Electronic Supplementary Information (ESI)

for

Target-triggered enzyme-free amplification for highly efficient AND-gated bioimaging in living cells

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1 Materials and reagent

DNA oligonucleotides were synthesized and purified by Shanghai Sangon Biological Engineering Technology & Services (Shanghai, China). The sequences of the used oligonucleotides (oligos) are listed in detail in Table S1. The human non-small cell lung cancer cells (A549 cells), human cervical carcinoma cells (HeLa cells), and human embryonic kidney 293T cells (HEK 293T cells) used in the experiment were obtained from the Shanghai Cell Bank of the Chinese Academy of Sciences. Glutathione (GSH), alpha-lipoic acid (ALA), and N-ethylmaleimide (NEM) were purchased from Aladdin (Shanghai, China). Histidine (His), leucine (Leu), isoleucine (Ile), lysine (Lys), methionine (Met), proline (Pro), and tyrosine (Tyr) were purchased from Sigma Aldrich (Shanghai, China). Cell Counting Kit-8 (CCK-8) was purchased from Shanghai Sangon Biological Engineering Technology & Services (Shanghai, China). Opti-MEM, Dulbecco's Modified Eagle Media (DMEM), Fetal Bovine Serum (Corning, New Zealand), and liposome 3000 were purchased from Thermo Fisher Scientific. Tris-HCl buffer (20 mM Tris-HCl, 150 mM NaCl, 5.0 mM MgCl2, pH 7.4) was prepared in the laboratory. Ultrapure water was used throughout the whole experiment, which was purified by a Milli-Q reference system (Millipore, USA).

2 Apparatus

Fluorescence measurements were recorded using an RF-6000 spectrophotometer (Tokyo, Japan) and Cell Counting Kit-8 (CCK-8) was performed at 450 nm using Thermo Fisher Scientific's Varioskan Flash microplate reader (Shanghai, China). The T20S Series Thermal Cycler for Porous Plates was purchased from Long Gene (Beijing, China) and the FireReader V10 Gel Imager was purchased from UVITEC (UK). Confocal fluorescence images of cells were obtained using LEICA TCS SP8 laser scanning confocal microscopy (Beijing, China).

3 Selective experiments

To evaluate the selectivity of the GHCR system, several different amino acids (His, Leu, Ile, Lys, Met, Pro, Tyr) were selected to replace GSH. Several amino acids were added to the GHCR system, miR-1246 was added, and incubated at 37°C for 35 min, and then the fluorescence intensity ratio of acceptor Cy5 to donor Cy3 (F_A/F_D) was measured. Similarly, other miRNAs (miR-21, miR-26a, miR-29a, miR-203) were selected to replace miR-1246, these miRNAs were added to the system, GSH was added, incubated at 37°C for 35 min, and then the fluorescence intensity ratio of acceptor Cy5 to donor Cy3 (F_A/F_D) was measured.

4 Cell culture and cytotoxicity

A549 cells were cultured using RPMI 1640 medium containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin solution (100 ×) and Hela and HEK 293T cells were cultured using DMEM medium containing 10% FBS and 1% penicillin-streptomycin solution (100 ×). All cells were cultured in an incubator containing 5% CO_2 at 37 °C. Cytotoxicity of the GHCR system was assessed using the CCK-8 trial. A549 and HEK 293T cells were transplanted into 96-well plates overnight at a density of 2×10^4 cells/well. Add GH1 and H2 at different concentrations (0~200 nM) to 100 μL of DMEM complete medium. After incubation for different times (24 h and 48 h), the cell culture medium was removed and washed with Dulbecco phosphate-buffered saline (DPBS). Subsequently, 90 μL of new complete medium and 10 μL of DMSO dissolved CCK-8 were added, mixed well, and incubated for 35 min. Cytotoxicity data were obtained by measuring the absorbance value (450 nm) and untreated cells were used as negative controls.

5 Supplementary scheme and figures

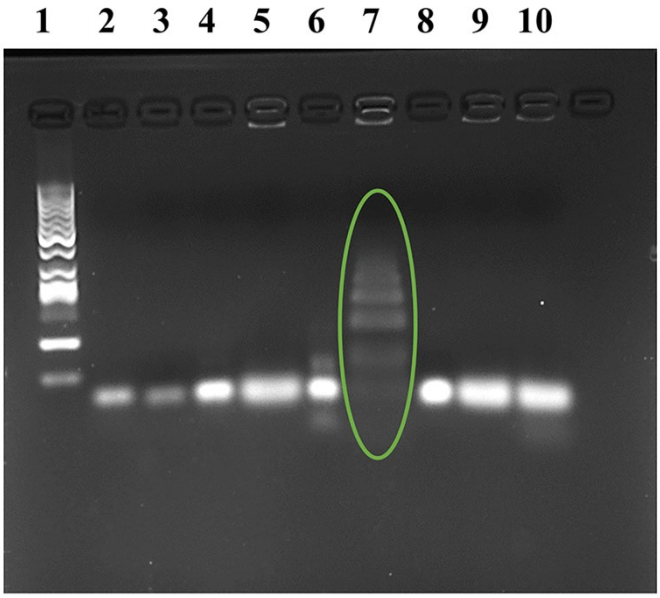

Fig. S1. Agarose gel electrophoresis image assay for monitoring the HCR process. lane1: DNA marker, lane2: GH1, lane3: H2, lane4: GH1+H2, lane5: GH1+H2+GSH, lane6: GH1+H2+miR-1246, lane7: GH1+H2+GSH+miR-1246, lane8: NH1+H2+GSH, lane9: NH1+H2+miR-1246, lane10: NH1+H2+GSH+miR-1246.

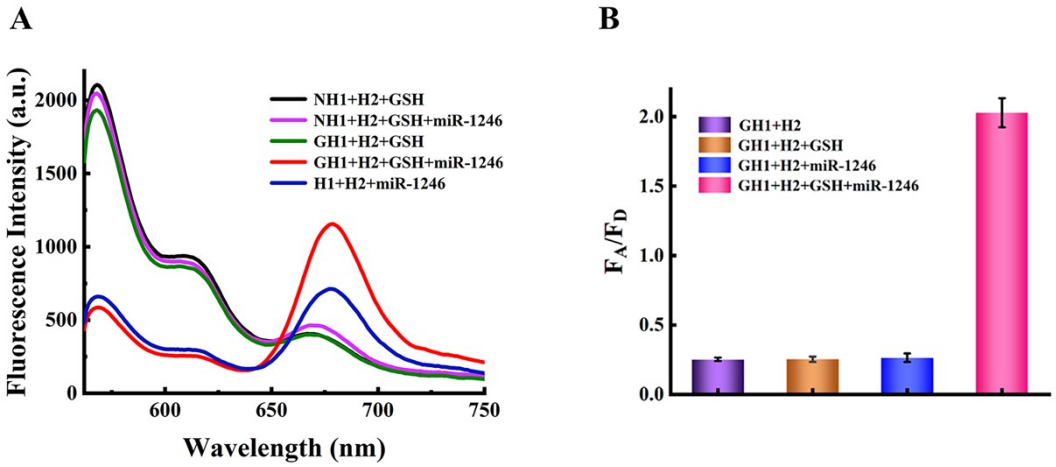

Fig. S2. (A) Fluorescence spectra of the GHCR system (GH1 and H2), HCR system (H1 and H2), and NHCR system (NH1 and H2) for miR-1246 (20 nM) in the presence of 1.5 mM GSH. (B) Fluorescence ratio (F_A/F_D) of the GHCR system and HCR system (H1 and H2) for miR-1246 (20 nM) in the presence of 1.5 mM GSH. The reactions were performed with 50 nM GH1, NH1, H1; 50 nM H2, 1.5 mM GSH, and 20 nM miR-1246 in Tris-HCl buffer (20 mM Tris-HCl, 150 mM NaCl, 5.0 mM $MgCl_2$, pH 7.4).

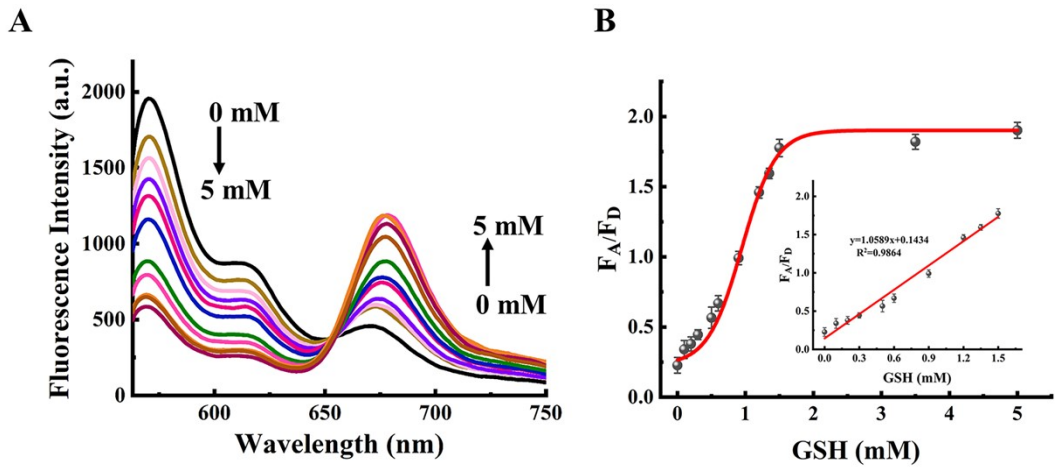

Fig. S3. (A) Fluorescence spectra of the GHCR system in response to different concentrations of GSH (0~5.0 mM). (B) F_A/F_D of the GHCR system in response to different concentrations of GSH (0~5.0 mM); the inset shows the linear relationship between F_A/F_D and the corresponding GSH concentrations from 0 mM to 5.0 mM. The reactions were performed with 50 nM GH1, 50 nM H2, 20 nM miR-1246, and GSH from 0 mM to 5.0 mM in Tris-HCl buffer (20 mM Tris-HCl, 150 mM NaCl, 5.0 mM $MgCl_2$, pH 7.4).

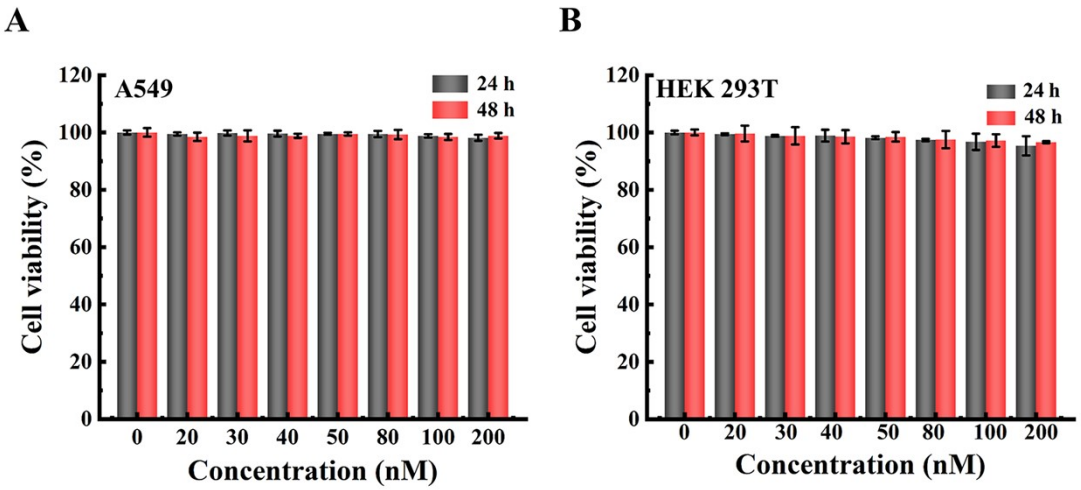

Fig. S4. Cell viability of A549 cells (A) and HEK 293T cells (B) incubated with different concentrations of GHCR system (0, 20, 30, 40, 50, 80, 100, 200 nM) for 24 h and 48 h. The cell viability was analyzed by CCK-8 assays.

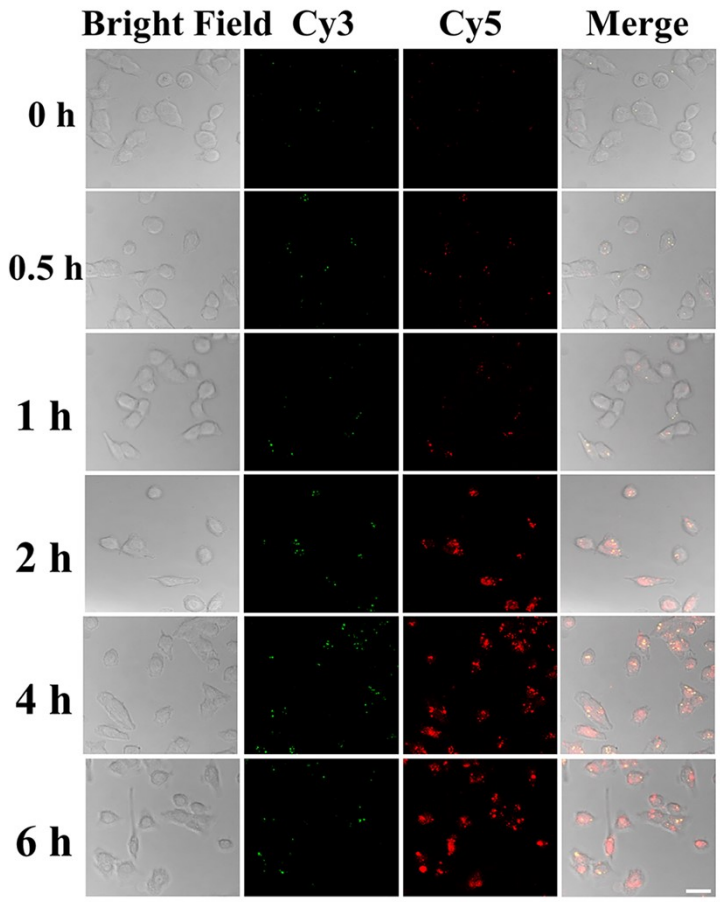

Fig. S5. Confocal fluorescence images of the GHCR system incubated with A549 cells for different times (50 nM GH1, 50 nM H2, λ_{ex}=552 nm; λ_{em}=560~590 nm (green), λ_{em}=640~700 nm (red); scale bar =30 μm).

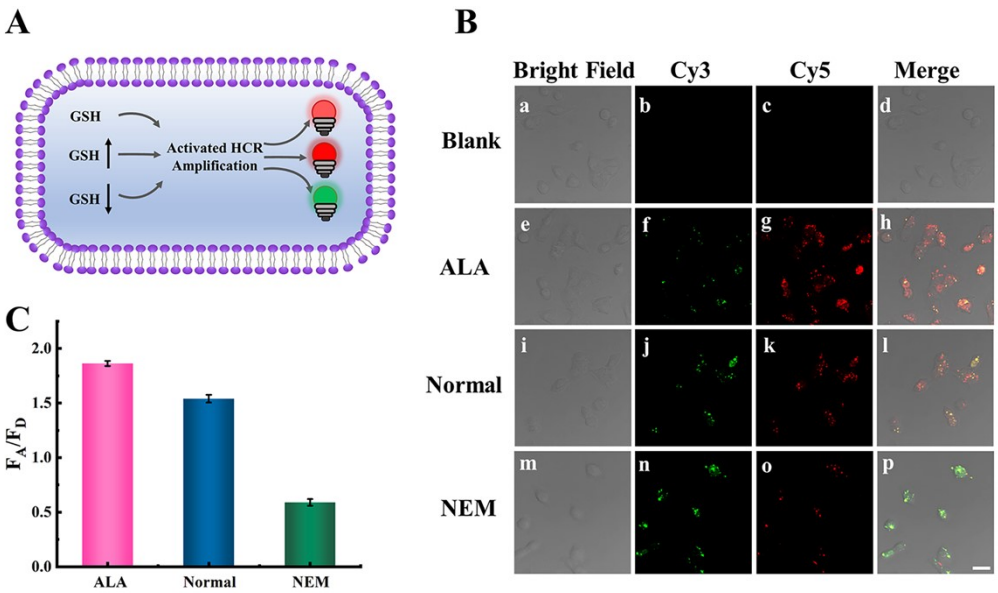

Fig. S6. (A) Schematic illustration of GHCR system in A549 cells with different GSH expression levels. (B) Confocal fluorescence images of the GHCR system of endogenous GSH, the "blank" means the individual buffer solution(a~d), after treatment with ALA (e~h), without any treatment (i~l), and NEM (m~p) in A549 cells. (50 nM GH1, 50 nM H2, λ_{ex}=552 nm; λ_{em}=560~590 nm (green), λ_{em}=640~700 nm (red); scale bar =30 μm). (C) The F_A/F_D ratio is generated from the red/green channel images.

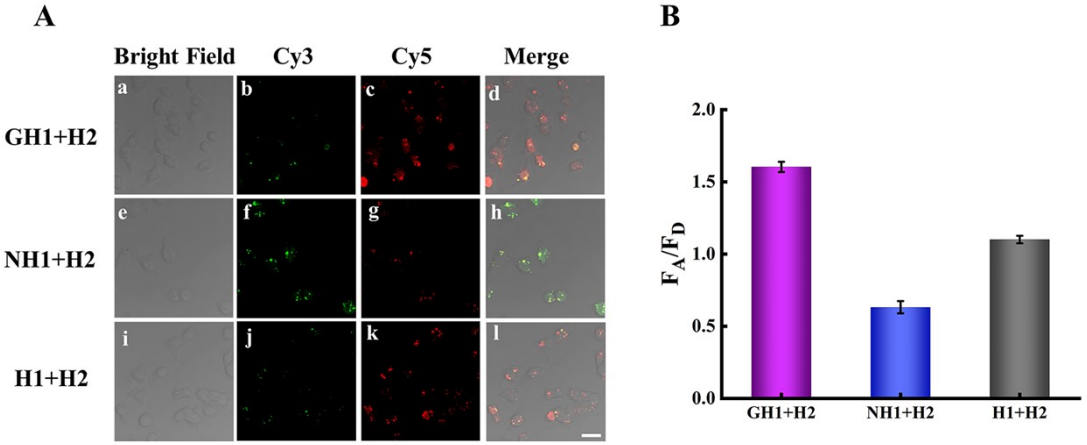

Fig. S7. (A) Confocal fluorescence images of A549 cells were treated with the GHCR system (a~d), the NH1/H2 system (e~h), and the HCR system (i~l), respectively. (50 nM GH1, NH1, H1, 50 nM H2, λ_{ex}=552 nm; λ_{em}=560~590 nm (green), λ_{em}=640~700 nm (red); scale bar =30 μm). (B) The F_A/F_D ratio is generated from the red/green channel images.

Table S1. The sequences of DNA used in this study

	The sequence of DNA (from left to right:5' to 3')
GH1	AATGG/iHS-SH/ATTTTTGGAGCAGGCACACCCTGCTCCAAAAATCCATT-Cy3
H2	GTGTGCCTGCTCCAAAAA/iCy5dT/AATGGATTTTTGGAGCAGG
NH1	AATGGATTTTTGGAGCAGGCACACCCTGCTCCAAAAATCCATT-Cy3
miR-1246	AATGGATTTTTGGAGCAGG
miR-21	TAGCTTATCAGACTGATGTTGA
miR-29a	TAGCACCATCTGAAATCGGTTA
miR-26a	TTCAAG TAATCCAGGATAGGCT
miR-203	GTGAAATGTTTAGGACCACTAG
Anti-miRNA-1246	CCTGCTCCAAAAATCCATT
Forward primer	TACGGAAGTGCTTCGATTTTG
Reverse primer	GTGCAGGGTCCGAGGT

Table S2. Comparison of GHCR system with other methods

Method	Advantages of GHCR system compared with this method[1-5]
Electrochemical method	The GHCR system enables real-time, non-invasive monitoring of dynamic miRNA changes in cells without requiring special electrodes or conditions. It can also simultaneously track multiple targets without harming the cells.
Traditional PCR method	The GHCR system offers simplified primer design, eliminates temperature cycling, requires no specific equipment, and enables real-time intracellular monitoring.
Mass spectrometry	GHCR simplifies sample prep, avoids radioisotopes/markers, and integrates fluorescent markers directly, while mass spectrometry requires expensive equipment and specialized support.
Colorimetric method	Colorimetry is less sensitive, needs high miRNA concentrations for visible changes, is affected by sample impurities, and lacks real-time intracellular monitoring. Subjective color judgment reduces accuracy, but GHCR overcomes these limitations.
Conventional fluorescence method	Traditional fluorescence is weak, needs high miRNA levels for reliability, lacks signal amplification, and has longer detection times with weaker signal quality and reliability. GHCR excels with strong signal amplification, shorter detection times, and improved signal quality and reliability.
Surface-Enhanced Raman Scattering (SERS)	SERS demands special nanostructures, laser equipment, complex operation, advanced instruments, higher technical expertise, and greater expenses, whereas GHCR system overcomes these limitations.

6 References

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