Supporting Information

Quantitative Detection and Real-Time Monitoring on Endogenous mRNA in Single Live Cell Level using Ratiometric Molecular Beacon

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Sequences

In our research, we designed two ratiometric molecule beacons (RMBs) targeting different regions of Hsp27 mRNA and a control RMB (random RMB) randomly selected by targeting *Haemophilus parasuis*; their sequences are listed in Table S1. Moreover, the sequences of FISH probes and primers are also listed in Table S1. All the RMBs, FISH probes, and primers were obtained from Sangon Biotech (Shanghai, China).

Table S1. Sequences of RMBs, FISH probes and primers. For RMBs, the bases modified by Cy5 are shown in *italics*; BHQ-1: Black Hole Quencher 1; FAM: Carboxyfluorescein; _: stem sequence; *: phosphorothioate modification; m: 2-O-methyl modification. For primers, lowercase letters: restriction site, lowercase letters in *italics*: T7 promoter sequence.

Probe	Sequence (5'---3')
RMB1	5'- /FAM/ mGmCmAmGmGmCmG*mG*mU*mG*mU*mU*mU*mG*mA* mG*mG*mU*mC*mU*mC*mU*mA*mU*mU*mA*mA*mGmCmCmU mGmC /BHQ-1/ -3'
RMB2	5'- /FAM/ mCmCmGmUmGmGmC*mU*mA*mG*mC*mU*mU*mG*mG* mG*mC*mA*mU*mG*mG*mG*mG*mG*mC*mU*mU*mCmCmAmC mGmG/BHQ-1/ -3'

Random RMB	5’- /FAM/ mCmCmUmGmCmUmG*mC*mC*mA*mC*mA*mG*mU**mU*** mA*mC*mA*mG*mA*mU*mU*mG*mA*mC*mAmGmCmAmGmG/BHQ-1/ -3’
Hsp27 FISH probe	5’- /Cy3/ CACAAA ACACGCTGCCCCCCGG -3’
Hsp27 FISH probe-2	5’- /Cy3/ CACAAA ACACGCTGCCCCCCGG/BHQ-2/ -3’
Control FISH probe	5’- /Cy3/ CAATTGTTCCAGGAACCAGG -3’
Control FISH probe-2	5’- /Cy3/ CAATTGTTCCAGGAACCAGG/BHQ-2/ -3’
Flag-Hsp27 F	5’- GCgaattcTGGGGAGGGGCGTCCCTCAAA -3’
Flag-Hsp27 R	5’- GCctcgagTAGGTGGGTTACTTGGAAC -3’
Flag-GAPDH F	5’- GCgaattcATGGGGAAGGTGAAGGTCGG -3’
Flag-GAPDH R	5’- GCctcgagTTACTCCTTGGAGGCCATGTGGG -3’
Q-Hsp27 F	5’- GGACGAGCATGGCTACATCT -3’
Q-Hsp27 R	5’- GGATGGTGATCTCGTTGGAC -3’
Q-GAPDH F	5’- GTCAGTGGTGGACCTGACCT -3’
Q-GAPDH R	5’- GAAGAGTGGGTGTCGCTGTT -3’
T7-Hsp27 F	5’- *taatacgactcactatagg*GTGGGGAGGGGCGTCCCTCAAA -3’
T7-Hsp27 R	5’- TAGGTGGGTTACTTGGAAC -3’

Optimization of SLO for RMB Delivery

The concentration and incubation time of SLO solution were optimized to achieve rapid RMB delivery as well as maintain high cell viability. CCK-8 (Cell Counting Kit-8) assay was performed to determine the cytotoxic effect of the SLO solution [S1]. 1×10^4 Vero cells in suspension were first seeded into each well of a 96-well culture plate and incubated for 24 h at 37 °C in a 5% CO_2 atmosphere incubator. After being washed with sterilized 1×PBS three times, the cells were treated with serum-free medium containing various concentrations of SLO from 0.25 HU/mL to 32 HU/mL. After 15 min, the SLO solution was removed, and the cells were washed with typical growth medium three times. Next, 100 μL typical growth medium with 10 μL CCK-8 was added into each well, followed by further incubation for 2 h in a 5% CO_2 atmosphere incubator. Absorbance at the wavelength of 450 nm was finally measured using a microplate reader (Tecan, Infinite F200, Swiss) for cell viability detection as shown in Figure S1(A). In order to deliver RMB into Vero cells rapidly, a high SLO concentration is preferred, but higher SLO concentration caused lower cell viability. According to Figure S1(A), when the SLO concentration was 1 HU/mL, the cell viability was still high, thus this SLO concentration was used for RMB delivery. After the determination of the SLO concentration, the incubation time of the SLO solution was optimized. 200 nM RMB (RMB1 or RMB2) and 1 HU/mL SLO solution were incubated with Vero cells for different incubation times, and Figure S1(B) illustrates the RMB delivery efficiency for RMB1 and RMB2, respectively. The results in Figure S1(B) show that in the period between 0 min to 20 min, both of the RMB's delivery efficiency increased with more incubation time, indicating that RMB was delivered into the Vero cells during this period. When the incubation time reached 10 min, the RMB delivery efficiency kept stable at ~95%, illustrating that most of RMB was delivered into Vero cells within the incubation

time of 12 min. Therefore, the incubation time of SLO solution was chosen as 12 min.

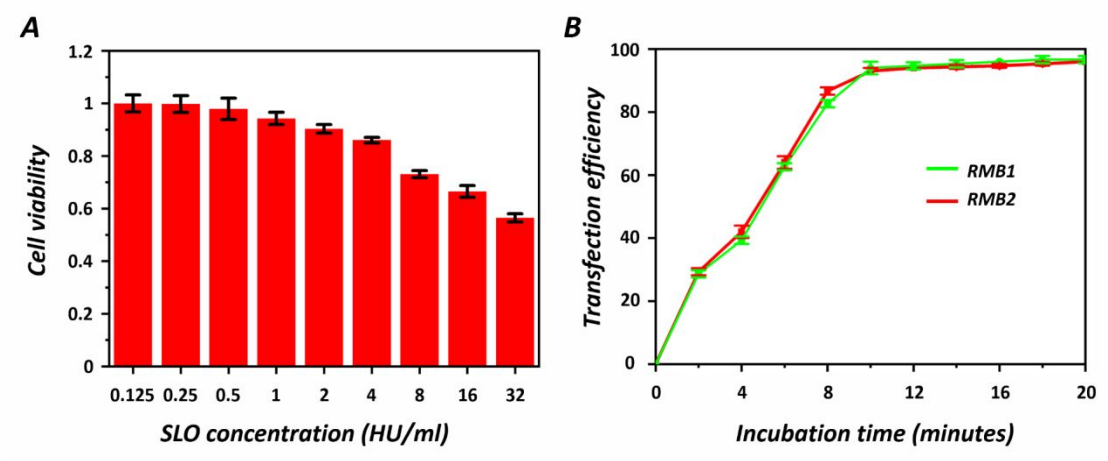

Figure S1. Optimization of SLO for RMB delivery. (A) Cell viability with different SLO concentrations from 0.125 HU/mL to 32 HU/mL. (B) Delivery efficiency of RMB1 and RMB2 with different SLO incubation times from 0 min to 20 min.

Co-localization of RMB1 signals and lysosome signals

We used LysoTracker Red DND-99 (Invitrogen™ L7528) to label the lysosome, according to the additional experiment in Figure S2, the co-localization coefficient of Cy5 and LysoTracker signals was 0.407, which was only slightly higher than the background signals of the random probe, indicating no much RMB was in the lysosome. Two representative fluorescent intensity plots along the white dotted Line 1 and Line 2 in Figure S2A are also presented in Figure S2B, showing that where FAM and Cy5 signals co-localized, there were no many lysosome signals, and also where lysosome signals were high, there were no many FAM or Cy5 signals, indicating the RMB did not enter into the lysosome. Therefore, FAM used in the RMB was suitable in our research.

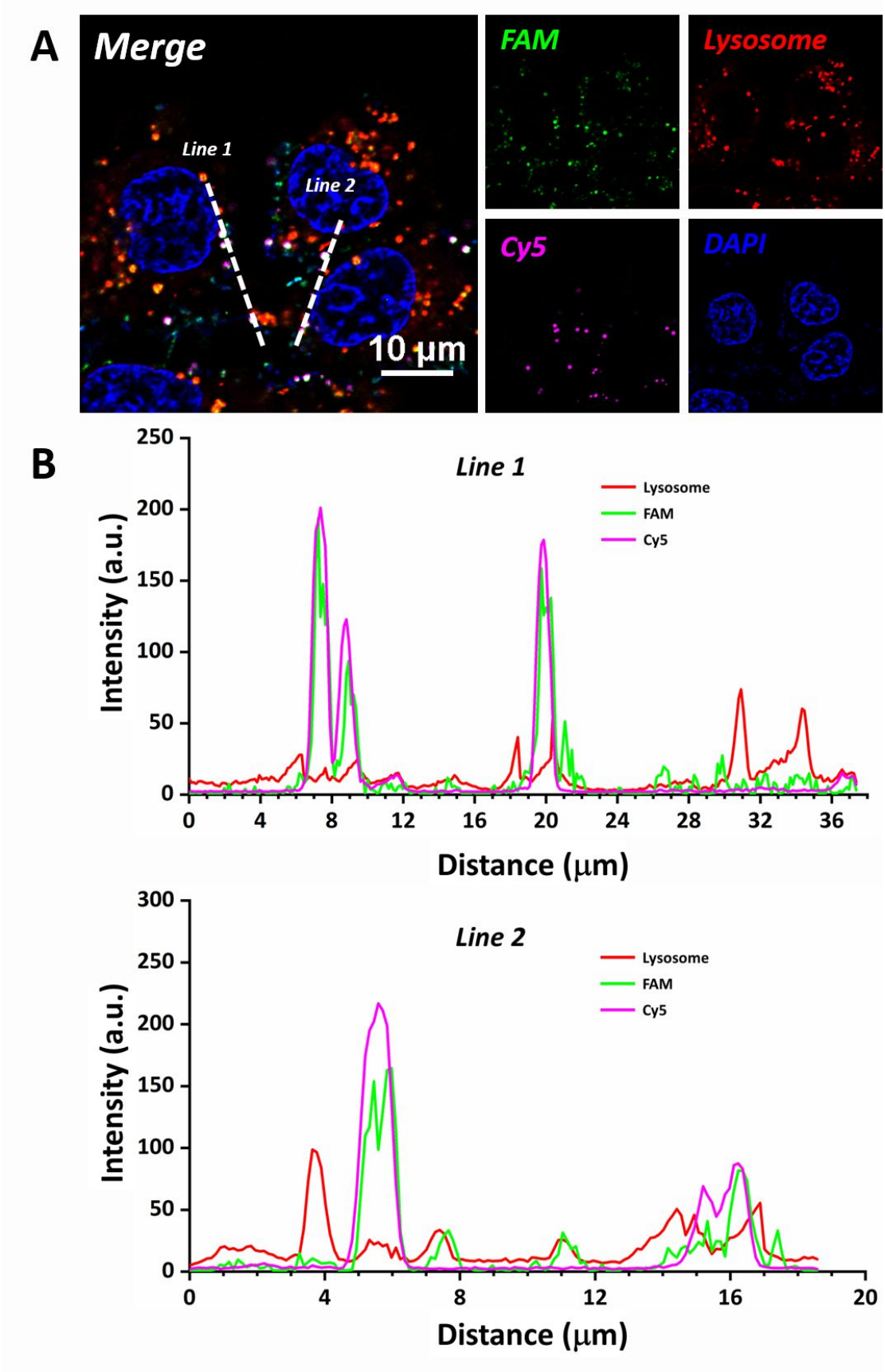

Figure S2. Analysis of the co-localization of RMB1 and lysosome. (A) Multimode imaging on live

Vero cells. The separated small figures list the FAM, Cy5, DAPI and LysoTracker Red DND-99 (lysosome) signals, respectively. (B) Fluorescent intensity plots along the white dotted Line 1 and Line 2 in (A), respectively.

Co-localization of RMB1 signals and FISH signals

We used fixed cells to show co-localization of RMB signals and FISH (Hsp27 FISH probe-2) signals in Figure S3. The co-localization coefficient of Cy5 and probe signals was 0.784. Therefore, RMB signals were real and they co-localized very well with FISH signals and detected the same mRNA targets as FISH.

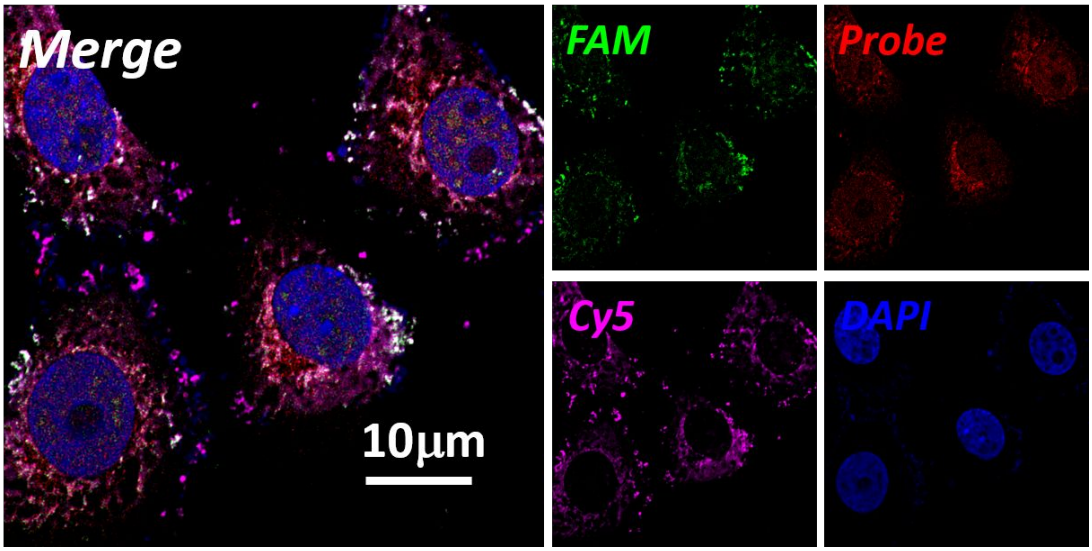

Figure S3. Analysis of the co-localization of RMB1 and FISH signals. Multimode imaging on fixed Vero cells. The separated small figures list the FAM, Cy5, DAPI and FISH probe signals, respectively.

GAPDH and Hsp27 Measurements using Real-Time PCR

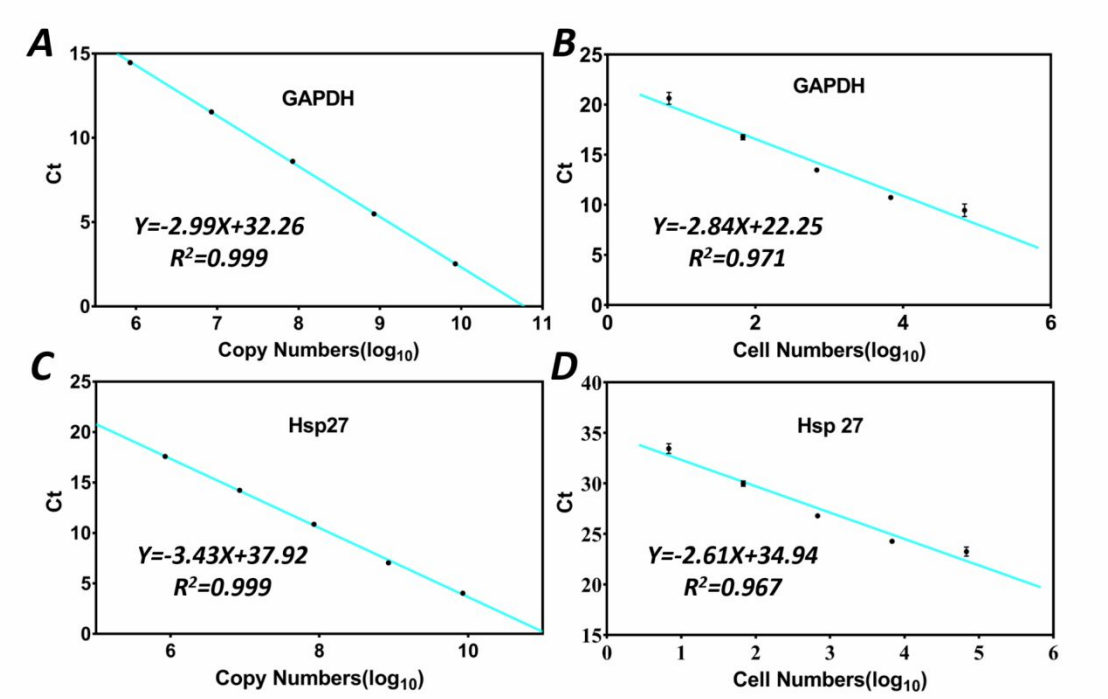

Figure S4. GAPDH and Hsp27 measurements using real-time PCR. (A) Linear fitted relation between the GAPDH copy numbers and the Ct values using a pair of specific primers Q-GAPDH F/R. (B) Linear fitted relation between the GAPDH cell numbers and the Ct values using a pair of specific primers Q-GAPDH F/R. (C) Linear fitted relation between the Hsp27 copy numbers and the Ct values using a pair of specific primers Q-Hsp27 F/R. (D) Linear fitted relation between the Hsp27 cell numbers and the Ct values using a pair of specific primers Q-Hsp27 F/R.

Real-Time Quantitative Hsp27 mRNA Measurements at the Single Live Cell Level

Video S1. Merged fluorescent images of a field of view in DIC, DAPI, FAM, and Cy5 channels on pCMV-flag-hsp27 transfected Vero cells after RMB1 was delivered at different times during the observation period of 4 h.

References

(S1) Santangelo, P. J.; Nix, B.; Tsourkas, A.; Bao, G. Dual FRET Molecular Beacons for mRNA Detection in Living Cells. *Nucleic Acids Res.* 2004, *32*, e57.