{-\Delta\Delta Ct}$ method.

The primers used for qRT-PCR were following:

miR-21 forward, 5'-ACA CTC CAG CTG GGT AGC TTA TCA GAC TG-3'

miR-21 reverse, 5’-CTC AAC TGG TGT CGT GGA GTC GGC AAT TCA GTT GAG TCA ACA TCA-3’

miR-155 forward, 5’-ACA CTC CAG CTG GGT TAA TGC TAA TCG TG-3’

miR-155 reverse, 5’-CTC AAC TGG TGT CGT GGA GTC GGC AAT TCA GTT GAG ACC CCT AT-3’

U6 forward, 5’-CTC GCT TCG GCA GCA CA-3’

U6 reverse, 5’-AAC GCT TCA CGA ATT TGC GT-3’

Table S1. Sequences of synthesized oligonucleotides. [a]

Name	Sequence (5'-3')
L1	CGTGCTCAACTCGTGCCCGAATGCCGTAAGCCTTTTTGACA GTAGCCAGT
L2	CGCAATCCCCTAACGCGCACGAGTTGAGCACGTTTTTTACG ACAGACAGGT
L3	TGATACCGGCCATAGTGCGTTAGGGGATTGCGTTTTTGAATG CTGCGTGT
L4	GGCTTACGGCATTCGGACTATGGCCGGTATCATTTTTT**GGTG GTGGTGGTTGTGGTGGTGGTGG**
L5	CGTGCTCAACTCGTGCCCGAATGCCGTAAGCCTTTTTACAC GCAGCATTC
L6	CGCAATCCCCTAACGCGCACGAGTTGAGCACGTTTTTTAAT CCGTCTGTCC
L7	TGATACCGGCCATAGTGCGTTAGGGGATTGCGTTTTTGAGTA CTAGAGGA
H1	TCAGACTGATGTTCTGTGTGTAGCTCAACATCAGTCTGATAA GCTATTTTTACTGGCTACTGTC
H2	**BHQ2**-CTGTGTGTAGCTCTCAGACTGATGTTGAGCTACACA CAG/**iCy5**/AACATCATTTTTACCTGTCTGTCGT
H3	TAATCGTGATAGGCTGTGTGTAGCTCCCTATCACGATTAGCA TTAATTTTTGGACAGACGGATT
H4	**Dabcyl**-CTGTGTGTAGCTCTAATCGTGATAGGGAGCTACACAC AGCC/**i6FAMdT**/ATCATTTTTTCCTCTAGTACTC
H1'	TCAGACTGATGTTCTGTGTGTAGCTCCTAACCGTCTAACATT GGACTTTTTACTGGCTACTGTC
H3'	TAATCGTGATAGGCTGTGTGTAGCTCTACGAGTCGTTAACGT ATATTTTTTGGACAGACGGATT
L4'	GGCTTACGGCATTCGGACTATGGCCGGTATCATTTTTTTTTTT TTTTTTTTTTTTTTTTTTTT
miRNA-21	UAGCUUAUCAGACUGAUGUUGA
miRNA-155	UUAAUGCUAAUCGUGAUAGGGGU
miRNA-10b	UACCCUGUAGAACCGAAUUUGUG

miRNA-151	CUAGACUGAAGCUCCUUGAGG
let-7b	UGAGGUAGUAGGUUGUGUGGUU
MM1-21	UAGCUUAUCAGACUGAUCUUGA
MM2-21	UAGCUUAUCAUACUGAUCUUGA
MM3-21	UAGAUUAUCAUACUGAUCUUGA
MM1-155	UUAAUGUUAAUCGUGAUAGGGGU
MM2-155	UUAAUGUUAAUCUUGAUAGGGGU
MM3-155	UUAAUGUUAAUCUUGAUAGUGGU
Antisense miRNA-21	UCAACAUCAGUCUGAUAAGCUA
Antisense miRNA-155	ACCCCUAUCACGAUUAGCAUUAA

[a]L1 to L7 are used to assemble the cross-shaped scaffold. AS1411 aptamer sequence is highlighted in red. H1 to H4 are used for hybridization with the cross-shaped scaffold to form the cruciate DNA probe. H1' and H3' are mismatched versions of H1 and H3, the underlined parts represent mismatched sequences. L4' replaces the aptamer sequence of L4 with poly-T for the control experiment. The single-base, two-base, and three-base mismatched sequences of miRNA-21 and miRNA-155 are represented by MM1-21, MM2-21, MM3-21, and MM1-155, MM2-155, MM3-155. The mismatched bases are underlined in blue.

Table S2. Comparison of the cruciate DNA probe with other strategies for miRNAs detection.

Detection method	Target	Linear range	Detection limit	Reference
DNA strand displacement cascades	let-7a	0 nM-8 nM	17.8 pM	Ref. 35
Toehold-mediated strand displacement reaction	let-7a	0.5 nM-25 nM	22.35 pM	Ref. 36
Entropy-driven catalytic reaction	miRNA-21	20 pM-5 nM	8 pM	Ref. 37
Ratiometric fluorescence probe	miRNA-141	5 nM-100 nM	2.5 nM	Ref. 38
Enzyme-powered DNA circuit	miRNA-21	0.2 nM-100 nM	0.2 nM	Ref. 39
Catalytic hairpin assembly	miRNA-21	5 pM-6 nM	3 pM	This work
	miRNA-155	5 pM-5 nM	2 pM	

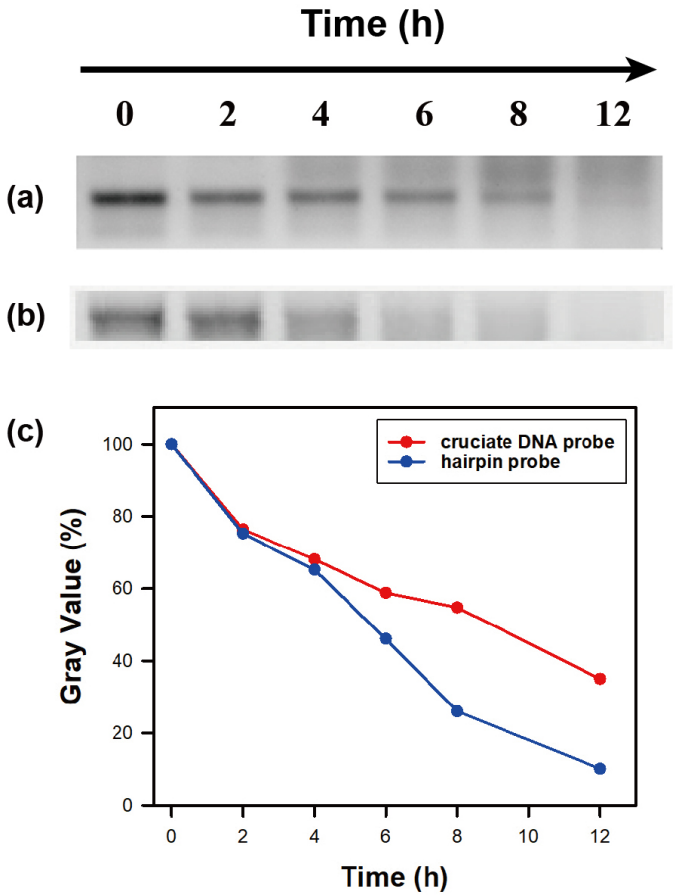

Fig. S1 Serum stability of cruciate DNA probe (a) and hairpin probe H1 (b). 3% agarose gel electrophoresis of probes after incubation with 10% FBS at 37 °C for 0 h, 2 h, 4 h, 6 h, 8 h, and 12 h. (c) The grayscale statistical result of degradation rates of cruciate DNA probe and hairpin probe corresponding to images a and b. The concentrations of cruciate DNA probe and hairpin probe were 1 μM.

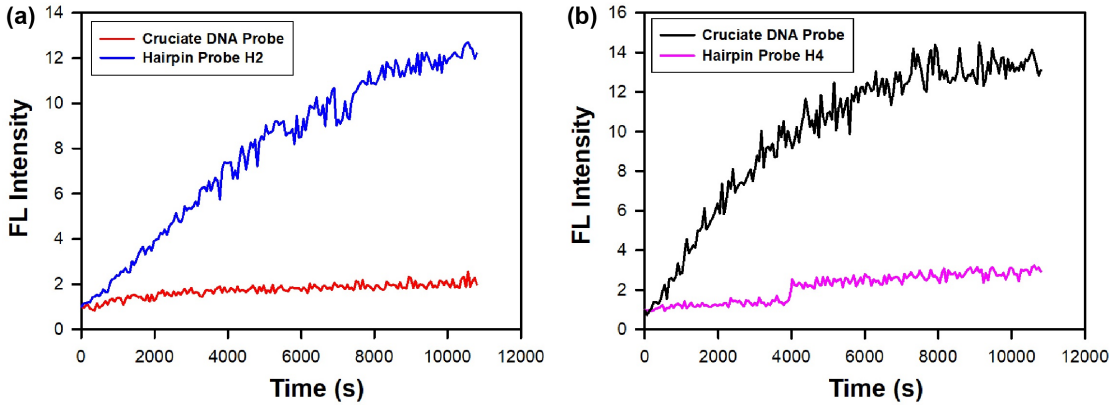

Fig. S2 Real-time fluorescence responses of cruciate DNA probe and hairpin probes in MDA-MB-231 cell lysate for stability analysis. (a) Fluorescence monitoring of 100 nM cruciate DNA probe and 100 nM hairpin probe H2 in the channel of Cy5 with 635 nm excitation. (b) Fluorescence responses of 100 nM cruciate DNA probe and 100 nM hairpin probe H4 in the FAM channel with 488 nm excitation.

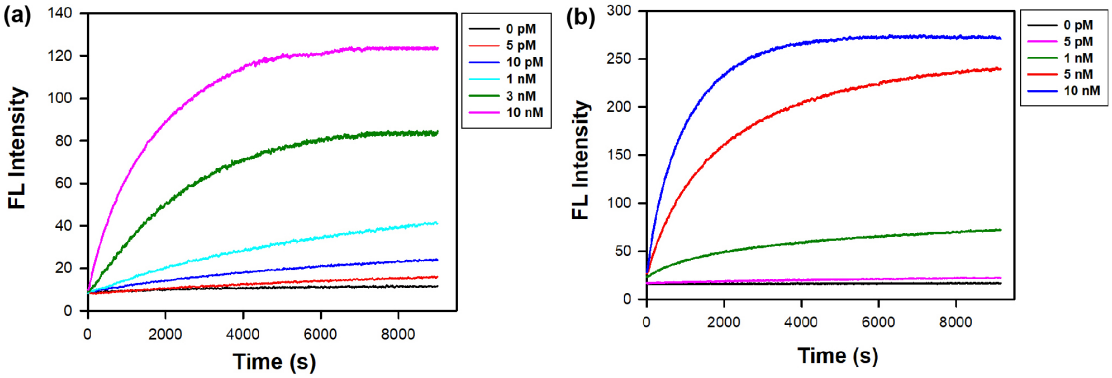

Fig. S3 Real-time fluorescence signals for 100 nM cruciate DNA probe to varying concentrations of target miRNA-21 (a) and miRNA-155 (b).

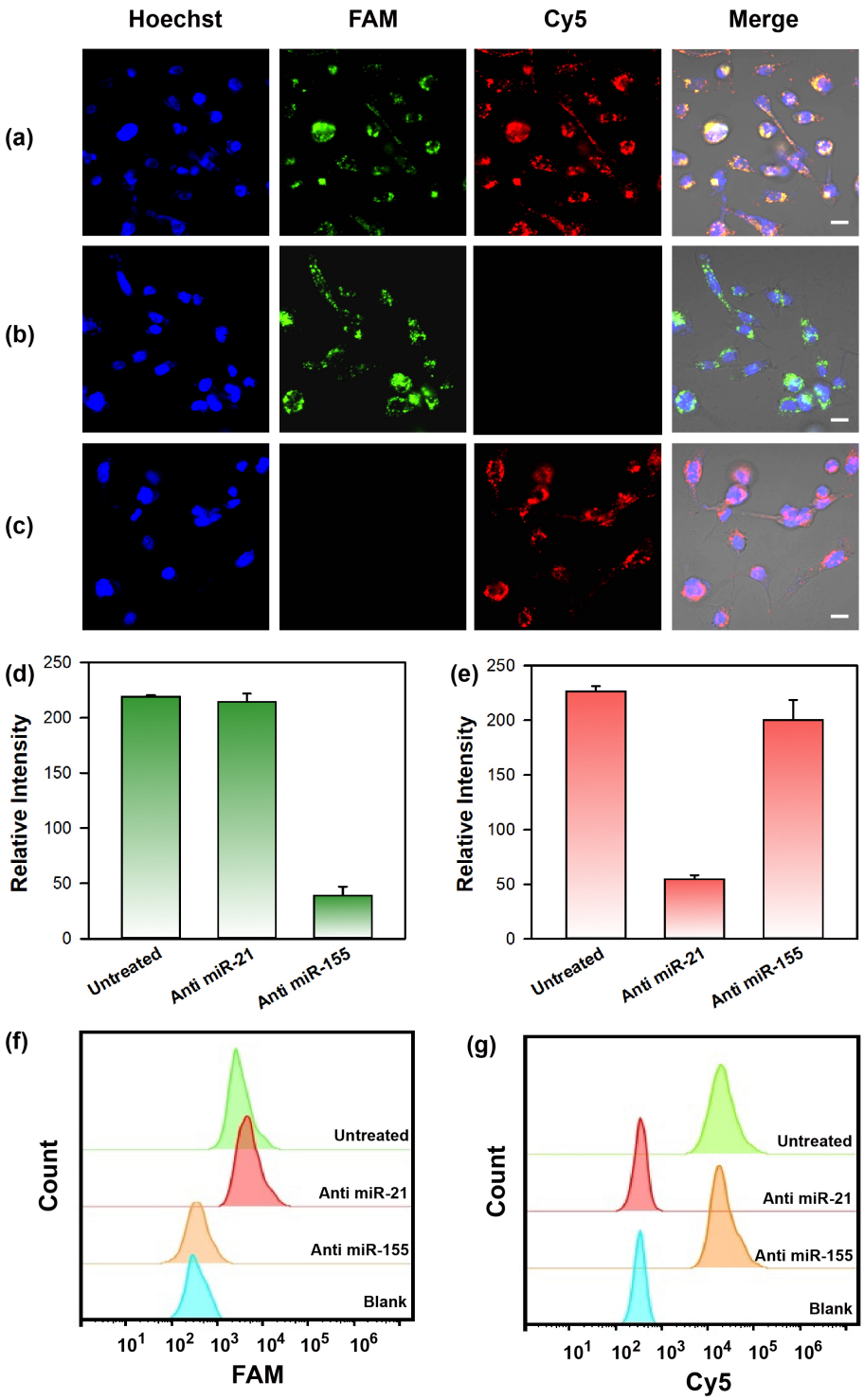

Fig. S4 Fluorescence images of untreated MDA-MB-231 cells (a), and MDA-MB-231 cells pretreated with 300 nM antisense miRNA-21 (b) or antisense miRNA-155 (c) followed by incubation with 100 nM cruciate DNA probe. Scale bar =10 μm. The mean fluorescence intensities (d, e) and flow cytometry analysis (f, g) of MDA-MB-231 cells corresponding to FAM (d, f) and Cy5 (e, g) channels in panels a, b, and c. Error bars represent standard deviations from three parallel assays.

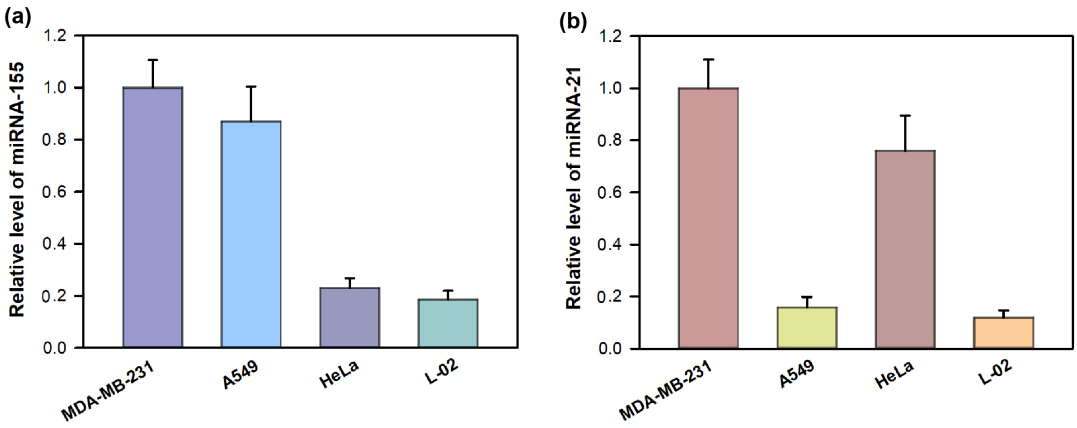

Fig. S5 Relative expression levels of miRNA-155 (a) and miRNA-21 (b) determined by qRT-PCR in MDA-MB-231, A549, HeLa, and L-02 cells. Error bars represent standard deviations from three parallel assays.

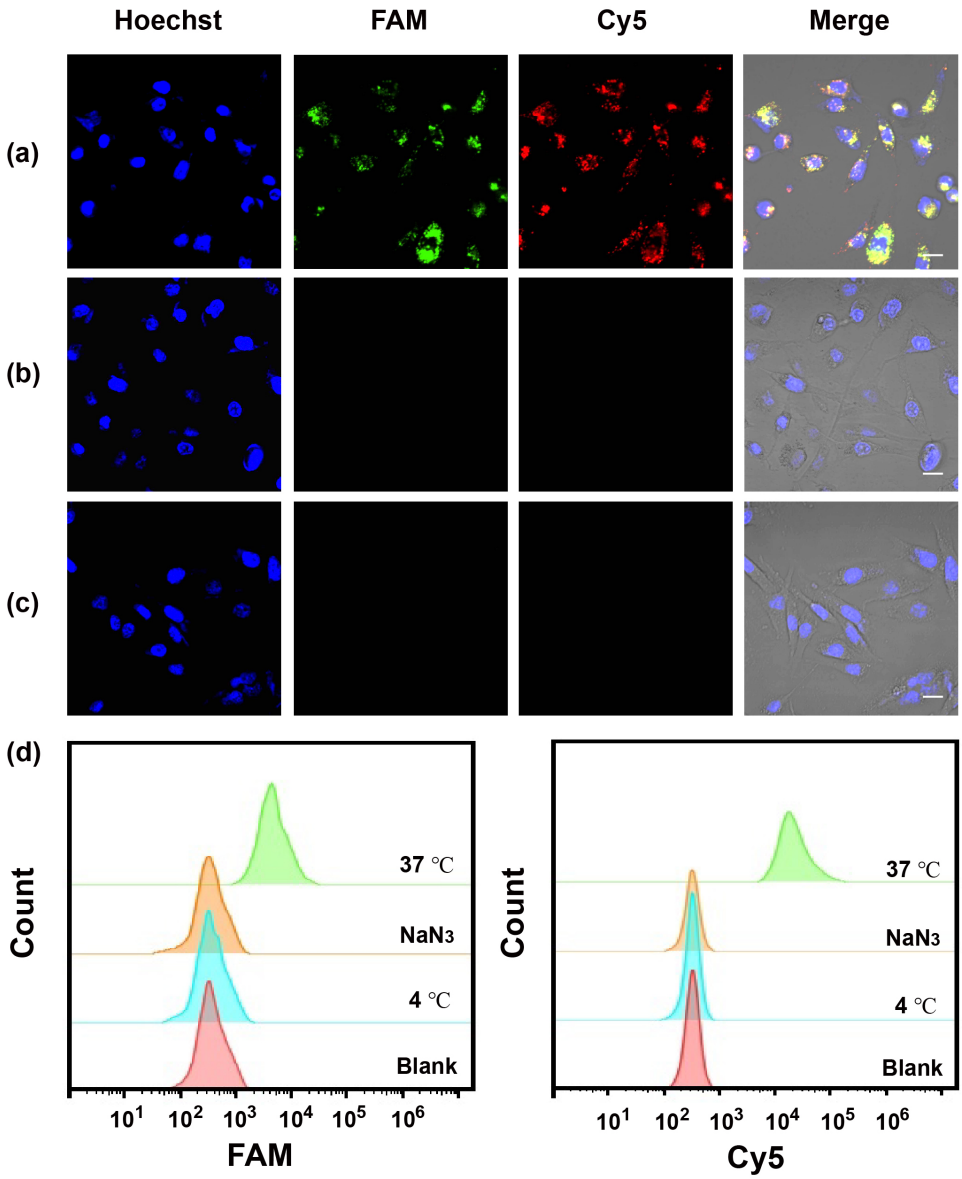

Fig. S6 Fluorescence images of MDA-MB-231 cells incubated with 100 nM cruciate DNA probe at 37 °C (a) and 4 °C (b), and MDA-MB-231 cells were pretreated with 0.1% NaN_3 followed by incubating with 100 nM cruciate DNA probe at 37 °C (c). (d) Flow cytometry analysis of MDA-MB-231 cells corresponding to panels a, b, and c. Scale bar =10 μm.

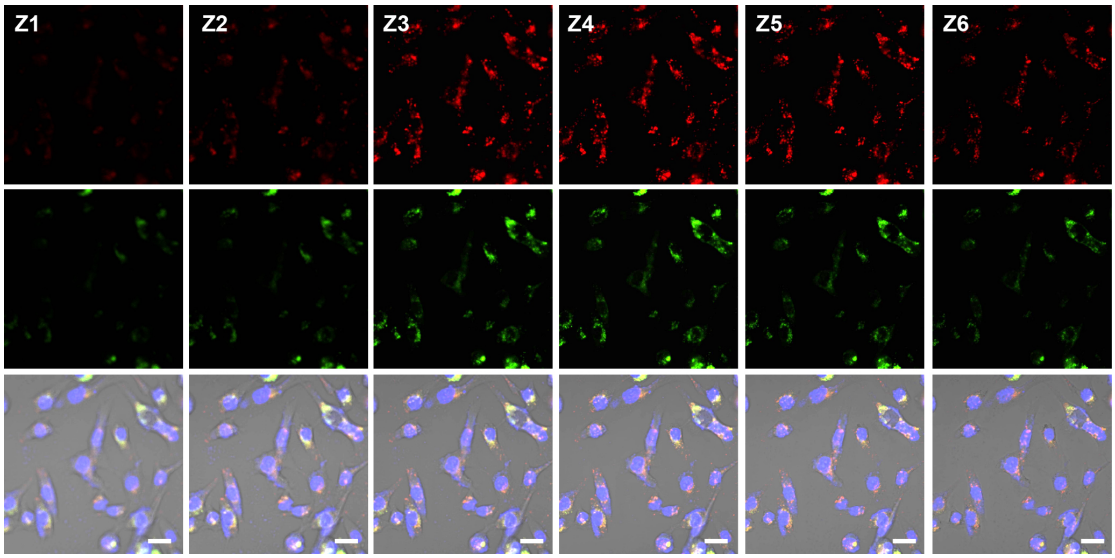

Fig. S7 Z-axis imaging of MDA-MB-231 cells after incubating with 100 nM cruciate DNA probe for 3 h. The images were taken at 2-μm z-axis intervals. Scale bar =10 μm.