Supplementary Material

In-situ imaging of mRNA transcripts based on split-aptamer and split protein in living cells

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Abstract

Messenger RNA (mRNA) is an essential component of cell development and growth. However, the detection of endogenous mRNA in living cells is currently limited. To address this issue, we have developed a novel strategy that comprises split-aptamer and split fluorescent protein dual-color, "turn-on" probes that specifically target mRNA with complementary sequences. Our split-aptamer and split-protein-initiated fluorescence complementation (sAPiFC) approach for live-cell imaging has demonstrated selectivity, stability, and capability for targeting various mRNAs.

Key Words: imaging; mRNA; split aptamer; split fluorescent protein; live-cell

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1.1 Experimental Procedures

1.1.1 Reagents

DNAs utilized in this study were synthesized and HPLC purified by Sangon Biotechnology Co., Ltd. (Shanghai, China). The DNA sequences are listed in Table S1. The stock solution of each DNA (100 μM) was prepared with TE buffer containing 10 mM Tris-HCl, 1 mM EDTA, and 12.5 mM $MgCl_2$ (pH 7.4). The 40% acrylamide mix solution, ammonium persulfate (APS), 1,2-bis(dimethylamino)- ethane (TEMED), and DNA ladder were acquired from Sangon Biotechnology Co., Ltd. (Shanghai, China). All the chemicals were of analytical grade and utilized as received without further purification. All oligonucleotides were purchased from Sangon Biotechnology Co., Ltd. (Shanghai, China). All sequencing experiments were performed at Sangon Biotechnology Co., Ltd. (Shanghai, China). DFHBI-1 T (MedChemExpress) and BI were synthesized by our laboratory, SDS-PAGE gel preparation kit, DAPI, and agarose were obtained from Beyotime (Shanghai, China). The HeLa cells (human cervical carcinoma cell line), and CHO cells (Chinese Hamster Ovary) were purchased from Procell, Inc.

1.1.2 Equipment

The Leica TCS SP8 laser confocal microscope (Leica Microsystems Inc., Exton, PA), 20 mm glass-bottom dish (Cellvis, Shanghai), Thermo Scientific™ Varioskan™ LUX (Thermo Scientific), and the UV-Vis absorption spectra were recorded on a UV-Vis spectrometer UV-3600 (Shimadzu, Japan).

1.1.3 RNA Transcription

All RNA sequences for in vitro assays were transcribed in vitro using DNA templates that contained a T7 promoter. The DNA template sequences can be found in Table S1. In vitro transcription was carried out following the instructions provided by the transcription kit. The transcription reaction contained T7 RNA polymerase, in vitro transcription buffer (20 mM Tris-HCl pH 7.5, 15 mM $MgCl_2$, 5 mM DTT, and 2 mM spermidine), and 2 mM of each ATP, CTP, GTP, and UTP was used in the transcription procedures (New England Biolabs). The reaction was incubated at 37°C for at least 4 hours, followed by stopping with RNA loading solution (95% formamide and 5 mM EDTA, with trace amounts of Xylene Cyanol FF and Bromophenol Blue). RNA purification was carried out using denaturing polyacrylamide gel electrophoresis (0.75 mm 6% TBE-PAGE, 8 M urea), and the bands corresponding to the expected product size were extracted and eluted by tumbling overnight in 300 mM Sodium Acetate pH 5.4. The elutes were precipitated in ethanol, resuspended in buffer (1 mM EDTA, 10 mM Tris-HCl pH 8.0), and the purified RNA was diluted to 50 μM and stored at -20 °C for further use.

1.1.4 In Vitro Assays

For in vitro assembly reactions on ice, a buffer (1 mM $MgCl_2$, 100 mM KCl, 40 mM HEPES pH 7.5) of 50 μL was prepared. Purified RNA products were also prepared on ice in the same buffer (1 mM $MgCl_2$, 100 mM KCl, 40 mM HEPES pH 7.5) containing BI at 10 μM. The fluorescence spectrum of the RNA products was compared to the fluorophore alone using a Multimode plate reader (ex = 472 nm). The samples were then preincubated at 37°C before being analyzed for fluorescence (ex = 472 nm, em = 490-700 nm or ex = 472 nm, em = 507 nm) using a Multimode plate reader at room temperature. Additional information regarding FISH probe sequences can be found in Table S2.

The purification of in vitro transcribed RNA was carried out using Trizol reagent, following the instructions provided by the manufacturer. 100 ng RNA was placed into a well of precast 10% TBE-Urea Gel and run at 300 V in 1 × TBE buffer. Following electrophoresis, the gel was rinsed three times for five minutes with water before being stained with 50 μM BI solution containing 1 mM $MgCl_2$, 100 mM KCl, and 40 mM HEPES pH 7.5. Visualization of the RNA was performed using a ChemiDoc MP (Bio-Rad) with excitation at 470 ± 15 nm and emission at 532 ± 14 nm. Subsequently, the gel was imaged using the SYBR Green channel (497 nm excitation and 520 ± 55 nm emission) on the same instrument.

1.1.5 Plasmid construction

The plasmid that expresses sAPiFC probes is obtained from the pSilence 2,1-U6 and contains a U6 promoter and pcDNA3.1+ plasmid. Before plasmid extraction and subsequent experiments, all constructs are accurately sequenced by Sangon Biotechnology Co., Ltd. (Shanghai, China), as documented in the Recombinant Vector Construction Information presented in Table S2.

1.1.6 Cell culture

Plasmid transfection and expression were carried out in HeLa or CHO cells at 37 °C in a humidified incubator with 5% CO_2. Cells were cultured in MEM medium (Gibco), supplemented with 100 unit/mL penicillin, 100 g/mL streptomycin sulfate, 2 mM L-glutamine, and 10% (v/v) fetal bovine serum (Invitrogen). Cells used for imaging analysis should be no more than 10 passages old. When cells reached 70-80% confluence, they were harvested by washing with 1 × PBS buffer and incubated with 1 mL of Trypsin-EDTA (0.25%) for 3-5 minutes at 37°C. The cells were then resuspended in 2 mL pre-warmed media and seeded in 12-well plates at a density of 8×10^5 cells/mL. After 24 hours of incubation, the cells should be 50% confluent.

1.1.7 Staining assay

On day 0, the cells were seeded into 12-well plates at a density of 1.0×10^5 cells/mL. On day 1, the cells were transfected with two vectors encoding sAPiFC probes. On day 2, the cells were detached with 0.25% trypsin and re-seeded

at 1.5-3.0 × 10^3 cells per dishes, precoated with fibronectin solution in PBS (50 μg/mL) in the complete growth medium, and then incubated at 37°C for 10 min in a humidified 5% CO_2 incubator. Cy3-DNA probes were co-transfected into cells, and target gene information is presented in Table S3, while Table S3 contains the sequence for Cy3-DNA probe information. After attaching the cells to the plate, they were stained with BI (final concentration: 10 μM), either with 10 μM BI with or without 10 μM DAPI (final concentration: 1 μg/mL, Life Technologies) by simply adding 1 from a stock solution in DMSO (1 mM) and DAPI from a stock solution in H_2O (1 mg/mL), growth medium (60 μL), and incubated at 37 °C for 10 min in a humidified 5% CO_2 incubator, and subsequently imaged on a Leica TCS SP8 confocal microscope (Leica Microsystems Inc., Exton, PA).

1.1.8 Observation of fluorescence using Confocal Laser Scanning Microscope (CLSM)

To stabilize the microscope, activate the temperature control system of the incubation chamber at least 30 minutes before imaging. Before imaging with the final imaging solution, which may consist of PBS buffer or MEM supplemented with 1 mM $MgCl_2$, 100 mM KCl, and 40 mM HEPES pH 7.5), cells should be incubated for 10 minutes. The imaging solution should include 10 μM BI, with or without 10 μM DAPI (Life Technologies). Images should be captured at 37°C using a laser confocal microscope, specifically a Leica TCS SP8 fitted with a HyD CCD camera with a 40 oil objective. The Green channel (Ex: 470 ± 15 nm; Em: 510 ± 20 nm) is utilized to activate the reassembled aptamer, with an exposure duration of 500 ms. The Red channel (Ex: 579 ± 12 nm; Em: 599 ± 20 nm), exposure time (100 ms); Blue channel (Ex: 358 ± 15 nm; Em: 461 ± 20 nm), exposure time: 50 ms. To avoid overexposure, the laser light intensity should be set to 10-15%. Cells should be seeded and transfected with plasmid in a glass-bottom dish for 24 hours before the *UHRF1* or *survivin* mRNA imaging experiment (refer to Table S4).

1.2 Results

1.2.1 Signal to Noise ratio of Broccoli with DFHBI-1T or BI (Figure. S1)

Purified RNA products of Broccoli at 1 μM concentrations were prepared on ice independently, in buffer (40 mM HEPES pH 7.5, 100 mM KCl, 5 mM $MgCl_2$) containing DFHBI-1T or BI at 10 μM, and the spectrum was compared to that of fluorophore alone by Multimode plate reader (ex = 472 nm). Samples were added to a clear, black-bottom 96-well plate and preincubated at 37°C, and then measured for fluorescence (ex = 472 nm, em = 490-700 nm) on a Multimode plate reader (Thermo Fisher Scientific) at room temperature.

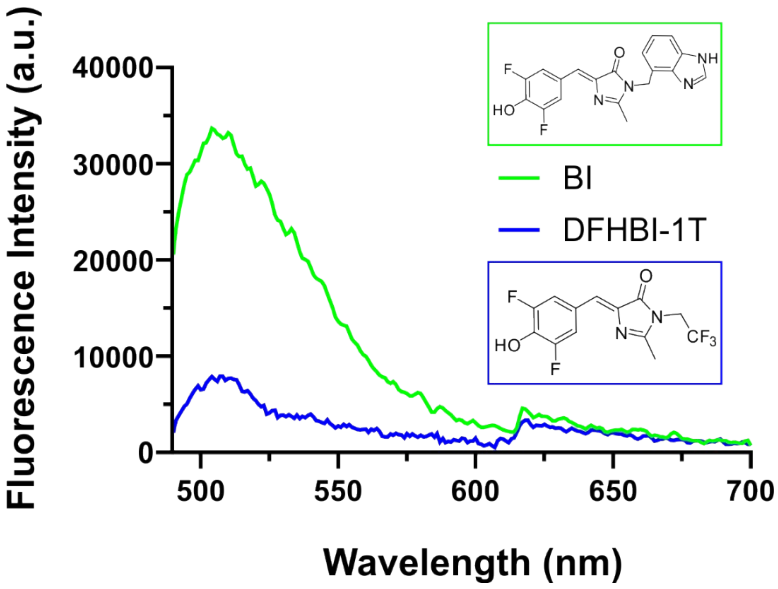

Figure. S1. The fluorescence intensity of 1 μM Broccoli was obtained in the presence of 10 μM BI or DFHBI-1T fluorophore, respectively.

1.2.2 Signal to Noise ratio of sAPiFC probe in vitro (Figure. S2)

In vitro assembly reactions were prepared on ice in buffer (40 mM HEPES pH 7.5, 100 mM KCl, 1 mM $MgCl_2$) at a final volume of 50 μL. Purified RNA products of RNA at 500 nM concentrations were prepared on ice independently, in buffer (40 mM HEPES pH 7.5, 100 mM KCl, 5 mM $MgCl_2$) containing BI at 1 μM and the spectrum was compared to that of fluorophore alone by Multimode plate reader (ex = 472 nm). Samples were added to a clear, black-bottom 96-well plate and preincubated at 37°C, and then measured for fluorescence (ex = 472 nm, em = 507 nm) on a Multimode plate reader (Thermo Fisher Scientific) at room temperature.

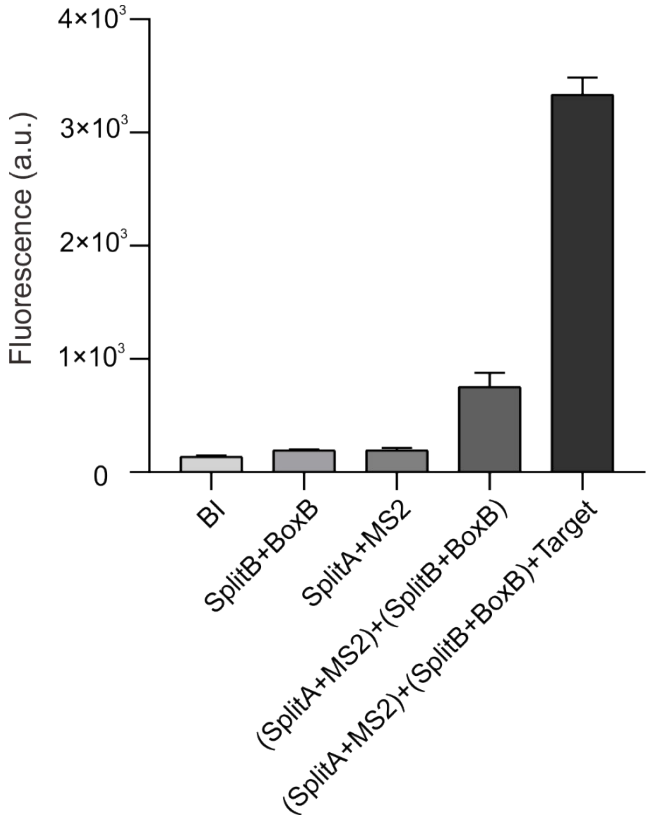

Figure. S2. The signal-to-noise ratio of sAPiFC probe (500 nM) in the presence of BI (1 μM).

1.3 Supplementary Tables

Table S1. T7 DNA template and reverse sequence used for in vitro T7 transcription. The underlined parts are the recognition sequence after transcription.

T7 transcription (DNA template and reverse sequence) used in the experiment (5'-3')	
SplitA-MS2-5 (target *β-actin*)	CGAAATTAATACGACTCACTATAGGGGGAGACGGTCGGGTCCAGTATGTCACCT
SplitA-MS2-3 (target *β-actin*)	TCAGGATTTAACATGGGTGATCCTCATGTACTGGAACGGTGAAGGTGACATACT
SplitB-BoxB-5 (target *β-actin*)	CGAAATTAATACGACTCACTATAGGGTGTGAACTTTGGCCCTGAAAAAGGGCCGGA
SplitB-BoxB-3 (target *β-actin*)	GGAGCCCACACTCTACTCGACAGTACGTCGGTTGGAGCGAGCATCCGGCCCTTTT
SplitA-MS2-5 (target *UHRF1*)	CGAAATTAATACGACTCACTATAGGGGGAGACGGTCGGGTCCAGTATGTCAGCC
SplitA-MS2-3 (target *UHRF1*)	GGTGGAGGAGACATGGGTGATCCTCATGTGACTCGCTGTCCAGGCTGACATACT
SplitB-BoxB-5 (target *UHRF1*)	CGAAATTAATACGACTCACTATAGGGCTCCTCCACCGGCCCTGAAAAAGGGCCGTC
SplitB-BoxB-3 (target *UHRF1*)	GGAGCCCACACTCTACTCGACAGTACGACTCGCTGTCCAGGCTGACGGCCCTTTT
SplitA-MS2-5 (target *survivin*)	CGAAATTAATACGACTCACTATAGGGGGAGACGGTCGGGTCCAGTATCTTATTG
SplitA-MS2-3 (target *survivin*)	AAAGAATTTGACATGGGTGATCCTCATGTCAAAGGAAACCAACAATAAGATACT
SplitB-BoxB-5 (target *survivin*)	CGAAATTAATACGACTCACTATAGGGTTCCAGCTCCGGCCCTGAAAAAGGGCCGGG
SplitB-BoxB-3 (target *survivin*)	GGAGCCCACACTCTACTCGACAGTACTGGGAGCCAGATGACGACCCGGCCCTTTT
β-actin target-5	CGAAATTAATACGACTCACTATAGGGTCAGGATTTAAAAACTGGAACGGTGAAG
β-actin target-3	TGTGAACTTTGGGGGATGCTCGCTCCAACCGACTGCTGTCACCTTCACCGTTCC
UHRF1 target-5	CGAAATTAATACGACTCACTATAGGGGGTTCGGACCATGGACGGGAGGCAGACC
UHRF1 target-3	CTCCTCCACCTTGGTCAGCCTGGACAGCGAGTCCACCGTGTGGGTCTGCCTCCC
survivin target-5	CGAAATTAATACGACTCACTATAGGGGGAGCTGGAAGGCTGGGAGCCAGATGAC
survivin target-3	CAAATTCTTTCTTCTTATTGTTGGTTTCCTTTGCATGGGGTCGTCATCTGGCTC

Table S2. Recombinant Vector Construction Information (Brackets represent restriction sites, yellow fonts represent MCP coat protein or λN protein fusion, gray represents linker sequences, and red represents the N or C terminus of mcherry)**.** Sequence alignment results are at the back of the table.

SplitA-MS2 (target *β-actin*)	GCAGGATCCCGAAATTAATACGACTCACTATAGGGGGAGACGGTCGGGTCCAGTATGTCACCTT CACCGTTCCAGTACATGAGGATCACCCATGTTAAATCCTGAAAGCTTACG
SplitB-BoxB (target β-actin)	GCAGGATCCCGAAATTAATACGACTCACTATAGGGTGTGAACTTTGGCCCTGAAAAAGGGCCGG ATGCTCGCTCCAACCGACGTACTGTCGAGTAGAGTGTGGGCTCCAAGCTTACG
SplitA-MS2 (target UHRF1)	GCAGGATCCCGAAATTAATACGACTCACTATAGGGGGAGACGGTCGGGTCCAGTATGTCAGCCT GGACAGCGAGTCACATGAGGATCACCCATGTCTCCTCCACCAAGCTTACG
SplitB-BoxB (target UHRF1)	GCAGGATCCCGAAATTAATACGACTCACTATAGGGCTCCTCCACCGGCCCTGAAAAAGGGCCGT CAGCCTGGACAGCGAGTCGTACTGTCGAGTAGAGTGTGGGCTCCAAGCTTACG
SplitA-MS2 (target *survivin*)	GCAGGATCCCGAAATTAATACGACTCACTATAGGGGGAGACGGTCGGGTCCAGTATCTTATTGT TGGTTTCCTTTGACATGAGGATCACCCATGTCAAATTCTTTAAGCTTACG
SplitB-BoxB (target *survivin*)	GCAGGATCCCGAAATTAATACGACTCACTATAGGGTTCCAGCTCCGGCCCTGAAAAAGGGCCGG GTCGTCATCTGGCTCCCAGTACTGTCGAGTAGAGTGTGGGCTCCAAGCTTACG
MCP coat protein sequence+(GGGGS)3+ N-Mcherry (DNA sequences)	GCAAAGCTTATGGCTTCTAACTTTACTCAGTTCGTTCTCGTCGACAATGGCGGAACTGGCGACGT GACTGTCGCCCCAAGCAACTTCGCTAACGGGATCGCTGAATGGATCAGCTCTAACTCGCGTTCA CAGGCTTACAAAGTAACCTGTAGCGTTCGTCAGAGCTCTGCGCAGAATCGCAAATACACCATCA AAGTCGAGGTGCCTAAAGGCGCCTGGCGTTCGTACTTAAATATGGAACTAACCATTCCAATTTTC GCCACGAATTCCGACTGCGAGCTTATTGTTAAGGCAATGCAAGGTCTCCTAAAAGATGGAAACC CGATTCCCTCAGCAATCGCAGCAAACTCCGGCATCTACGGAGGAGGGGGATCCGGTGGAGGCG GCAGTGGAGGCGGGGGATCTGTGAGCAAGGGCGAGGAGGATAACATGGCCATCATCAAGGAGT TCATGCGCTTCAAGGTGCACATGGAGGGCTCCGTGAACGGCCACGAGTTCGAGATCGAGGGCGA GGGCGAGGGCCGCCCCTACGAGGGCACCCAGACCGCCAAGCTGAAGGTGACCAAGGGTGGCCC CCTGCCCTTCGCCTGGGACATCCTGTCCCCTCAGTTCATGTACGGCTCCAAGGCCTACGTGAAGC ACCCCGCCGACATCCCCGACTACTTGAAGCTGTCCTTCCCCGAGGGCTTCAAGTGGGAGCGCGT GATGAACTTCGAGGACGGCGGCGTGGTGACCGTGACCCAGGACTCCTCCCTGCAGGACGGCGA GTTCATCTACAAGGTGAAGCTGCGCGGCACCAACTTCCCCTACGACGGCCCCGTAATGCAGAAG AAGACCATGGGCTGGGAGGCCTCCTCCGAGCGGATGTACCCCGAGGACGGCGAATTCACG
λN coat protein sequence+(GGGGS)3+ C-Mcherry (DNA sequences)	GCAAAGCTTAATGCGCGGACCCGCCGACGTGAACGGCGAGCAGAAAAACAGGCGCAATGGAA AGCAGCCAACGGCGGCGGTGGAAGTGGAGGTGGTGGAAGCGGAGGCGGTGGTAGTTACTTGAA GCTGTCCTTCCCCGAGGGCTTCAAGTGGGAGCGCGTGATGAACTTCGAGGACGGCGGCGTGGTG ACCGTGACCCAGGACTCCTCCCTGCAGGACGGCGAGTTCATCTACAAGGTGAAGCTGCGCGGCA CCAACTTCCCCTACGACGGCCCCGTAATGCAGAAGAAGACCATGGGCTGGGAGGCCTCCTCCGA GCGGATGTACCCCGAGGACGGCGCCCTGAAGGGCGAGATCAAGCAGAGGCTGAAGCTGAAGGA CGGAGGCCACTACGACGCTGAGGTCAAGACCACCTACAAGGCCAAGAAGCCCGTGCAGCTGCC CGGCGCCTACAACGTCAACATCAAGTTGGACATCACCTCCCACAACGAGGACTACACCATCGTG GAACAGTACGAACGCGCCGAGGGCCGCCACTCCACCGGCGGCATGGACGAGCTGTACAAGTAA GAATTCACG

Table S3. FISH probe information.

Cy3 labeled target *β-actin* probe	5'-Cy3- CTGCTGTCACCTTCACCGTTCCAGT -3'
Cy3 labeled target *UHRF1* probe	5'-Cy3- GACCTCGTAGTCGAAGAGGGTATGG -3'
Cy3 labeled target *survivin* probe	5'-Cy3- TCTGGCTCGTTCTCAGTGGGGCAGT -3'

Table S4. The target mRNA sequence for sAPiFC recognition. The cDNA sequence of the following human mRNA was obtained from NCBI. The mRNA sequence which is complementary to the pair of sAPiFC recognition is underlined.

Target mRNA	NCBI Reference Sequence	mRNA sequence (5'-3') complementary to the FaApt recognition sequence
β-actin	NM_001369512.1	CTATGACTTAGTTGCGTTACACCCTTTCTTGACAAAACCTAACTTGCGCA
UHRF1	NM_001048201.3	GGTTCGGACCATGGACGGGAGGCAGACCCA
survivin	NM_001012270.2	GGAGCTGGAAGGCTGGGAGCCAGATGACGA