Supplementary Information

Surface charge tunable fluorescent protein-based logic gates for smart delivery of nucleic acids

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1. Experimental section

1.1. Materials

The H_{39}GFP gene was synthesized by Takara Co. (Dalian, China). Other reagents including Tris, NaCl, $NiSO_4$ and imidazole were purchased from Sangon Inc. (Shanghai, China). Human cervical cancer cells (HeLa) were obtained from the Cell Bank of Xiangya Central Experiment Laboratory of Central South University (Changsha, China). All oligonucleotides were synthesized and purified by Sangon Inc. (Shanghai, China). Other chemicals were all of analytical grade and used without further purification. All solutions were prepared with ultra-pure water (18.25 MΩ·cm) from a Millipore system.

1.2. Apparatus

Fluorescence measurements were carried out with a 1-cm path length quartz cuvette on a Quanta Master TM 4 fluorescence spectrometer (Photo Technology International, Inc., NJ, USA). UV/Vis absorption spectra were performed at 25 °C on a Beckman DU-800 spectrophotometer (Beckman Coulter, Inc., USA). The zeta potential (ζ-potential) measurements were performed by a Zeta sizer Nano-ZS (Malvern Instruments Ltd, UK). The confocal imaging was performed and analyzed with a TI-E+A1 SI laser scanning confocal microscope (Nikon, Japan). The flow cytometry was performed on a Beckman Coulter Gallios (USA). The atomic force microscopy (AFM) was performed on a Nanoscope IIIa (Veeco, USA) using ScanAsyst mode in ambient air.

1.3. Cloning, protein expression and purification

The amino acid sequence of H_{39}GFP was referred to the reported literature[1]. The gene sequence encoding H_{39}GFP was reversely translated from its amino acid sequence and optimized for *E. coli* codon usage. The full-length gene was synthesized and inserted in plasmid pUC19 by Takara (Dalian, China). The gene of H_{39}GFP was amplified by PCR from plasmid pUC19-H_{39}GFP, and then inserted to plasmid pET28a. The reconstructed plasmid pET28a-H_{39}GFP was transformed into *E. coli* BL21 (DE3) by electric shock. Cells were grown overnight at 37 °C in 3 ml Luria-Bertani (LB)

medium, and were transferred to 100 mL fresh LB for another 2 h till the OD_{600} (optical density at 600 nm) reached about 0.6. Then 0.2 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to induce H_{39}GFP expression for another 3 h at 25 °C. Cells were harvested by centrifugation and re-suspended in buffer (10 mM Tris pH 7.4, 2 M NaCl), and then lysed by sonication in an ice-water bath. After centrifugation at 12,000 rpm for 10 min to remove cell debris, the clear green supernatant containing H_{39}GFP was obtained. The supernatant was filtered through a mixed cellulose ester (MCE) syringe filter, and purified by Ni-NTA agarose chromatography (ÄKTA, GE). The purified protein was stored in the buffer (10 mM Tris-HCl, 100 mM NaCl, 5% glycerol, and pH 7.4) by desalination chromatography (ÄKTA, GE). The purified H_{39}GFP was then quantitated by the absorbance at 488 nm with an extinction coefficient of 8.33×10^4 $M^{-1}cm^{-1}$, and then stored at -20 °C before use.

1.4. Atomic force microscopy (AFM)

An atomic force microscope was used to characterize the H_{39}GFP/DNA complex. Samples containing the complexes in PBS (1.5 mM NaH_2PO_4, 8.1 mM Na_2HPO_4, 137.9 mM NaCl and 2.7 mM KCl; 5 μL with 100 nM H_{39}GFP and/or 100 nM DNA and/or 5 mM Ni^{2+} at different pH) were deposited onto the center of a freshly split untreated mica disk. Following adsorption for 10 min at room temperature, excess fluid was taken off by filter paper. The mica surface was dried at room temperature before imaging.

1.5. Fluorescence of H_{39}GFP quenched by BHQ1 labeled DNA (DNA-Q)

H_{39}GFP (100 nM) was mixed with DNA-Q (Table S1) solution with varying concentrations in PBS (1.5 mM NaH_2PO_4, 8.1 mM Na_2HPO_4, 137.9 mM NaCl, 2.7 mM KCl, pH 6.0 and 7.4) at room temperature. Then fluorescence spectrum of each sample was recorded on a PTI spectrometer. The excitation wavelength was 485 nm, and the emission wavelengths were in the range from 495 to 600 nm with both excitation and emission slits of 2.5 nm.

1.6. Confocal microscopy

The ability of H_{39}GFP to deliver nucleic acids into HeLa cells was assessed by confocal laser scanning microscopy (CLSM). For CLSM study, HeLa cells were plated

onto 35-mm glass-bottom microwell dishes with a cover glass (MatTek, USA) at a density of 5×10^4 cells each dish. They were incubated in humidity medium for 24 h at 37 °C and 5% CO_2 before treatment. The culture media (RPMI-1640 containing 10% FBS and 1% antibiotic (penicillin-streptomycin, 10000 U/mL)) was withdrawn and cells were washed three times with PBS (1.5 mM NaH_2PO_4, 8.1 mM Na_2HPO_4, 137.9 mM NaCl, 2.7 mM KCl, and pH 7.4). For each sample, DNA-C was added to the wells at a concentration of 200 nM, as well as 200 nM H_{39}GFP with or without 5 mM Ni^{2+}. The buffer is PBS at pH 6.0 or 7.4. After co–incubation with cells for 1.0 h, the buffer was removed and the cells were washed three times with 200 μL PBS containing 20 U mL^{-1} heparin to remove the membrane-bound protein[2]. Imaging was taken by a confocal scanning laser microscope. H_{39}GFP was observed by using a 488-nm laser, and the emission wavelength was read from 525 to 575 nm and expressed as green. DNA-C was observed by using a 639.5-nm laser, and the emission wavelength was read from 645 to 695 nm and expressed as red. Nuclei was stained with Hoechst (KeyGEN BioTECH, Nanjing, China), and shown as blue.

1.7. Flow cytometry

Cellular uptake efficiency of H_{39}GFP or H_{39}GFP with nucleic acids was quantitatively estimated by flow cytometry. HeLa cells were seeded in 12 well plates at a density of 2.0×10^5 cells each well and cultured with 500 μL of RPMI-1640 containing 10% FBS and 1% antibiotic for 24 h. H_{39}GFP/DNA-C (200 nM/200 nM) mixtures with or without 5 mM Ni^{2+} in PBS (1.5 mM NaH_2PO4, 8.1 mM Na_2HPO4, 137.9 mM NaCl, 2.7 mM KCl, pH 6.0 or 7.4) were prepared and added to the plates. After 1.0 h incubation, the buffer was discarded and the cells were washed three times with 400 μL PBS (pH 7.4) containing heparin. All the cells were digested by trypsin and collected in centrifuge tubes after centrifugation at 2000 rpm for 5 min. The supernatant was discarded and the bottom cells were washed twice with PBS (pH 7.4). The suspended cells were then filtered and examined by flow cytometry. The instrument was calibrated with non-treated cells as controls, and the cells were determined from a fluorescence scan performed with 1.0×10^4 cells using the FL1-H and FL4-H channels.

1.8. Western Blotting

2 × 10^5 cells were collected and suspended with different samples (500 nM H_{39}GFP, and 500 nM H_{39}GFP with 100 pmol siRNA or negative control RNA in pH 6.0 PBS). After incubation for 10 min at room temperature, the cells were added to a 35-mm dishes containing 2 mL RPMI-1640 with 10% FBS. After 72 h, Cells were washed three times with PBS and lysed with 80 μL RIPA buffer containing 1× protease inhibitor cocktail, 1 × phosphatase inhibitor cocktail A and 1× phosphatase inhibitor cocktail B for 2 minutes. The resulting cell lysate was centrifuged at 4 ºC at 14000 RCF for 10 min. The supernatant of cells lysate was analyzed by SDS-PAGE on an 8% acrylamide gel.

The proteins on the gel were transferred by electroblotting onto a PVDF membrane pre-soaked in methanol. Membranes were blocked in 5% BSA for 1 h, and incubated in the primary antibody solution with 5% BSA overnight at 4 ºC. The membrane was washed three times with PBST (4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.4, 137 mM NaCl, 2.7 mM KCl, 0.2% Tween 20) and treated with the secondary antibodies (GAPDH and α-tubulin) in blocking buffer for 1 h. The membrane was washed three times with PBST and imaged using MicroChemi 4.2 with bio-imaging systems. Images were analyzed by Image Studio LiteVer 3.1 software. GAPDH protein expression was measured by Western blotting and normalized to that of α-tubulin. The GAPDH and α-tubulin primary antibody were purchased from CMCTAG and the secondary antibodies were from Bioworld. All siRNAs were purchased from GenePharma.

1.9. Cell viability assays

Cytotoxicity of H_{39}GFP in HeLa cells was analyzed by MTT assay, and performed according to the protocol. Briefly, HeLa cells were seeded onto a 96-well plate at a density of 10000 cells/well, and incubated in 100 μL of RPMI-1640 medium containing 10% FBS and 1% antibiotic for 24 h. Then, the original medium was replaced with H_{39}GFP-containing PBS buffer (pH 6.0), or with PBS buffer only as the blank. After incubation for 4 h at 37 ºC, the buffer was discarded and MTT reagent solution (5 mg/mL, 20 μL each well) was added to each well, and the cells were incubated for another 4 h. Subsequently, the supernatant was removed, and 150 μL DMSO was added

to each well to dissolve formazane of MTT at 150 rpm for 30 minutes. The optical density (OD) of each well was determined at 570 nm by a microplate reader (BioTek, Synergy, USA). The relative cell viability was calculated according to the following equation: cell viability (%) = OD (sample) × 100/OD (blank). Each value was averaged from five independent experiments.

1.10 Quantitative reverse transcription PCR (RT-qPCR)

6×10^5 cells were incubated with H_{39}GFP (500 nM) and siRNA (100 pmol) for 2 h under different stimuli. Then, cells were washed three times with PBS containing 20 U/mL heparin and two times with PBS after transfection. 35-mm dishes were treated with 1 mL TRlzol Reagent (Thermo) after transfection 72 h. The cells were lysed directly in the culture dish by pipetting the cells up and down several times and stored at -80 ºC. RNA was extracted by RNASimple Total RNA kit (Tian Gen) following the manufacturer's protocol. The complementary cDNA was generated from 500 ng of RNA using the PrimeScripTM RT Reagent Kit with gDNA Eraser (TaKaRa) following the manufacturer's protocol. QPCR reactions contained 1 × SYBR Premix DimerEraser (TaKaRa), 1 μL cDNA (<100 ng) and 400 nM both forward and reverse primers (see Suppporting Information for sequences).

qPCR reactions were subjected to the following program on a QuantStudioTM 7 Flex Real-Time PCR System (Life Technologies): 15 seconds at 95 ºC, then 39 cycles of amplification (5 s at 95 ºC, 30 s at 55 ºC, and 30 s at 72 ºC). The amplification was quantified during the 72 ºC step. Dissociation curves were obtained by subjecting samples to 15 s at 95 ºC, 1 min at 60 ºC and 15 s at 95 ºC and monitoring fluorescence during heating 60 ºC to 95 ºC. Gene expression values were determined using QuantStudioTM Real-Time PCR software and analyzed by the comparative CT (ΔΔCt) method.

Supplementary table

Table S1 Sequences of the oligonucleotides used in the manuscript.

	Sequence
DNA-Q	5'-BHQ1-GTGAGCAAGGGTCAGACTGATCGGAAGTTC-3'
DNA-C	5'-Cy5-GTGAGCAAGGGTCAGACTGATCGGAAGTTC-3'
siRNA-GAPDH (sense)	5'-UGACCUCAACUACAUGGUUdTdT-3'
siRNA-GAPDH (antisense)	5'-AACCAUGUAGUUGAGGUCAdTdT-3'
Forward β-actin	5′-TGGCACCCAGCACAATGAA-3′
Reverse β-actin	5′-CTAAGTCATAGTCCGCCTAGAAGCA-3′
Forward GAPDH	5′-CAACTCACTCAAGATTGTCAGCAA-3′
Reverse GAPDH	5′-GGGATGGACTGTGGTCATGA-3′

Supplementary Figures

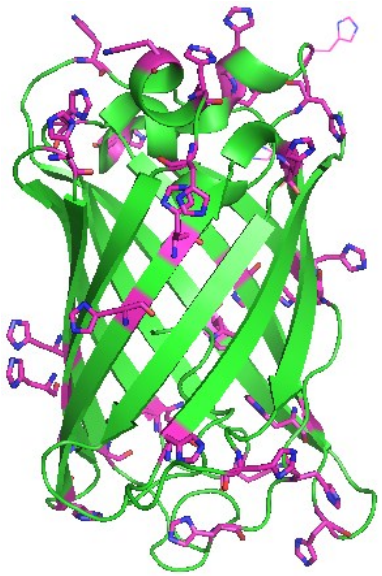

Figure S1. The overall structure of H_{39}GFP.

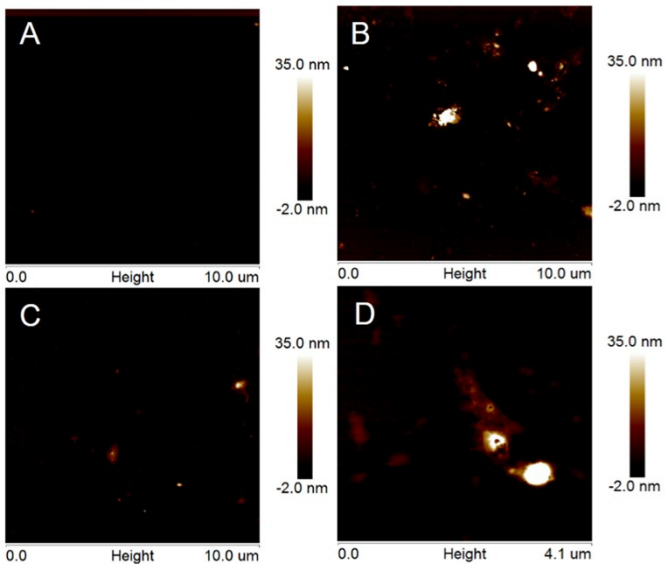

Figure S2. Characterization of H_{39}GFP/DNA nano-complex by atomic force microscopy. (A) H_{39}GFP; (B) H_{39}GFP/DNA at pH 6.0; (C) H_{39}GFP/DNA at pH 7.0; (D) H_{39}GFP/DNA/Ni^{2+} at pH 7.0. [DNA] = 100 nM, [H_{39}GFP] = 100 nM, [Ni^{2+}] = 5 mM.

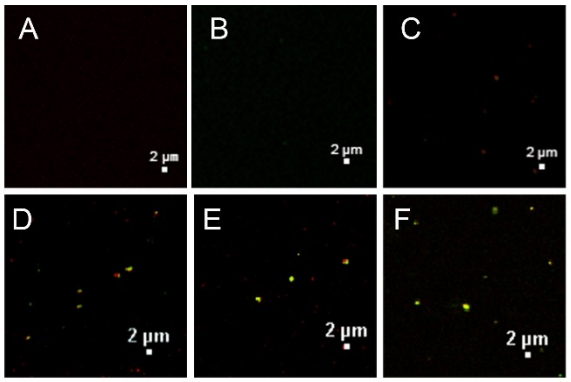

Figure S3. Fluorescence microscopy of 30-nt DNA-C (A) and H_{39}GFP alone at pH 7.4 (B); (C) DNA-C and H_{39}GFP were mixed at pH 7.4; (D) DNA-C and H_{39}GFP were mixed at pH 6.0; (E) DNA-C and H_{39}GFP were mixed at pH 7.4 with nickel ion; (F) DNA-C and H_{39}GFP were mixed at pH 6.0 with nickel ion. H_{39}GFP is shown in green, and DNA-C is in red.

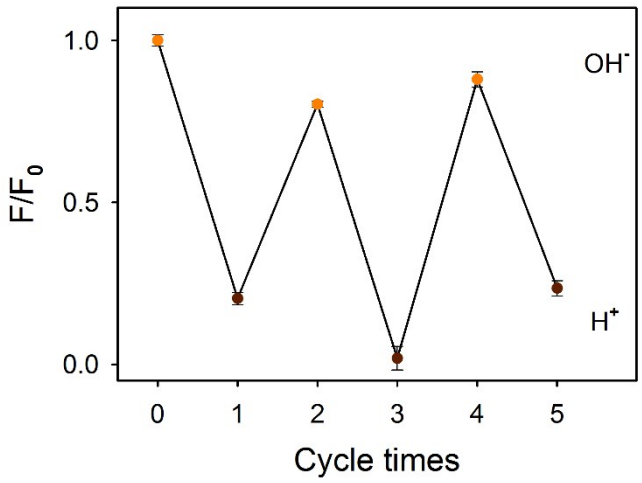

Figure S4. The change of fluorescent intensity after H^+ (at pH6.0) or OH^- (at pH 7.4) was added into the solution of H_{39}GFP (100 nM) and DNA-Q (100 nM).

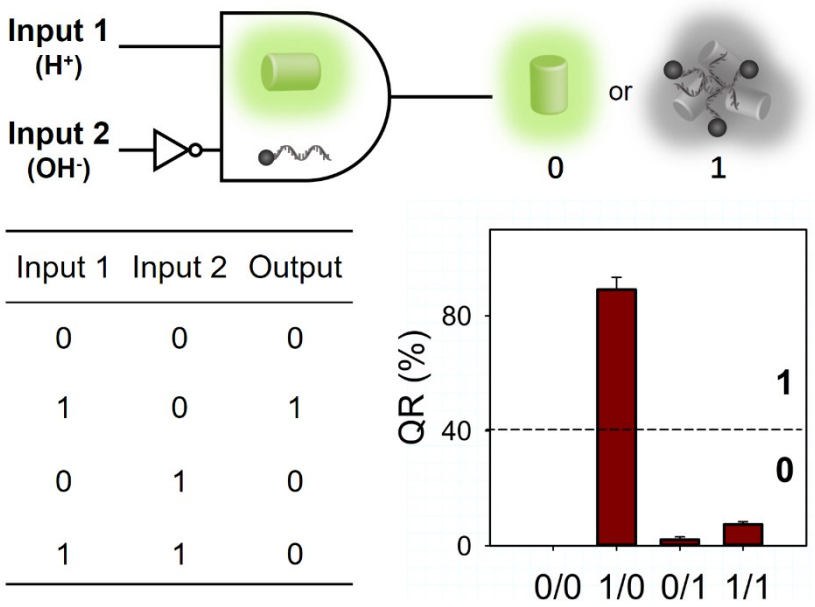

Figure S5. Schematic illustration of the INHIBIT logic gate based on H_{39}GFP, its truth table, and the fluorescence quenching rate (QR) of H_{39}GFP with different inputs. H_{39}GFP, 100 nM; DNA-Q, 100 nM; OH^-, pH 7.4; and H^+, pH 6.0.

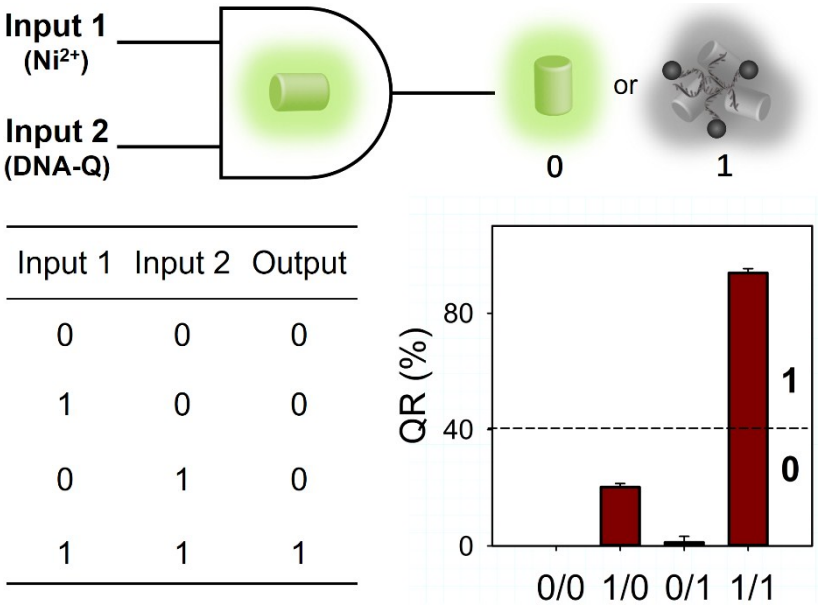

Figure S6. Schematic illustration of the AND logic gate based on H_{39}GFP, its truth table, and the fluorescence quenching rate (QR) of H_{39}GFP with different inputs. H_{39}GFP, 100 nM; DNA-Q, 100 nM; and Ni^{2+}, 5 mM.

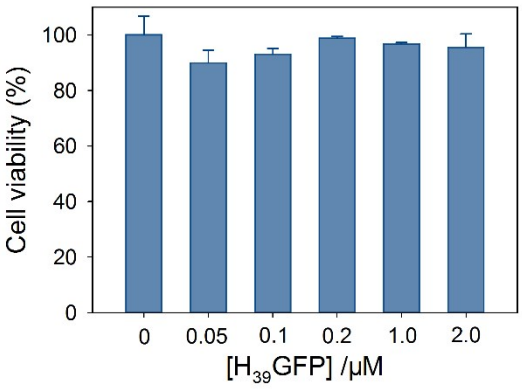

Figure S7. Cell viability of Hela Cell in the presence of different concentrations $H_{39}GFP$ was measured MTT assay.

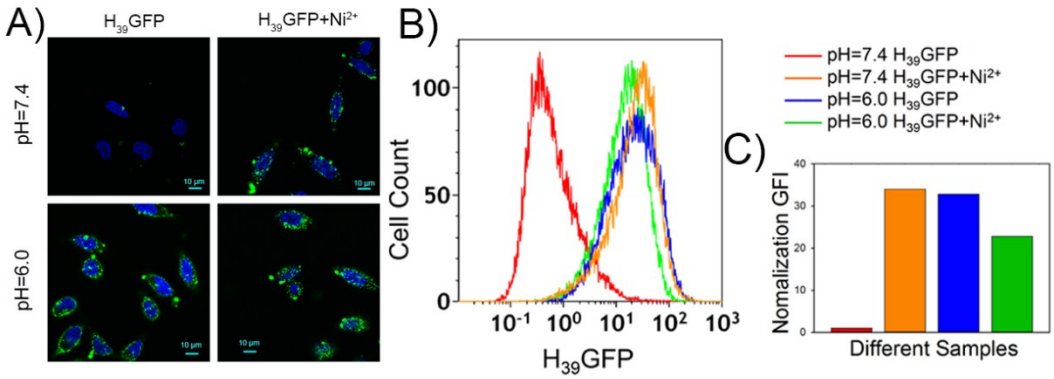

Figure S8. Internalization of $H_{39}GFP$ into Hela cells under different stimuli recorded by confocal laser scanning microscopy (A) and flow cytometry (B and C).

2. Reference

1 C. Lei, Z. Wang, H. Deng, H. Hu, Y. Huang, S. Yao, *Analytical Chemistry*, 2015, **87**, 1974-1980.

2 B. R. McNaughton, J. J. Cronican, D. B. Thompson, D. R. Liu, *Proceedings of the National Academy of Sciences of the United States of America*, 2009, **106**, 6111-6116.