Supplemental Information

Highly Selective DNA Aptamer Sensor for Intracellular Detection of Coenzyme A

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Experimental Procedures

Chemicals and Materials. Coenzyme A, (R)-Pantetheine, adenine, adenosine-5'-triphosphate (ATP), Pantothenic acid, Bis-Tris, sodium chloride, magnesium chloride, calcium chloride, zinc chloride, potassium chloride, ethylenediaminetetraacetic acid disodium salt dihydrate were purchased from Sigma-Aldrich. Taq DNA polymerase, dNTP, and ATP, were purchased from New England BioLabs (NEB). Small molecules used for the selectivity test were purchased from Cayman Chemical. PZ-2891 (Item No. 37309) was purchased from Cayman Chemical. Calcium hopanate was purchased from LGC Group. All the oligonucleotide sequences were purchased from Integrated DNA Technologies and were purified by high-performance liquid chromatography or polyacrylamide gel electrophoresis and confirmed by mass spectrometry (Table S3). All other reagents and solvents were obtained from the domestic suppliers and were used as received.

SELEX Proceedings. For the first round of SELEX, the initial 1 nmol ssDNA library was used to pair with five times of complementary strands in 250 μL of selection buffer (50 mM Bis-Tris-HCl, 150 mM KCl, 10 mM NaCl, 1 mM $CaCl_2$, 9 mM $MgCl_2$, 180 μM $ZnCl_2$, pH 7.0). The mixture was added to a streptavidin agarose column and incubated for 10 minutes.[1–3] Afterward, the outflow was collected from the column and applied to the column two more times. Then, the column was washed ten times with selection buffer. Afterwards, 250 μL of selection target (R)-Pantetheine used in SELEX rounds 1-4, and CoA used in SELEX rounds 5-14) dissolved in selection buffer was added to the column and incubated for 30 minutes at room temperature. Thereafter, the outflow was collected, and the elution step was repeated two more times.

The solutions eluted by CoA from three times were combined and then amplified as the template for PCR [1 cycle of 95 °C, 2 minutes; N cycles of (95 °C, 15 seconds; 60 °C, 30 seconds; and 72 °C, 45 seconds), and 1 cycle of 72 °C]. The PCR amplicons were incubated with a streptavidin agarose column for about 10 minutes. Thereafter, the elution was collected and incubated with streptavidin agarose column

for two more times to ensure all amplicons were connected to the beads. Afterward, the resin was washed ten times with 1 × PBS. To elute ssDNA containing the aptamer library, 300 μL of 0.2 M NaOH was added to the column and incubated for 10 minutes. Then, an additional 100 μL of 0.2 M NaOH to was added to collect the residual amount left in the column. All the NaOH outflow was collected, neutralized with 0.1 M HCl, and concentrated for the next round of C-SELEX as the library. From the fifth round of SELEX, adenosine, adenosine triphosphate, pantetheine, and pantothenic acid were added as counter targets. After 14 rounds of selection, selection pools were analyzed by high throughput sequencing.

High throughput sequencing of selection rounds. Selected DNA pools from rounds 5, 9, 10, 11, 12, 13, and 14 were prepared for high-throughput sequencing (HTS) analysis on the Illumina HiSeq 4000 platform. The sequencing was performed by the DNA Services Lab at the Roy J. Carver Biotechnology Center, University of Illinois at Urbana-Champaign (UIUC). The DNA sequencing libraries were prepared by the Ovation® Ultralow V2 DNA-Seq Library Preparation Kit from NuGEN Technologies, following the manufacturer's instruction.

The DNA library preparation process included several key steps. First, adaptors were ligated to the DNA fragments, which allowed the sequences to bind to the sequencing platform. Then the sequences were amplified by PCR to generate enough of the final DNA libraries. Thereafter, the PCR products were purified using Agencourt AMPure XP Beads from Beckman Coulter. DNA quantification was then performed using Qubit dsDNA Broad Range Kit to ensure accurate measurement. To create the sequencing pool, approximately equal amounts of each indexed library were combined. Quality control was conducted by the DNA Services Lab, which included qPCR quantification and fragment analysis to assess the size and concentration of the DNA.

The sequencing was carried out using 100-base pair single-end reads. The resulting HTS data were analyzed using the FASTAptamer software suite.[4] FASTAptamer-Count was employed to tally the occurrences of each sequence in the population, enabling ranking and sorting by abundance. Meanwhile, FASTAptamer-Enrich was utilized to calculate fold enrichment for sequences present across multiple

rounds by comparing their reads per million (RPM) between different rounds of selection.

Fluorescence Measurement. CoA-seq 10 aptamer was labeled with FAM at the 5′ terminal and the capture strand was labeled with BHQ1 at the 3′ terminal. Initially, aptamers and the capture strands were mixed and denatured at 95 °C for 5 minutes, followed by annealing at room temperature for 2 hours to produce sensors. Different concentrations of CoA were added to the sensor, followed by incubation at room temperature for 2 hours. The fluorescence was detected using a Microplate System (Biotek, H1M) with a 480 nm excitation wavelength and 520 nm emission wavelength for FAM, respectively. All fluorescence experiments were performed with at least three biological replicates, each with three technical repeats. Aptamer K_d values were determined as previously published methods.[5]

ITC. To study the K_d between CoA and CoA-seq10 aptamer, ITC was performed using a VP-ITC microcalorimeter instrument (MicroCal). CoA and CoA-seq10 aptamer were dissolved in 1× aptamer selection buffer, respectively. Before ITC analysis, the pH of the CoA and CoA-seq10 aptamer solutions were carefully titrated to be the same, which was very important for successful ITC analysis. The solution of CoA and its aptamer were degassed for 10 minutes before subjecting them to ITC. CoA-seq10 aptamer (300 μM) was loaded in the cell, and 3.2 mM CoA in the same buffer was loaded into the syringe. The syringe injected 4 μl of CoA into the cell each time. Each titration was performed three times with separately prepared samples.

To study the K_d between ATP and CoA-seq10 aptamer, ITC was performed using a Malvern MicroCal PEAQ-ITC microcalorimeter instrument. CoA and CoA-seq10 aptamer were dissolved in 1× aptamer selection buffer, respectively. Before ITC analysis, the pH of the ATP and CoA-seq10 aptamer solutions were carefully titrated to be the same, which was very important for successful ITC analysis. The solution of CoA and its aptamer were degassed for 10 minutes before subjecting them to ITC. CoA-seq10 aptamer (300 μM) was loaded in the cell, and 3.2 mM ATP in the same buffer was loaded into the syringe. The syringe injected 0.5 μl of ATP into the cell each time. Each titration was performed three times with separately prepared samples. Through measuring the heat changes and fitting the titration

curves to a one-site binding model, thermodynamic data, including K_d, enthalpy change, entropy change, free energy change and binding stoichiometry, were obtained.

Cell Culture. The HeLa (CCL-2, ATCC) cell line was cultured in DMEM supplemented with 10% fetal bovine serum (FBS; GeminiBio), 100 U ml^{-1} penicillin and 100 U ml^{-1} streptomycin. The cells were cultured at 37 °C in a humidified incubator with 5% CO_2.

HeLa Cell CoA Regulation. HeLa cells were plated at 1 x 10^5 cells per 35 mm poly-D-lysine coated with imaging dishes and were cultured at 37 °C in a humidified incubator with 5% CO_2. Three treatment groups were established: an untreated control in DMEM supplemented with 10% FBS and antibiotics, a group treated with 3 μM PZ-2891 in DMEM supplemented with 10% FBS and antibiotics for 1 day, and a group DMEM without vitamin B5 and with 1 mM hopantenate for 2 days. Following incubation, the cells were washed 3 times with 1 × PBS. The 5' Cy5.5-labeled CoA-seq10 aptamer sensor and the 3' BHQ1-labeled capture strand were delivered into HeLa cells using Lipofectamine 3000 at 37 °C in a humidified incubator with 5% CO_2, following the manufacturer's protocol. As a negative control (NC), we delivered 5' Cy5.5-labeled oligo containing the scrambled sequence (see Fig 2b) and the 3' BHQ1-labeled capture strand. 3.5 hours post-CoA aptamer delivery, the cells were washed 3 times with 1 × PBS. Then, the cells were stained with Hoechst 33342 for nuclear visualization. The cells were washed 3 times with 1 × PBS and incubated with 1 × HBSS for imaging. Microscopy images were captured using either a Zeiss Observer 7 (Zen 3.1 pro) microscope or a Nikon W1 spinning-disk microscope.

Microscopy and image analysis. Images in Figure S6 and S8 were taken on a Zeiss Observer 7 (Zen 3.1 pro) microscope with a x40 oil immersion objective. Images in Figure 5 were taken on a Nikon W1 spinning-disk microscope. To accomplish the imaging, a ×60 water immersion objective was applied, and the fluorophore was excited with a 640-nm laser and Cy5 filter (emission of 672–712 nm). The images were taken with monochromatic Andor EMCCD cameras and were processed using ImageJ (Fiji). More than five frames of each imaging group were processed for further statistical analysis, and more than three biological replicates were performed and validated, showing similar trends.

Data analysis. All experiments were performed with at least three biological replicates. For each individual biological replicate, three technical repeats were performed in cell imaging experiments. The results of each test are displayed as the mean ± s.d. For comparison of two independent groups, a two-tailed unpaired Student's t-test was performed. All the statistical calculations and graph making were performed with GraphPad Prism 8. Statistical significance was determined by t-test as not significant, $P < 0.05$ (*), $P < 0.01$ (**) and $P < 0.001$ (***). The schematics in Figs. 2a and 3d were created with BioRender.com. A BioRender academic license/proof for using these artworks for publication is in place.

Supporting Tables and Figures

Table S1. Counter targets and positive SELEX targets utilized across SELEX rounds. The presented strategy involves a progressive increase in counter-target concentrations alongside a reduction in CoA levels to enhance aptamer selectivity. For instance, in round seven, 5 mM CoA is paired with 10 mM of adenosine (1 mM Adenosine, 9 mM ATP) for counter selection, while subsequent rounds witness a gradual decrease in CoA concentration down to 0.1 mM by rounds 12 to 13. Concurrently, challenging SELEX conditions are introduced with elevated concentrations of counter-targets, aiming to isolate aptamers with heightened selectivity amidst stringent selection pressures.

SELEX round	Counter SELEX	Positive SELEX
1-4	N/A	5 mM (R)-Pantetheine
5	0.1 mM Adenine	5 mM CoA
6	1 mM Adenine	5 mM CoA
7	1 mM Adenine 9 mM ATP	5 mM CoA
8	1 mM Adenine 9 mM ATP 50 μM Pantetheine	5 mM CoA
9	1 mM Adenine 9 mM ATP 100 μM Pantetheine	4 mM CoA
10	1 mM Adenine 9 mM ATP 150 μM Pantetheine	1 mM CoA
11	1 mM Adenine 9 mM ATP 150 μM Pantetheine	300 μM CoA
12	1 mM Adenine 9 mM ATP 150 μM Pantetheine 150 μM Pantothenic acid	100 μM CoA
13	1 mM Adenine 9 mM ATP 150 μM Pantetheine 150 μM Pantothenic acid	100 μM CoA
14	1 mM Adenine 9 mM ATP 150 μM Pantetheine 150 μM Pantothenic acid	100 μM CoA

Table S2. The Top 10 Sequences Enriched from CoA aptamer SELEX.

Sequence Name	Sequence (5' to 3')
Sequence 1	GGGACGATGCCAACGCGAAAACGTGTCGCAAAGGCAAGGC
Sequence 2	GGACGACGACGCTGAAAGAGTACAACAGTGTACAGTGCAA
Sequence 3	GGCCAGACAGCCGCAGTGCTGAAGGTGTAGGAGACAGACC
Sequence 4	GGGACAGACAGATAACTGGTATGTGGGAGTTATCTGGAAC
Sequence 5	GGGCAGCCGACAAACGTGAGAATAACGTGCAGACGGAACA
Sequence 6	GGGACACGATGTACAGTCCGCAAGGACTAGCACAGCGGGA
Sequence 7	GGGCGACAACAAGTGCCTGCTAAAGGGGTACTGTACGGAC
Sequence 8	GGGCAGTAGAAGTGTACCACATAGTGATACACTTATACCG
Sequence 9	GGCAAGAGTCAGGCATAATCAGTATGATGCCCTGAGTCTG
Sequence 10	GCAAAGGGGAGAGTGATTATAATCACTCGGGACATGGGAT

Table S3. Oligonucleotides used in this study.

Name	Sequence (5' to 3')
SELEX library	GCAGTCGGCGTCGGACAG-N40-CTGTCCGACGATGTAACGCTTCAC
Forward primer	GCAGTCGGCGTCGGACAG
Reverse primer	GTGAAGCGTTACATCGTCGGACAG
5' Biotin-Reverse primer	/5BiosG/iSpC3/GTGAAGCGTTACATCGTCGGACAG
5' Biotin-Capture strand	/5BiotinTEG/CTGTCCGACGCCGACATA
5' FAM -CoA-seq10 aptamer	/56-FAM/ ATGCAGTCGGCGTCGGACAGGCAAAGGGGAGAGTGATT ATAATCACTCGGGACATGGGATCTGTCCGACGATGTAAC GCTTCAC
3'BHQ-Capture strand	CTGTCCGACGCCGAC/3BHQ1/
5' FAM -Negative control with scrambled sequences	/56-FAM/ ATGCAGTCGGCGTCGGACAGGGACGACGACGCTGAAAG TGTACAACAGTGTACAGTGCAACTGTCCGACGATGTAAC GCTTCAC
5' Cy5 -CoA-seq10 aptamer	/5Cy5/ATGCAGTCGGCGTCGGACAGGCAAAGGGGAGAGT GATTATAATCACTCGGGACATGGGATCTGTCCGACGATGT AACGCTTCAC
3' Iowa Black® RQ-Capture strand	CTGTCCGACGCCGAC/3IAbRQSp/
5' Cy5 -Negative control with scrambled sequences	/5Cy5/ATGCAGTCGGCGTCGGACAGGGACGACGACGCTG AAAGTGTACAACAGTGTACAGTGCAACTGTCCGACGATG TAACGCTTCAC
Mutation 1	/56-FAM/ ATGCAGTCGGCGTCGGACAGGCAAAGGGGAGAGTGATA ATAATCACTCGGGACATGGGATCTGTCCGACGATGTAAC GCTTCAC
Mutation 2	/56-FAM/ ATGCAGTCGGCGTCGGACAGGCAAAGGGGAGAGTGATT TTAATCACTCGGGACATGGGATCTGTCCGACGATGTAAC GCTTCAC
Mutation 3	/56-FAM/ ATGCAGTCGGCGTCGGACAGGCAAAGGGGAGAGTGATT AAAATCACTCGGGACATGGGATCTGTCCGACGATGTAAC GCTTCAC
Mutation 4	/56-FAM/ ATGCAGTCGGCGTCGGACAGGCAAAGGGGAGAGTGATT ATTATCACTCGGGACATGGGATCTGTCCGACGATGTAACG CTTCAC
Mutation 5	/56-FAM/ ATGCAGTCGGCGTCGGACTGGCAAAGGGGAGAGTGATT ATAATCACTCGGGACATGGGATCAGTCCGACG
Mutation 6	/56-FAM/ ATGCAGTCGGCGTCGGTCAGGCAAAGGGGAGAGTGATT ATAATCACTCGGGACATGGGATCTGACCGACG
Mutation 7	/56-FAM/

	ATGCAGTCGGCGTCGGACAGGCAAAGGGGAGAGAGATT ATAATCTCTCGGGACATGGGATCTGTCCGACG
Mutation 8	/56-FAM/ ATGCAGTCGGCGTCGGACAGGCAAAGGGGAGAGTGAAT ATATTCACTCGGGACATGGGATCTGTCCGACG
Mutation 9	/56-FAM/ ATGCAGTCGGCGTCGGACAGGCAAAGGGGAGAGTGTTT ATAAACACTCGGGACATGGGATCTGTCCGACG
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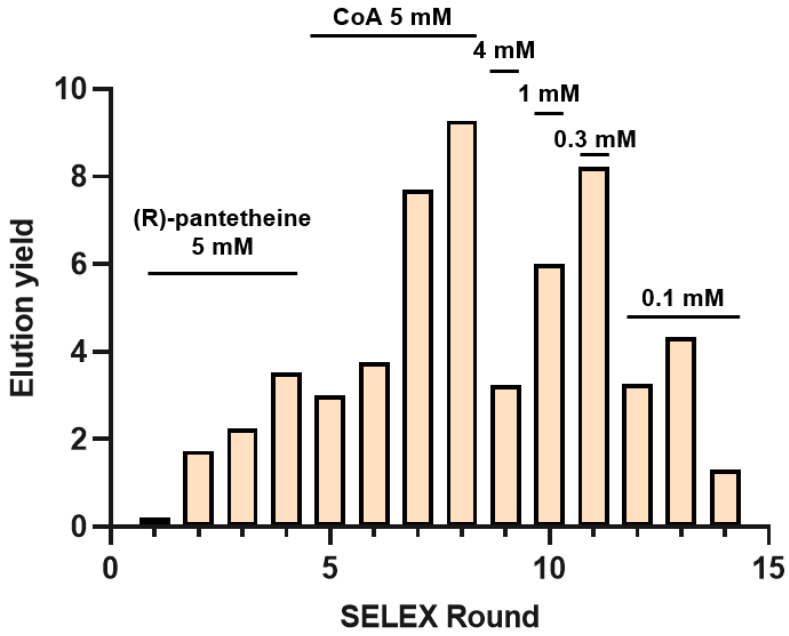

Figure S1. Monitoring the progress of the SELEX by quantification of the elution yield, *i.e.*, bound ssDNA over total added ssDNA, using qPCR.

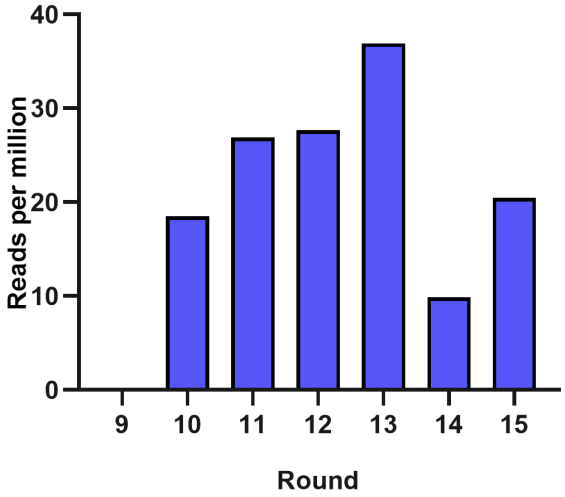

Figure S2. Reads per million (RPM) obtained from analysis of the HTS data for the CoA-seq10 sequence as a function of the selection rounds, using FASTAptamer-Count.[4]

CoA-seq10	ATGCAGTCGGCGTCGGACAGGCAAAGGGGAGAGTGATTATAATCACTCGGGACATGGGATCTGTCCGACGATGTAACGCTTCAC
Mutation 1	ATGCAGTCGGCGTCGGACAGGCAAAGGGGAGAGTGATAATAATCACTCGGGACATGGGATCTGTCCGACGATGTAACGCTTCAC
Mutation 2	ATGCAGTCGGCGTCGGACAGGCAAAGGGGAGAGTGATTTTAATCACTCGGGACATGGGATCTGTCCGACGATGTAACGCTTCAC
Mutation 3	ATGCAGTCGGCGTCGGACAGGCAAAGGGGAGAGTGATTAAAATCACTCGGGACATGGGATCTGTCCGACGATGTAACGCTTCAC
Mutation 4	ATGCAGTCGGCGTCGGACAGGCAAAGGGGAGAGTGATTATTATCACTCGGGACATGGGATCTGTCCGACGATGTAACGCTTCAC
Mutation 5	ATGCAGTCGGCGTCGGACTGGCAAAGGGGAGAGTGATTATAATCACTCGGGACATGGGATCAGTCCGACG
Mutation 6	ATGCAGTCGGCGTCGGTCAGGCAAAGGGGAGAGTGATTATAATCACTCGGGACATGGGATCTGACCGACG
Mutation 7	ATGCAGTCGGCGTCGGACAGGCAAAGGGGAGAGAGATTATAATCTCTCGGGACATGGGATCTGTCCGACG
Mutation 8	ATGCAGTCGGCGTCGGACAGGCAAAGGGGAGAGTGAATATATTCACTCGGGACATGGGATCTGTCCGACG
Mutation 9	ATGCAGTCGGCGTCGGACAGGCAAAGGGGAGAGTGTTTATAAACACTCGGGACATGGGATCTGTCCGACG
Mutation 10	ATGCAGTCGGCGTCGGACAGGCAAAGGGGAGTGTGATTATAATCACACGGGACATGGGATCTGTCCGACG

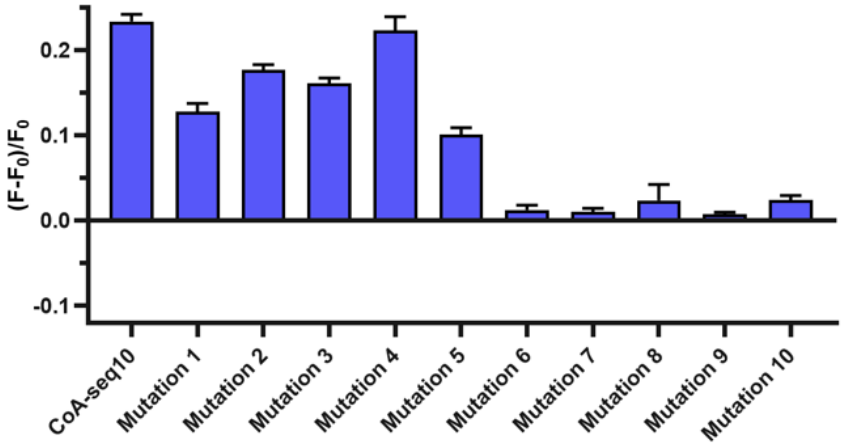

Figure S3. Normalized fluorescence of CoA-seq10 aptamer and its mutations.

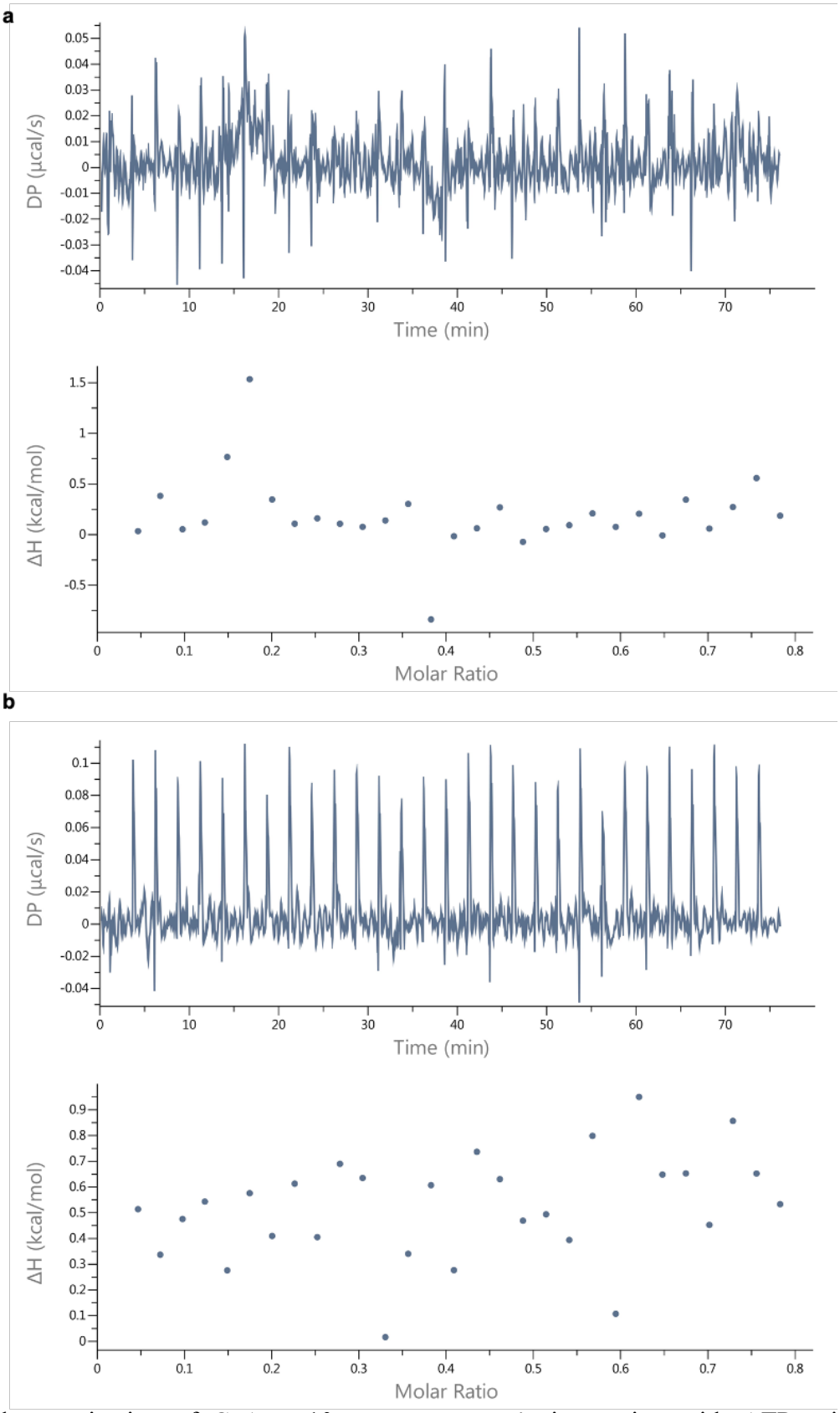

Figure S4. Characterization of CoA-seq10 aptamer sensor's interaction with ATP using ITC. (a) Thermogram of adding different volumes of 3.2 mM ATP in the aptamer binding buffer to aptamer binding buffer; (b) Thermogram of adding different volumes of 3.2 mM ATP to 300 µM CoA-seq10 in aptamer binding buffer to titrate different molar ratio between ATP and the aptamer buffer.

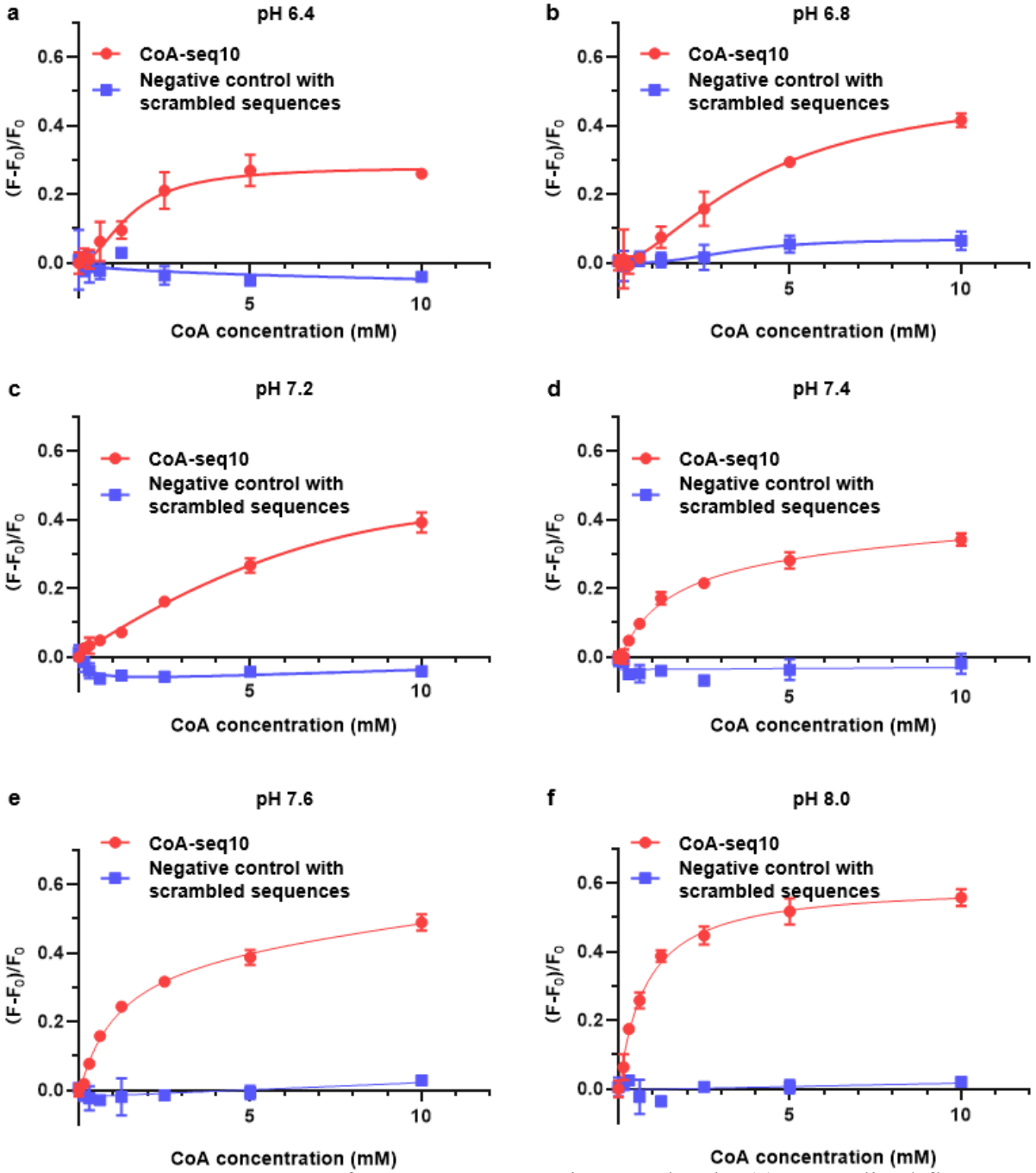

Figure S5. CoA aptamer sensor performance across various pH levels. (a) Normalized fluorescence of the CoA-seq10 aptamer sensor's activity versus the negative control with scrambled sequences at pH 6.4, (b) pH 6.8, (c) pH 7.2, (d) pH 7.4, (e) pH 7.6, and (f) pH 8.0.

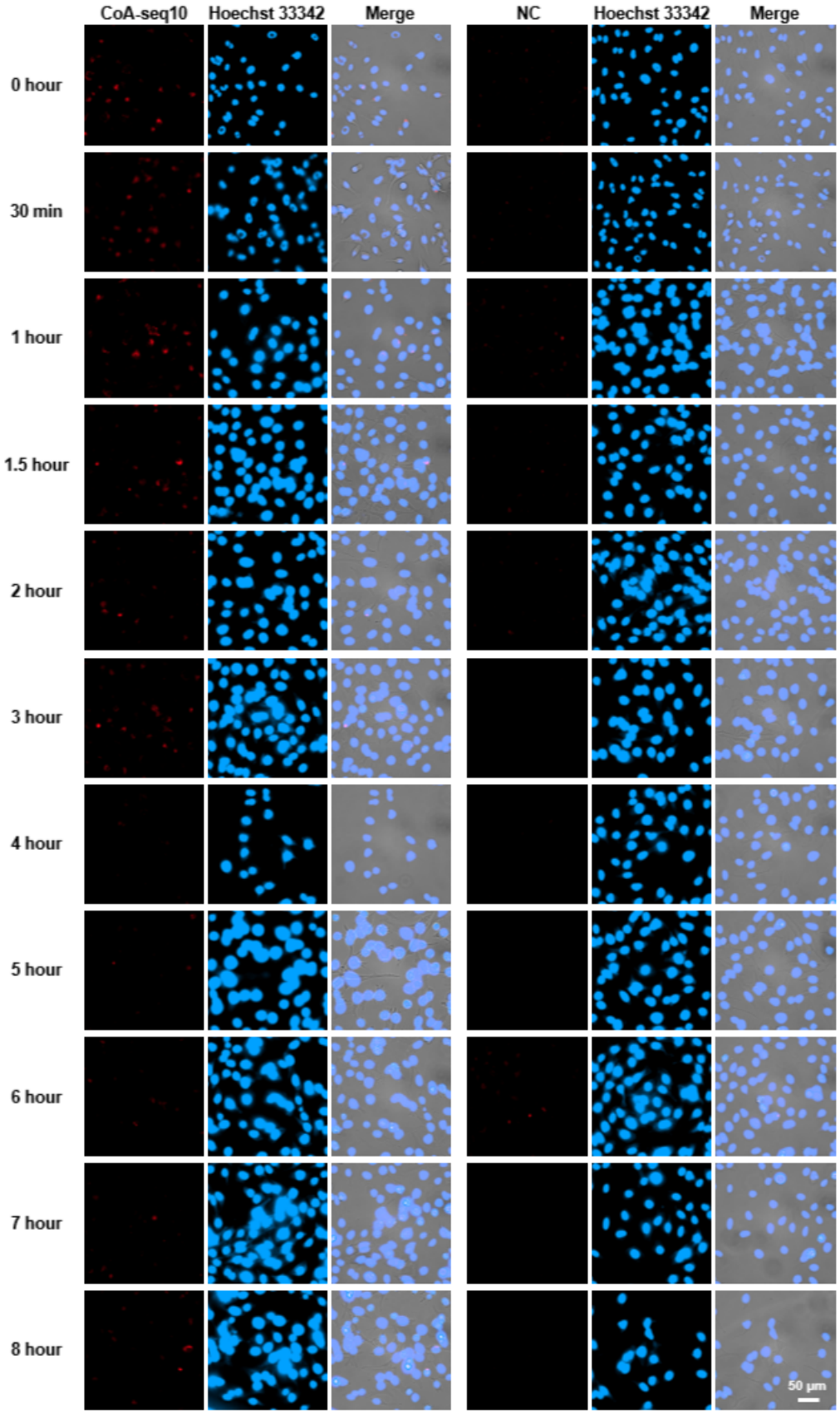

Figure S6. Time-course analysis post transfection of the CoA aptamer sensor in live HeLa cells. Representative microscopy images of HeLa cells with the CoA-seq10 sensor and the NC sensor. Data represent three independent experiments; n = 5 frames. Scale bar is 50 µm.

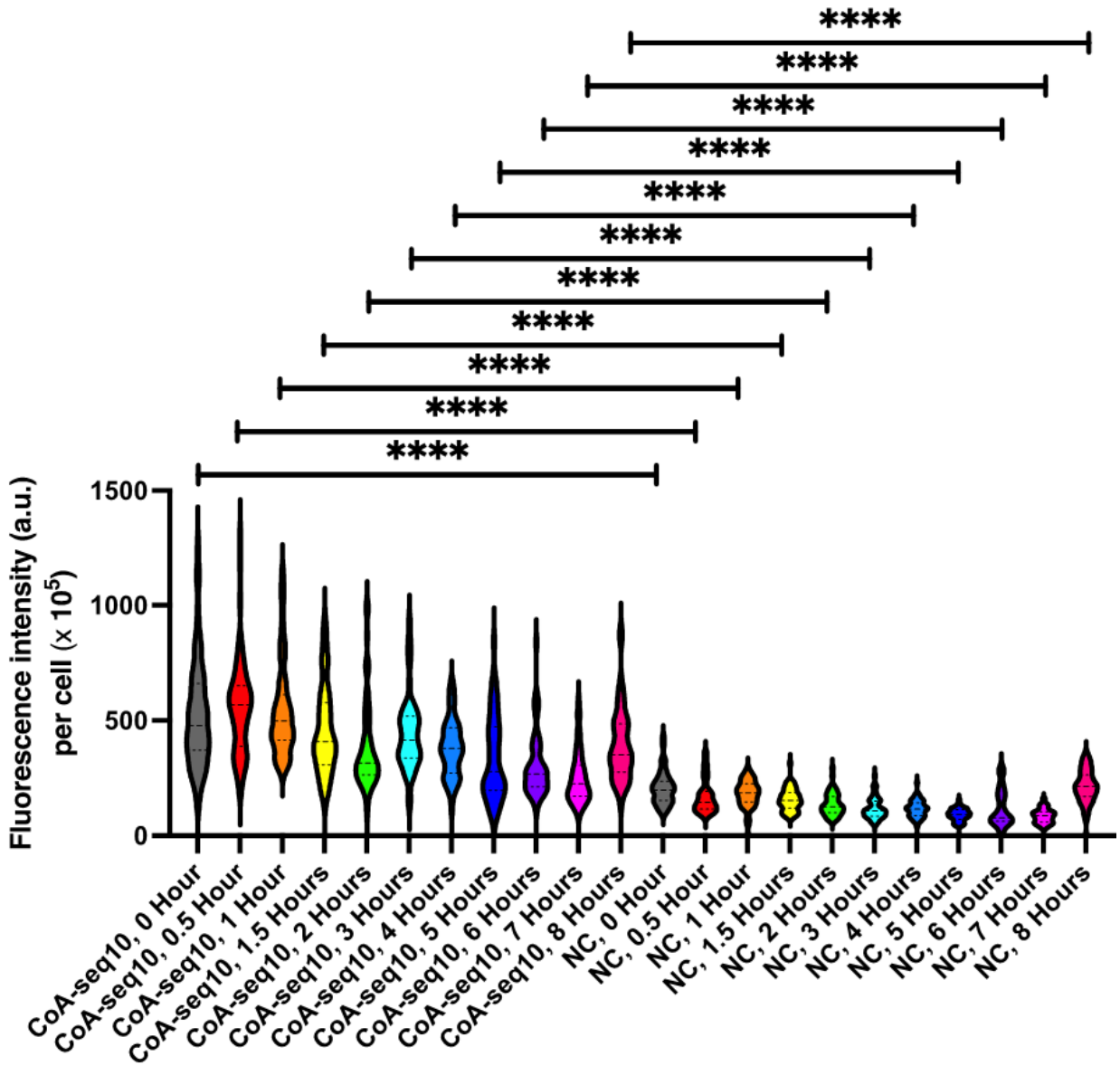

Figure S7. Time-course analysis post-transfection of the CoA aptamer sensor in live HeLa cells. Quantification of average fluorescence intensity (arbitrary units, a.u.) per cell shown in Figure S6; ***P < 0.0001. Data represent three independent experiments; n = 5 frames. Data are shown as mean ± s.d. Statistical significance was determined by unpaired two-tailed Student's t-test; NS, not significant (P>0.05); *** (P < 0.001).

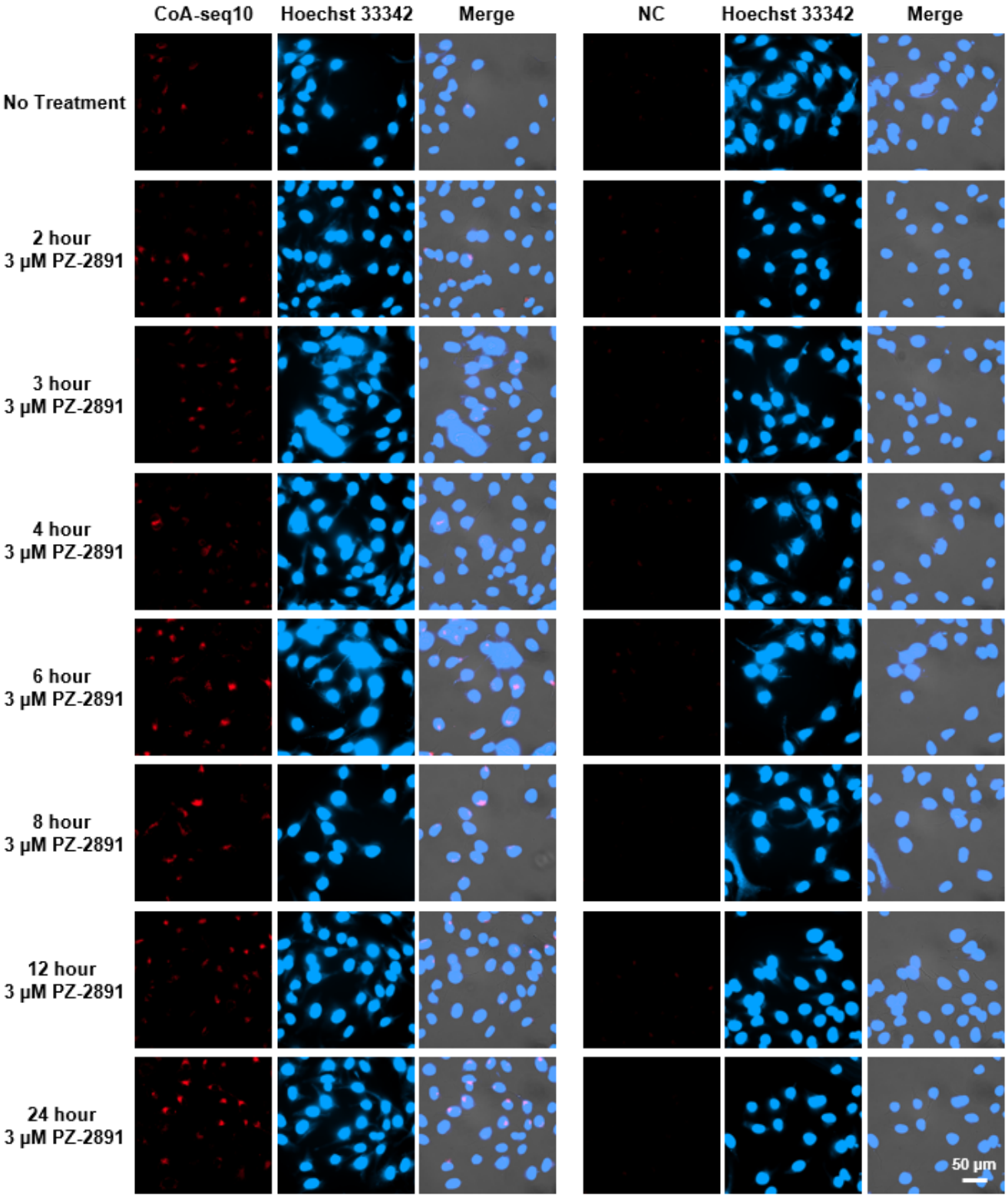

Figure S8. CoA aptamer sensor response in live HeLa cells following time-course treatment with 3 µM PZ2891. Representative microscopy images of HeLa cells with the CoA-seq10 sensor and the NC sensor. Data represent three independent experiments; n = 5 frames. Scale bar is 50 µm.

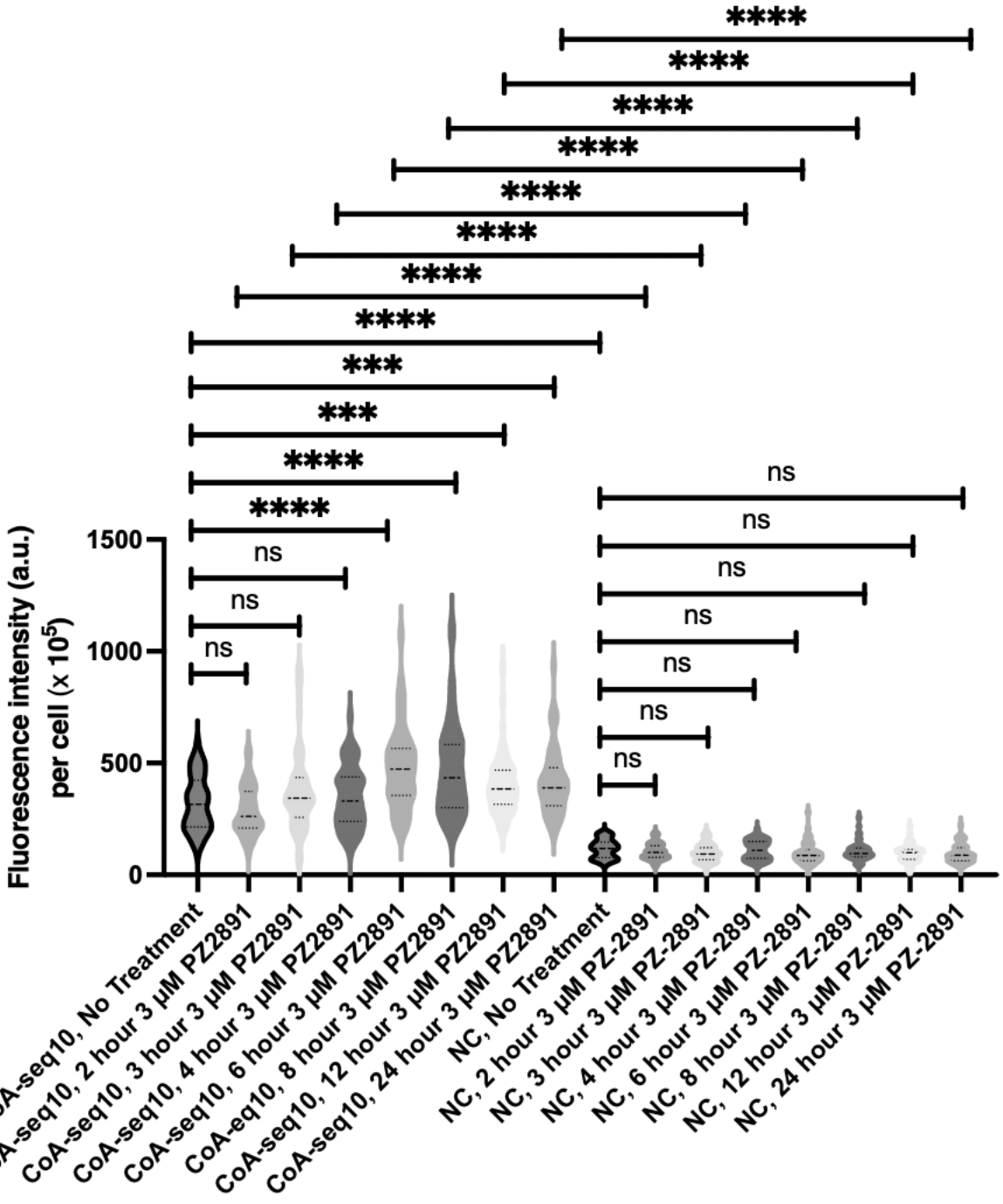

Figure S9. CoA aptamer sensor response in live HeLa cells following time-course treatment with 3 μM PZ2891. Quantification of average fluorescence intensity (arbitrary units, a.u.) per cell shown in Figure S8; ***$P < 0.0001$. Data represent three independent experiments; $n = 5$ frames. Data are shown as mean ± s.d. Statistical significance was determined by unpaired two-tailed Student's t-test; NS, not significant ($P>0.05$); *** ($P < 0.001$).

References

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