OMTN, Volume *27*

Supplemental information

Transferrin receptor-mediated internalization and intracellular fate of conjugates of a DNA aptamer

Nan Zhang, Junyan Wang, Tao Bing, Xiangjun Liu, and Dihua Shangguan

Supplemental Table

Table S1. DNA sequences used in this paper.

name	Sequences (5'-3')
HG1-9	GGATAGGGATTCTGTTGGTCGGCTGGTTGGTATCC
ctr-sq	AGAGCAGCGTGGAGGATAGTTGGGGTTTGGCAAGTATTG
sgc8c	ATCTAACTGCTGCGCCGCCGGGAAAATACTGTACGGTTAGA
c-HG1-9	GGATACCAACCAGCCGACCAACAGAATCCCTATCC
c-ctr-sq	CAATACTTGCCAAACCCCAACTATCCTCCACGCTGCTCT
mGC	TTCCATAGCCATTCTGTTCGTCGCCTGGTTCCTATCC

Note: Fluorophores, biotin or azide group were modified at the 5‘ end of DNA sequences, and a thymic nucleotide was added to link the modified functional group and the DNA sequence in the table.

Table S2. The Mander's colocalization coefficients (MCC) in TfR-EGFP /Rab 5B-mCherry and TfR-EGFP /Rab 11A-mCherry HeLa cells after incubation with HG1-9.

in TfR-EGFP /Rab 5B-mCherry co-expressed HeLa cells						
Mander's colocalization coefficient (MCC)	M1 (HG1-9 / TfR)	M2 (TfR/ HG1-9)	M1 (HG1-9 / Rab5B)	M2 (Rab5B / HG1-9)	M1 (TfR / Rab5B)	M2 (Rab5B/ TfR)
	0.642 ±0.125	**0.609 ±0.234**	**0.636 ±0.223**	**0.577 ±0.193**	**0.446 ±0.197**	**0.637 ±0.128**

in TfR-EGFP /Rab 11A-mCherry co-expressed HeLa cells						
Mander's colocalization coefficient (MCC)	M1 (HG1-9 / TfR)	M2 (TfR/ HG1-9)	M1 (HG1-9 / Rab11A)	M2 (Rab11A / HG1-9)	M1 (TfR / Rab11A)	M2 (Rab11A/ TfR)
	0.812 ±0.0573	**0.639 ±0.0852**	**0.828 ±0.023**	**0.585 ±0.0643**	**0.813 ±0.151**	**0.792 ±0.158**

note: (HG1-9 /TfR) means the fraction of HG1-9 overlapping TfR, and all that.

Supplemental Figures

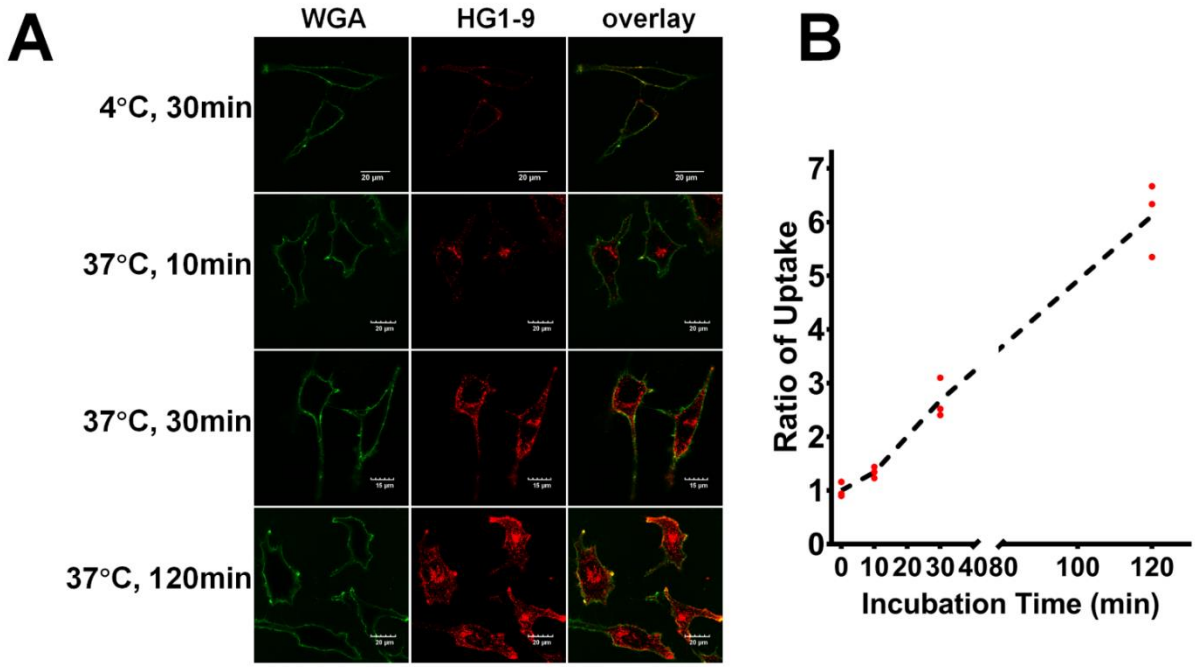


Figure S1. (A) Representative confocal images of cellular uptake of aptamer HG1-9. HG1-9 (labeled by rhodamine, 200 nM) was incubated with HeLa cells at 37 ℃ for 10 min, 30 min and 2 h. Incubation at 4 ℃ for 30 min was as beginning of cellular uptake. After incubation, cells were stained by membrane probe-FITC conjugated wheat germ agglutinin (WGA, 5 μg/mL) at 4°C for 10 min. (B) The enrichment of aptamer HG1-9 within cells along with incubation time. Three images of each time point were chosen, and quantification of fluorescence intensity was measured by ImageJ. The uptake ratio was calculated by $F_{(37\,℃,\,t)}$/ $F_{(4\,℃,\,30min)}$. Scale bars: 15 or 20 μm.

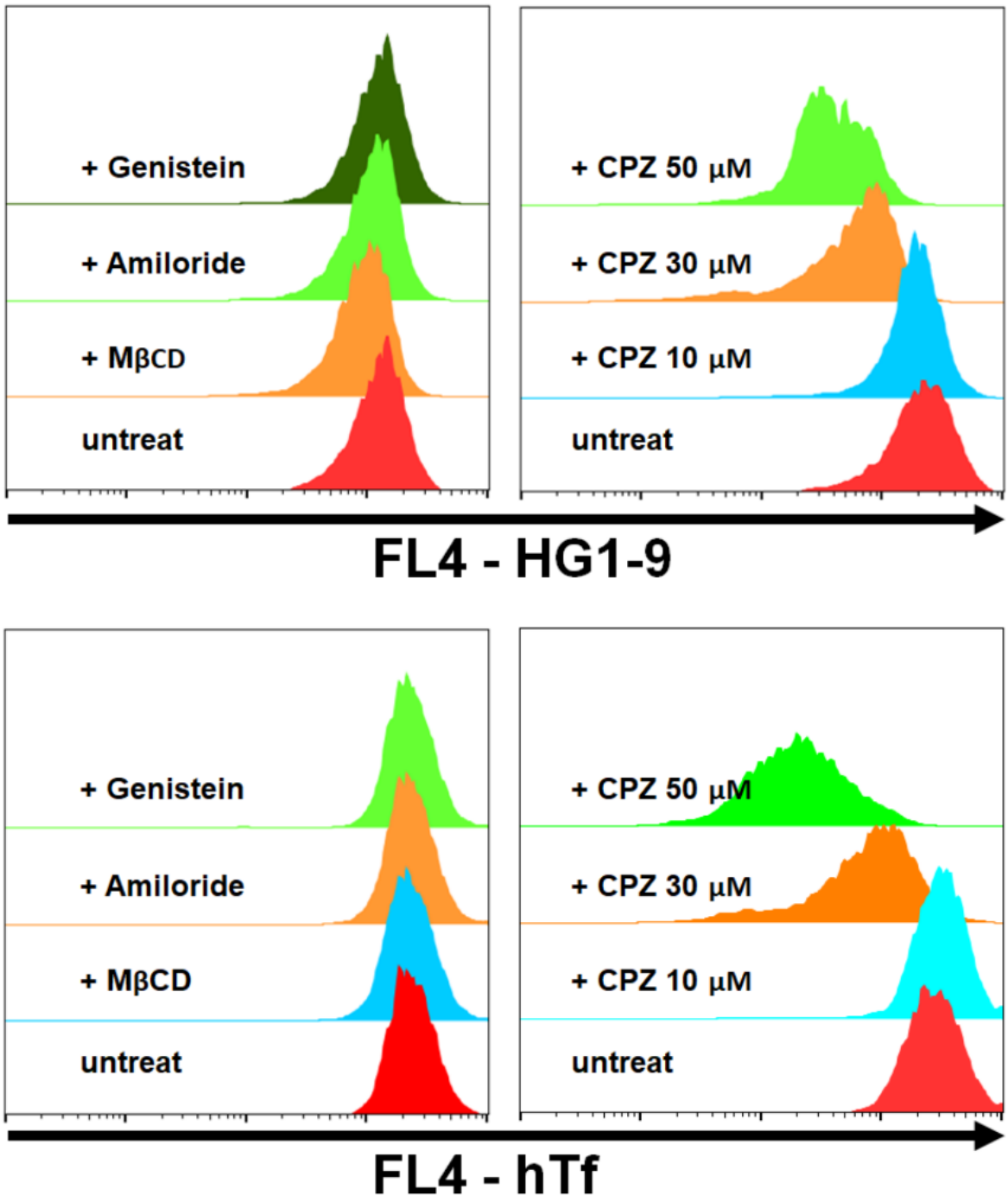


Figure S2. Flow cytometry analysis of the uptake of aptamer HG1-9 and hTf (200 nM, labled by AF647) by cells pre-treated with endocytosis inhibitors (50 μM Amiloride, 50 μM Genistein and and 5 mM Methyl-β-cyclodextrin (MβCD); Chlorpromazine (CPZ, 10, 30 and 50 μM)); the uptake by untreated cells was as positive control; at least two independent experiments were done.

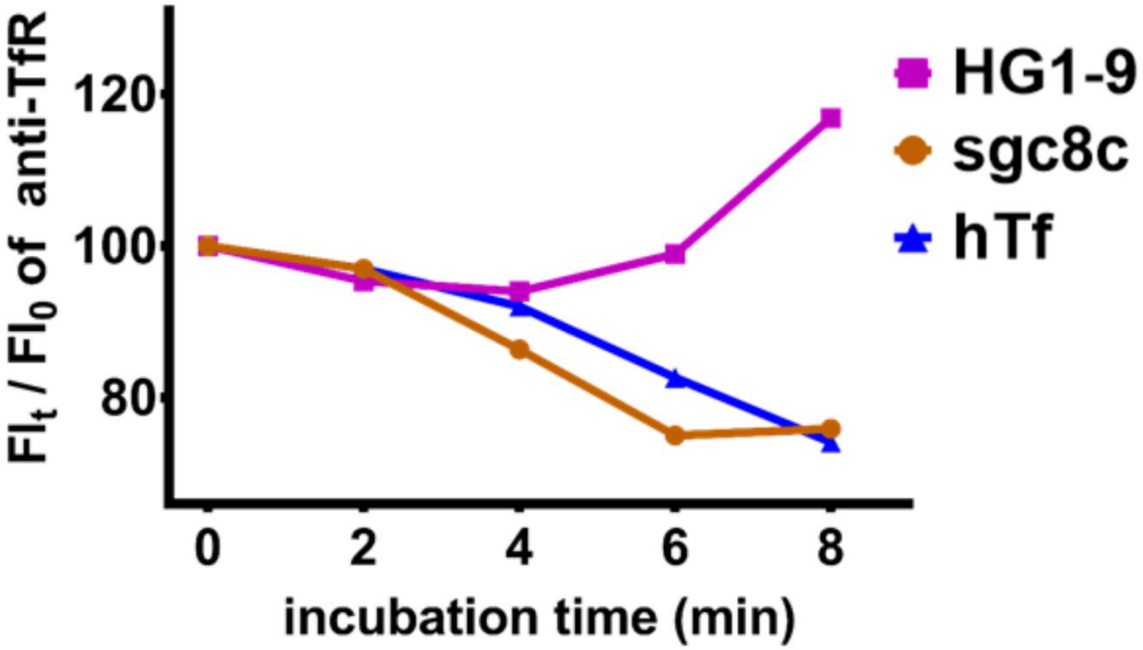


Figure S3. The flow cytometry analysis of the fluorescence of anti-TfR antibody on HeLa cell surface (representing the content of TfR on cell surface) after incubation with unlabeled hTf (200 nM), HG1-9 (200 nM) and sgc8c (200 nM) at 37 ℃ for a series of incubation times (0, 2, 4, 6, 8 min). The median fluorescence intensity of cells before incubation (0 min) was as 100%.

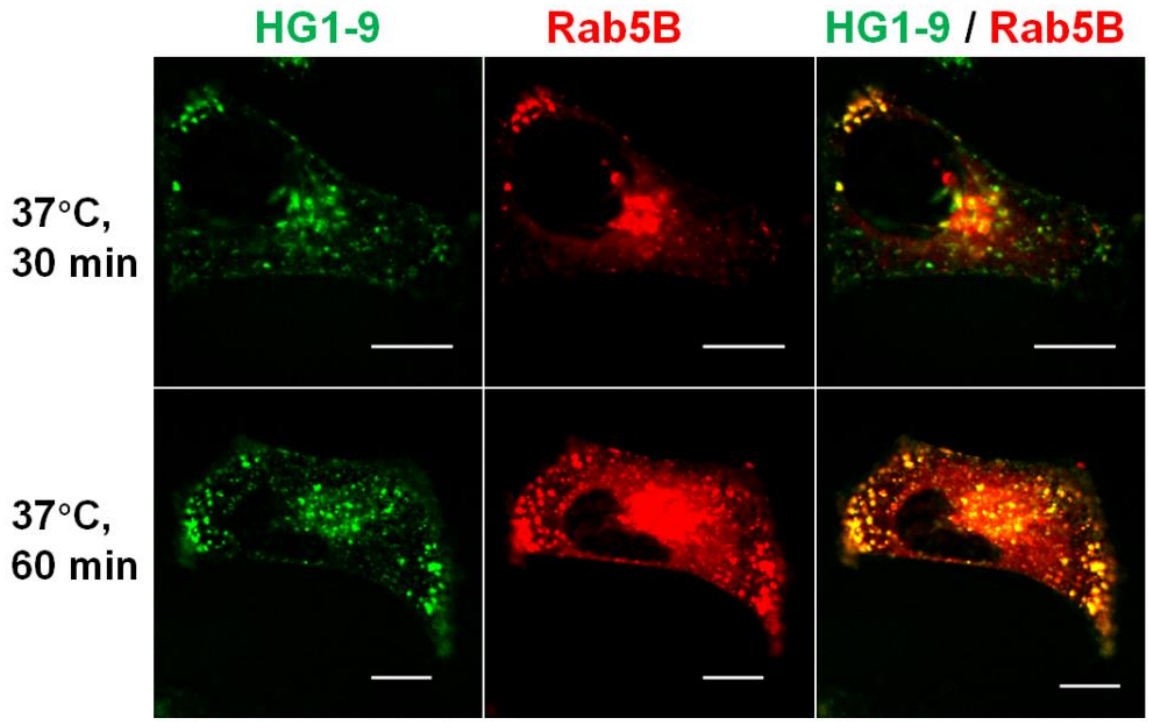


Figure S4. Confocal imaging of distribution of HG1-9 (AF488 labeled, 50 nM, shown in green) and Rab5B (shown in red) after incubation with Rab5B-mCherry transfected HeLa cells at 37 ℃ for 30 min and 60 min. Scale bars: 10 μm.

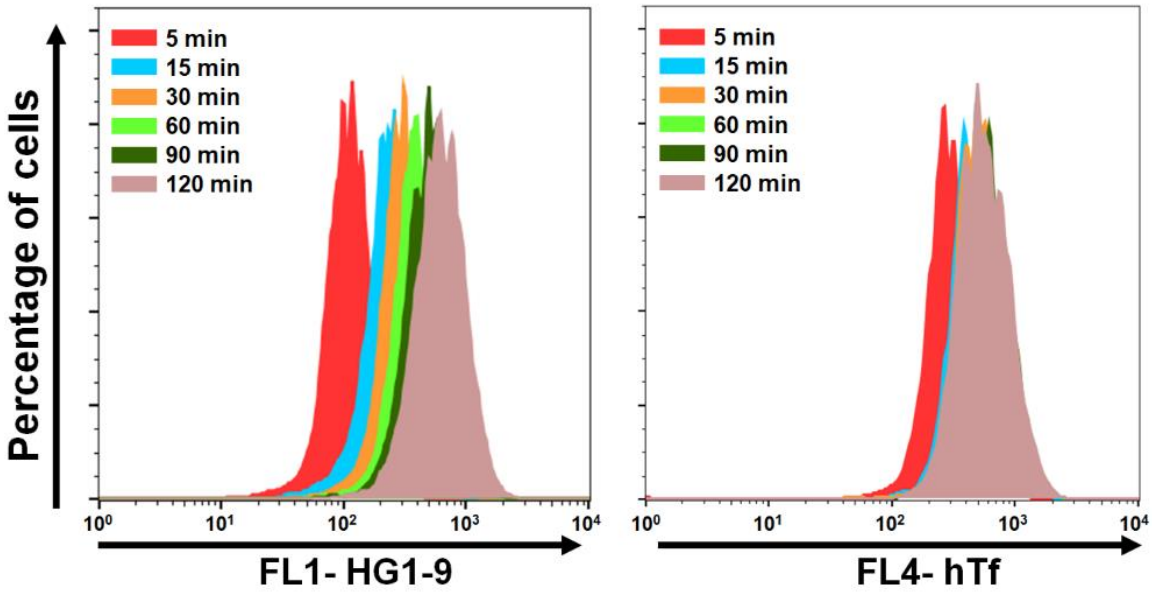


Figure S5. The enrichment of aptamer HG1-9 and hTf within cells along with incubation time. Jurkat cells were incubated with 150 nM AF488-HG1-9 or AF647-hTf at 37 ℃ for a series of incubation time (5, 15, 30, 60, 90, and 120 min). After incubation, the cells were washed and analyzed by flow cytometry, each sample repeated twice.

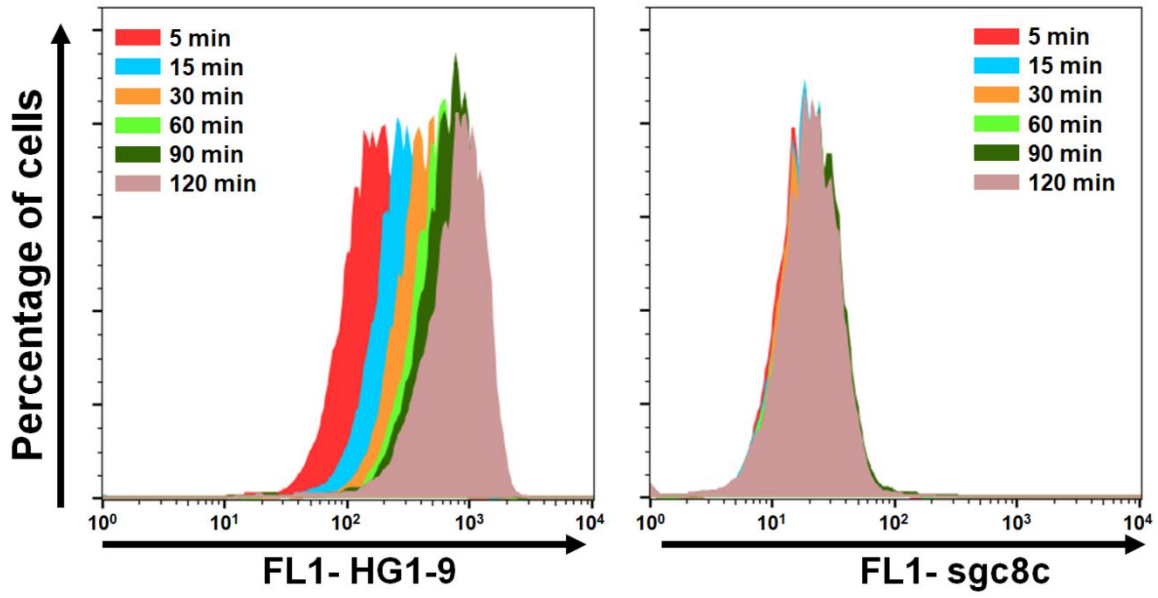


Figure S6. The enrichment of aptamer HG1-9 and aptamer sgc8c within cells along with incubation time. Jurkat cells were incubated with 150 nM AF488-HG1-9 or AF488-sgc8c at 37 ℃ for a series of incubation time (5, 15, 30, 60, 90, and 120 min). After incubation, the cells were washed and analyzed by flow cytometry, each sample repeated twice.

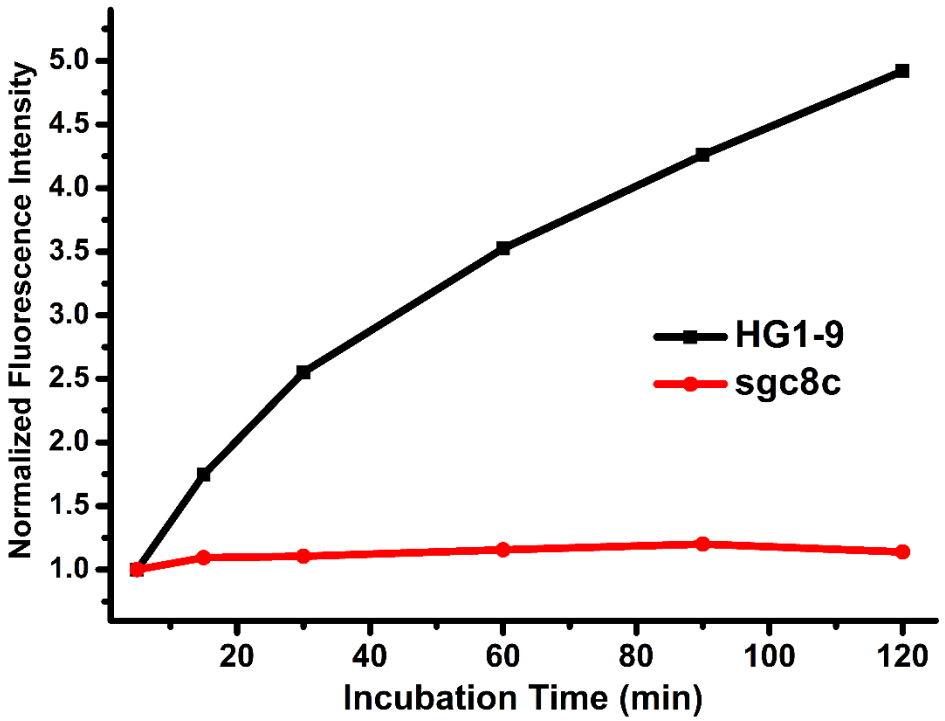


Figure S7. The enrichment of aptamer HG1-9 and aptamer sgc8c within cells along with incubation time. Jurkat cells were incubated with 150 nM AF488-HG1-9 or AF488-sgc8c at 37 ℃ for a series of incubation time (5, 15, 30, 60, 90, and 120 min). After incubation, the cells were washed and analyzed by flow cytometry. The uptake ratios were measured by dividing the fluorescence intensity of cells after incubation for a certain time by that after incubation for 5 min.

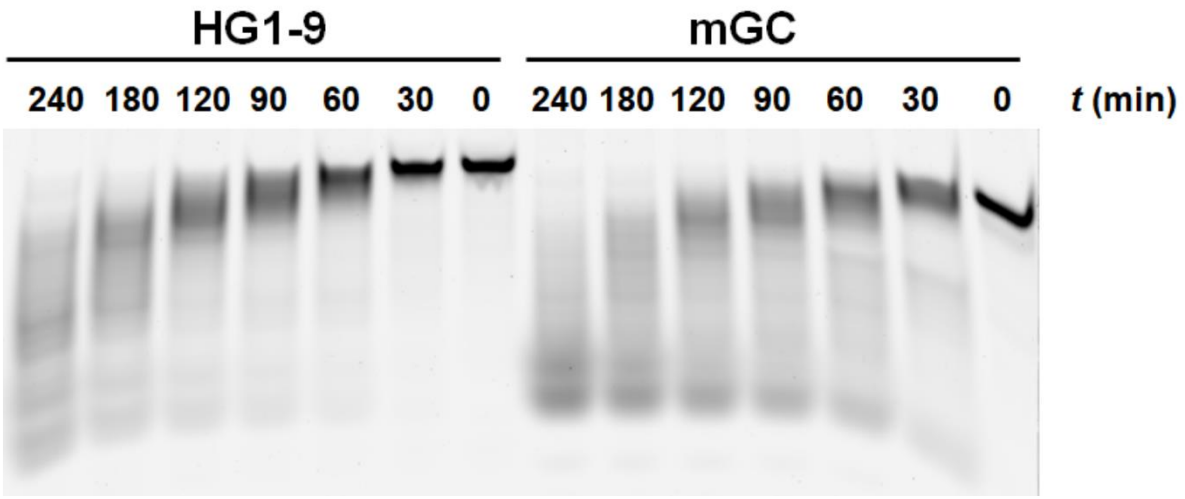


Figure S8. Nuclease resistance of 5'-end labeled HG1-9 and the control sequence mGC. Denaturing-polyacrylamide gel (20%) electrophoresis assay of 5'-FITC labeled HG1-9 (2 μM) and mGC (2 μM) after incubated in RPMI 1640 medium with 10% FBS at 37 ℃ for different time (0, 30, 60, 90, 120, 180, and 240 min), gels were exposed photographed by Typhoon V.

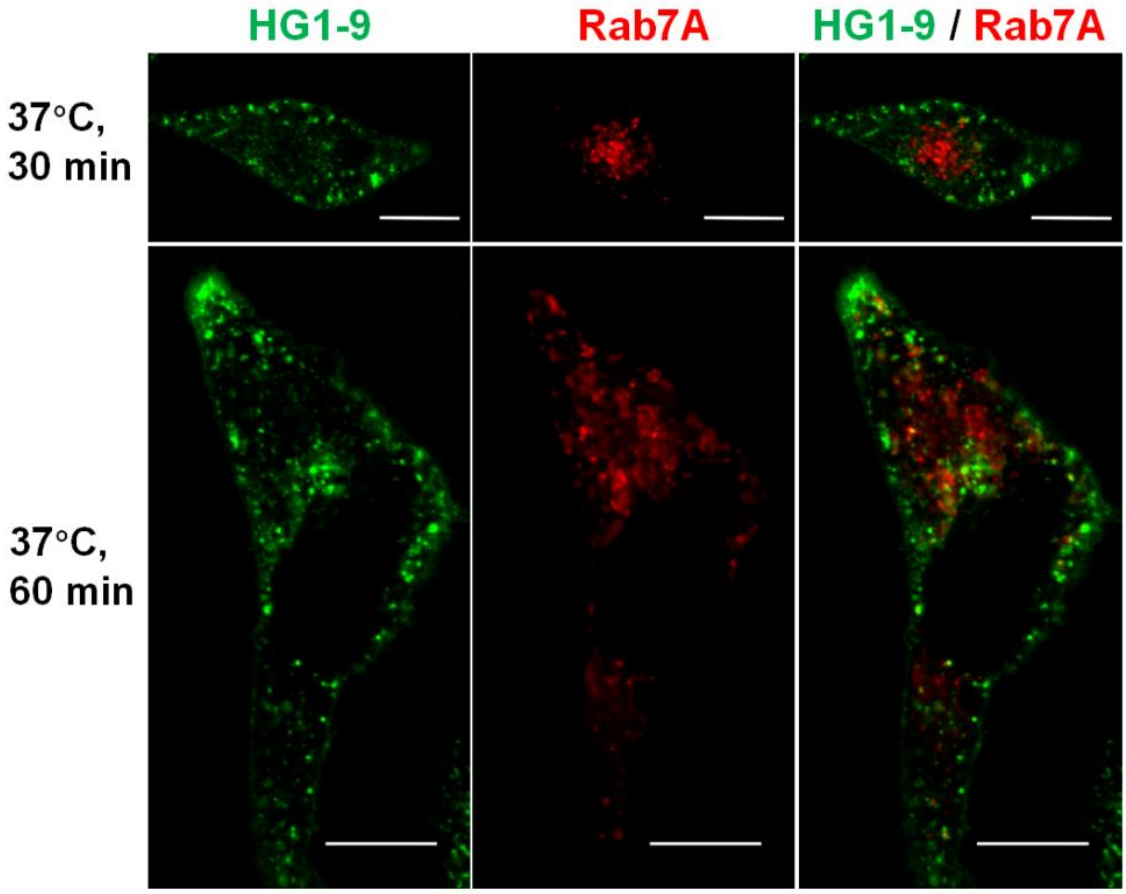


Figure S9. Confocal imaging of distribution of HG1-9 (AF488 labeled, 50 nM, shown in green) and Rab7A (shown in red) after incubation with Rab7A-mCherry transfected HeLa cells at 37 ℃ for 30 min and 60 min. Scale bars: 10 μm.

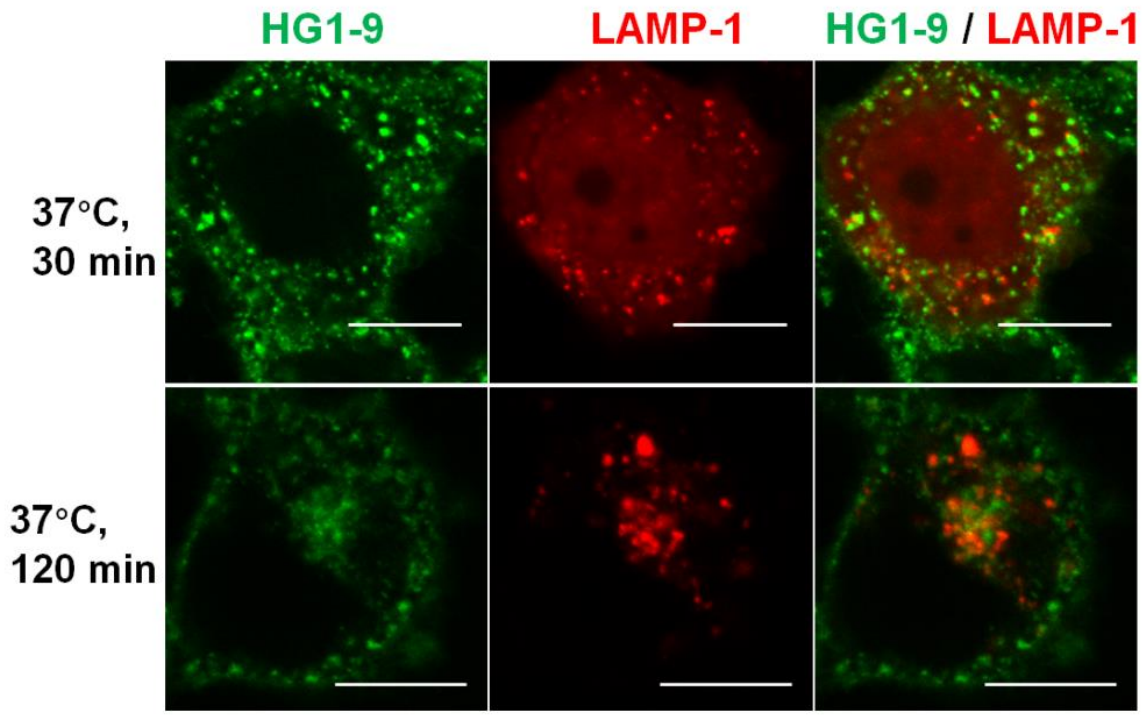


Figure S10. Confocal imaging of distribution of HG1-9 (AF488 labeled, 50 nM, shown in green) and LAMP-1 (shown in red) after incubation with LAMP-1-mCherry transfected HeLa cells at 37 ℃ for 30 min and 120 min. Scale bars: 10 μm.

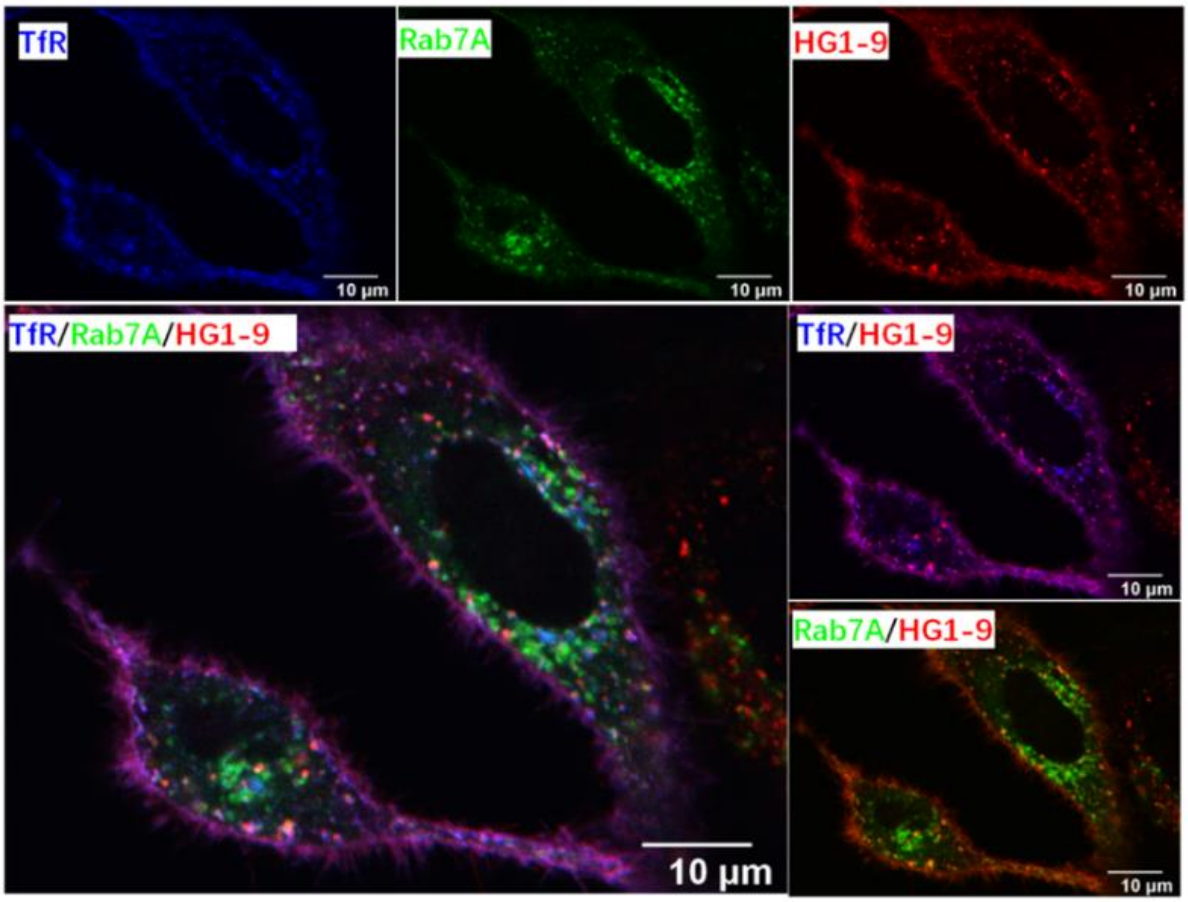


Figure S11. confocal imaging of the binding and distribution of aptamer HG1-9 (AF647 labeled, 50 nM) to TfR-EGFP and Rab7A-mCherry co-transfected HeLa cells after incubation for 60 min at 37 ℃. Confocal images showed that internalized HG1-9 co-located well with TfR-EGFP both on cell membrane and within cell; neither HG1-9 nor TfR-EGFP overlaid with Rab7A-mCherry, suggesting that HG1-9 accompanied with its target transferred along the way without late endosomes. Scale bars: 10 μm.

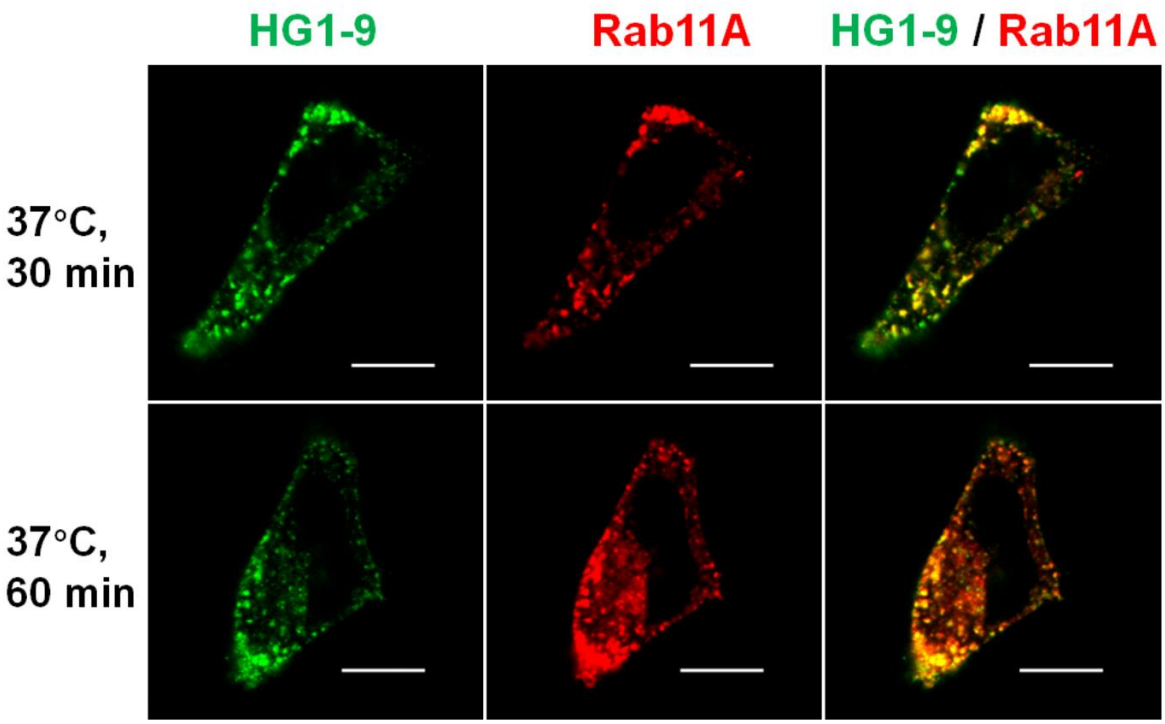
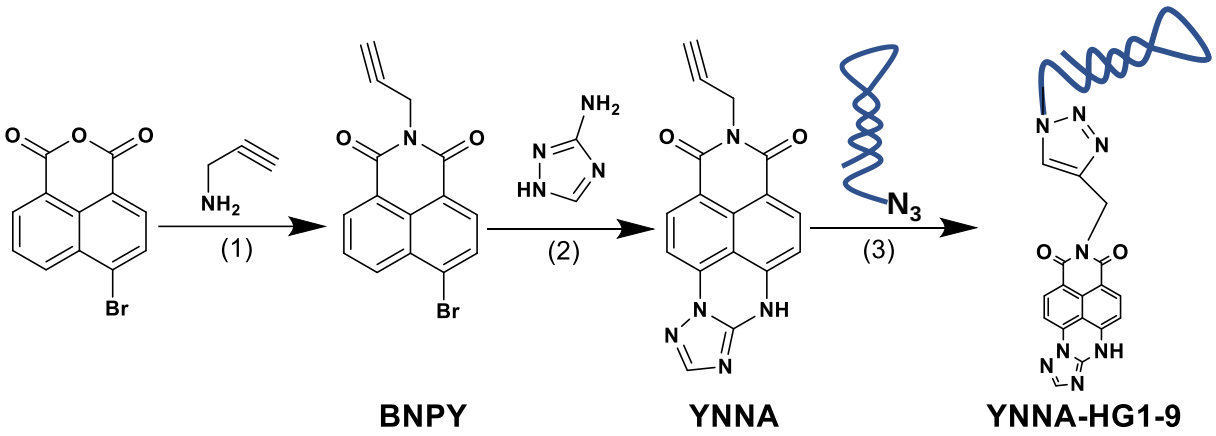


Figure S12. Confocal imaging of distribution of HG1-9 (AF488 labeled, 50 nM, shown in green) and Rab11A (shown in red) after incubation with Rab11A-mCherry transfected HeLa cells at 37 ℃ for 30 min and 60 min. Scale bars: 10 μm.



Scheme S1. The synthetic route of YNNA and YNNA-HG1-9. Reagents and conditions: (1) ethanol, reflux, 1 h; (2) NaOH, DMSO, 135 ℃, 12 h; (3) TBTA, $CuSO_4$, sodium ascorbate in DMSO/water (70%/30%, v/v), 40 ℃, 20 min.

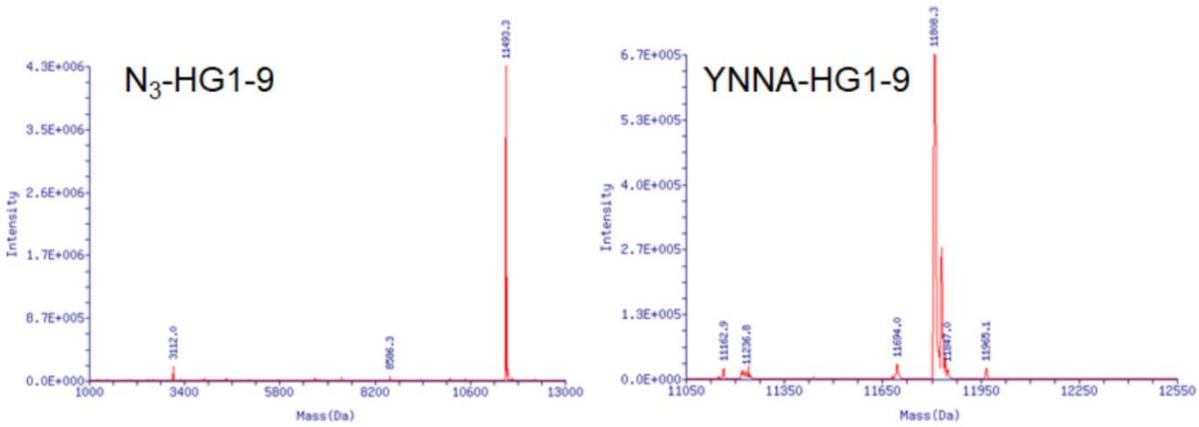


Figure S13. MS of azido-HG1-9 and YNNA-HG1-9.

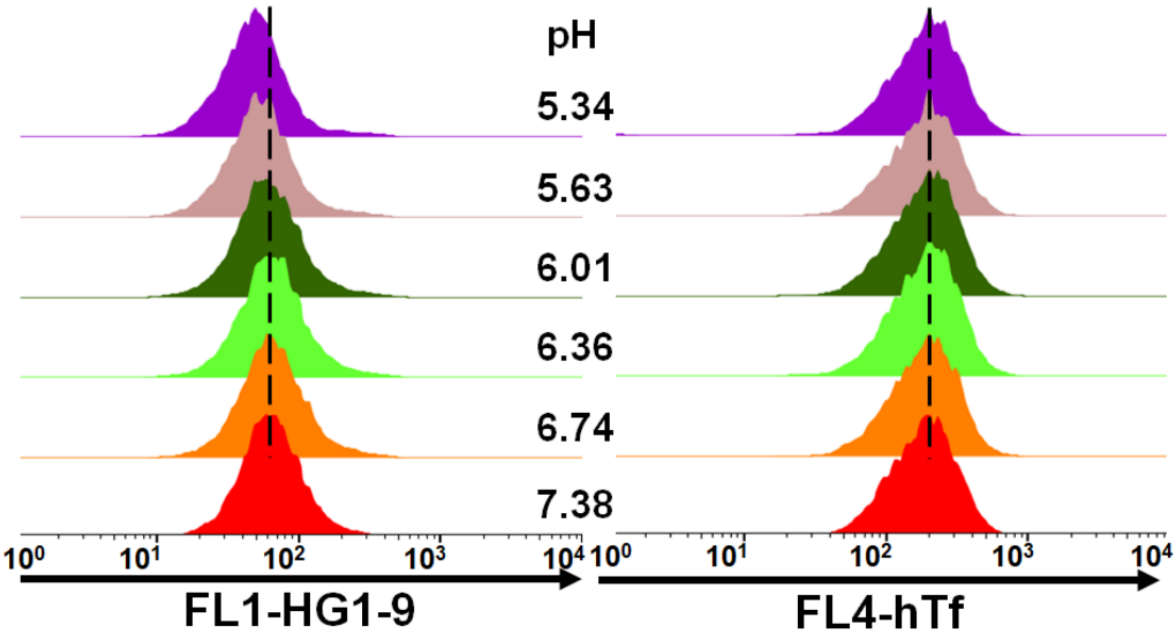


Figure S14. The flow cytometry analysis of binding of HG1-9 (AF488 labeled) and human Transferrin (hTf, AF647 labeled) to fixed HeLa cells in HEPES buffers (125 mM KCl, 20 mM NaCl, 0.5 mM $CaCl_2$, 0.5 mM $MgCl_2$, 5 mM glucose and 20 mM HEPES) with different pH values (pH 7.38, 6.74, 6.36, 6.01, 5.63, and 5.34).

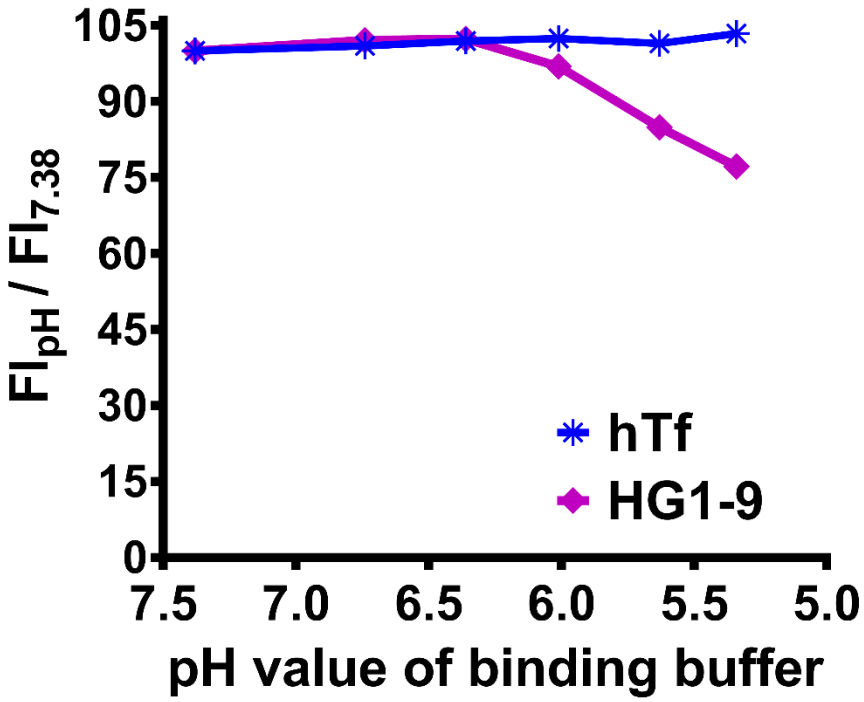


Figure S15. The binding ability of HG1-9 (AF488 labeled) and human Transferrin (hTf, AF647 labeled) to fixed HeLa cells in HEPES buffers (125 mM KCl, 20 mM NaCl, 0.5 mM $CaCl_2$, 0.5 mM $MgCl_2$, 5 mM glucose and 20 mM HEPES) with different pH values (pH 7.38, 6.74, 6.36, 6.01, 5.63, and 5.34).

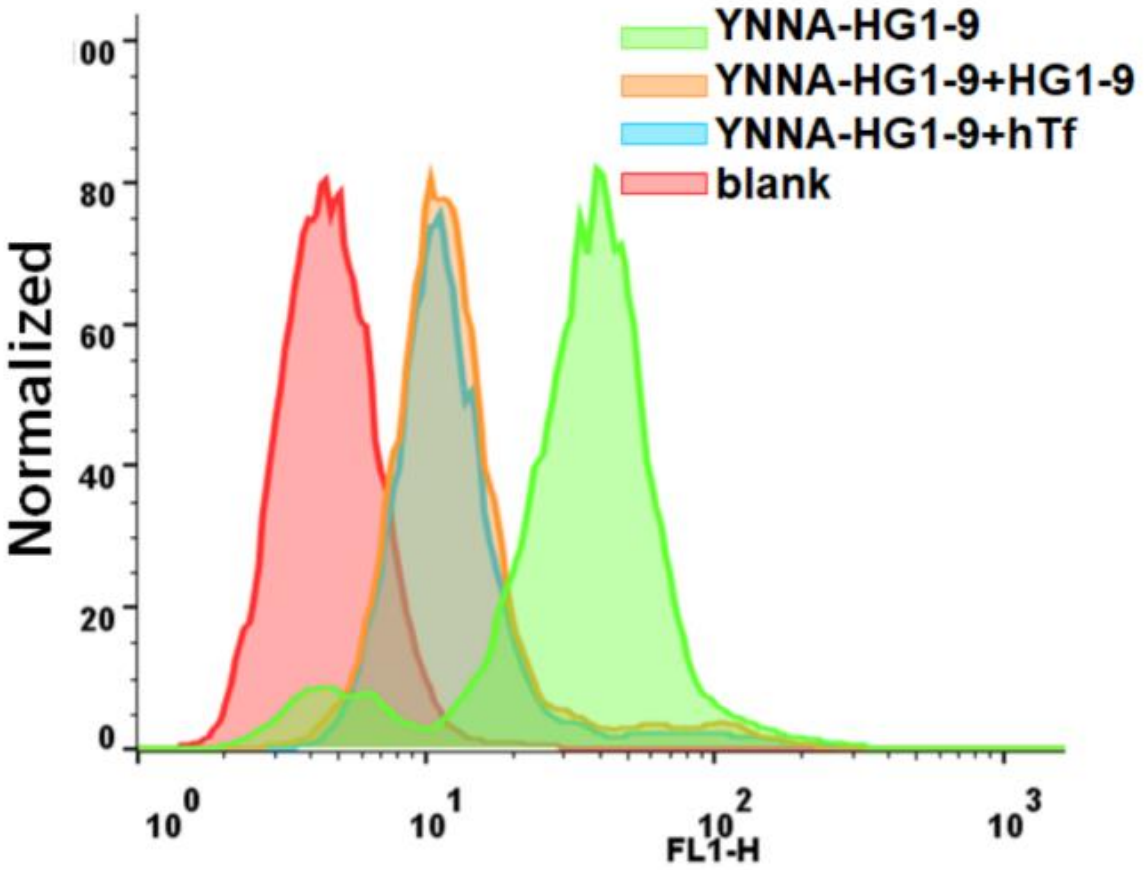


Figure S16. The binding ability of YNNA-HG1-9 to HeLa cells. HeLa cells were incubated with 100 nM YNNA-HG1-9, 100 nM YNNA-HG1-9 (containing 1 μM hTf), or 100 nM YNNA-HG1-9 (containing 1 μM unlabeled HG1-9) on ice for 30 min, then the cells were washed and analyzed by flow cytometry. The blank is cell only without being stained.

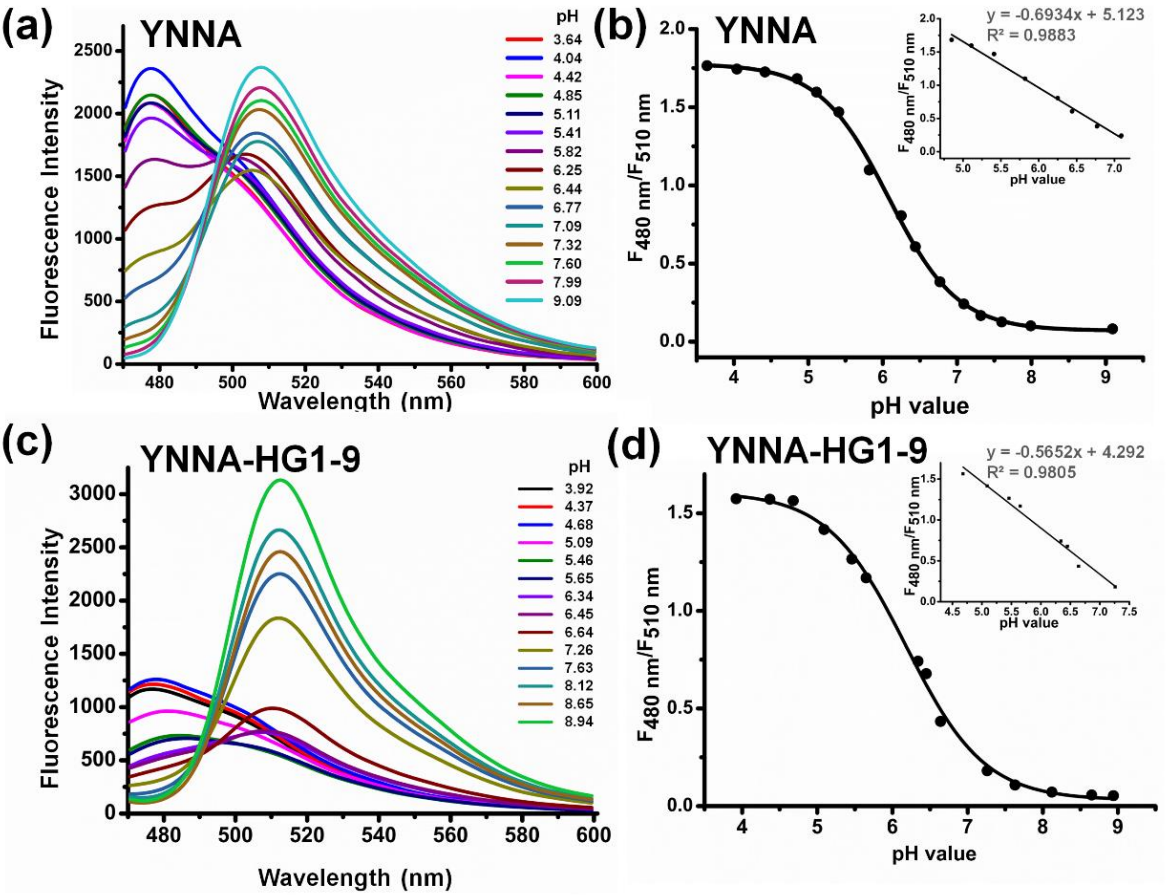


Figure S17. The pH response of YNNA and YNNA-HG1-9 in buffer. YNNA was diluted in HEPES buffers (125 mM KCl, 20 mM NaCl, 0.5 mM $CaCl_2$, 0.5 mM $MgCl_2$, 5 mM glucose and 20 mM HEPES) with different pH values (3.64, 4.04, 4.42, 4.85, 5.11 5.41 5.82, 6.52, 6.44, 6.77, 7.09, 7.32, 7.60, 7.99, and 9.09) to dilute a final concentration of 1 μM, YNNA-HG1-9 was diluted in HEPES buffers with different pH values (3.92, 4.37, 4.68, 5.09, 5.46, 5.65, 6.34, 6.45, 6.64, 7.26, 8.12, 8.65, and 8.94) to dilute a final concentration of 200 nM; then the fluorescence emission spectra were collected from 470 nm to 600 nm with excitation at 455 nm. As shown in the figure, the maximum emission wavelength of YNNA showed red shift from 480 nm to 510 nm with the buffer changed from acidic (3.64) to basic (9.09) (a). And the curve of ratios of the fluorescence intensities ($F_{480\ nm}$/ $F_{510\ nm}$) to pH values (Ratio-pH curve) showed that the ratios exhibited a linear response to pH from pH 4.89 to pH 7.05, indicating a pH-dependent response of YNNA in tube (b). Meanwhile, the pH response of YNNA after conjugation to aptamer HG1-9 in tube was also measured, the results showed that the fluorescence intensities of YNNA-HG1-9 in acidic and basic form were different from those of YNNA though, the ratios ($F_{480\ nm}$/ $F_{510\ nm}$) and pH still exhibited a linear relationship from pH 4.68 to pH 7.26, suggesting that YNNA-HG1-9 kept a pH response in a physiological pH range (c and d).

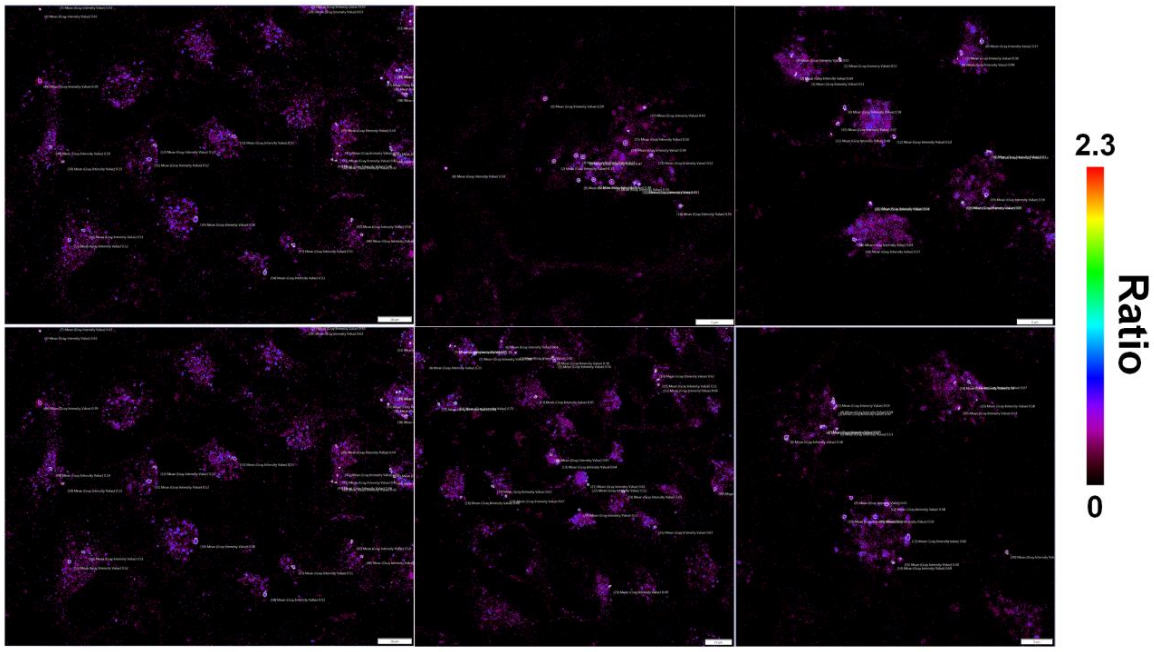


Figure S18. The pseudo-color ratio images of live cells after incubation with 500 nM YNNA-HG1-9 at 37 ℃ for 1 h. The total 151 regions of interest (ROIs) were randomly selected and marked with white circles, and the ratios of ROIs were marked in the figure. Scale bars: 5 or 10 μm; color bar represents fluorescence ratio (0-2.3).

Video Legends

Video S1 and **Video S2:** real-time imaging of internalization process of HG1-7 (AF488 labeled, 50 nM, shown in green) in Rab5B-mCherry (shown in red) transfected HeLa cell.

Video S3 and **Video S4:** real-time imaging of internalization process of HG1-7 (AF488 labeled, 50 nM, shown in green) in Rab7A-mCherry (shown in red) transfected HeLa cell.

Video S5: real-time imaging of internalization process of HG1-7 (AF488 labeled, 50 nM, shown in green) in Rab11A-mCherry (shown in red) transfected HeLa cell.