Electronic Supplementary Material (ESI) for Chemical Science.

A DNA-Mediated Crosslinking Strategy to Enhance Cellular Delivery and Sensor Performance of Protein Spherical Nucleic Acids

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Abstract: Intracellular delivery of enzymes is essential for protein-based diagnostic and therapeutic applications. Protein–spherical nucleic acids (ProSNAs) defined by protein core and dense shell of oligonucleotides have been demonstrated as a promising vehicle-free enzyme delivery platform. In this work, we reported a crosslinking strategy to vastly improve both delivery efficiency and intracellular sensor performance of ProSNA. By assembling individual ProSNA with lactate oxidase (LOX) core into a nanoscale particle, termed as crosslinked SNA (X-SNA), the enzyme delivery efficiency increased up to 5-6 times higher. The LOX X-SNA was later demonstrated as a ratiometric probe for quantitative detection of lactate in living cells. More importantly, X-SNA probe showed significantly improved sensor performance with signal-to-noise ratio 4 times as high as ProSNA when detecting intracellular lactate.

Experimental Procedures

Materials

Synthesized oligonucleotides, PAGE preparation kit, Sybrgeen II, Coomassie blue kit, 1M Hepes buffer and 1×TE (pH 8.0) buffer were obtained from Sangon Biotech Co., Ltd. (Shanghai, China). The sequences of the oligonucleotides were shown in Table S1. All the oligonucleotides were synthesized from Sangon Inc. and purified by HPLC. Lactate oxidase was purchased from Asahi Kasei Inc. Flavin adenine dinucleotide disodium salt hydrate (FAD), heterobifunctional cross-linker EMCS, lactate production inhibitor oxamate, and Sephadex G-15 were purchased from Sigma-Aldrich. Chemicals of CH_2Cl_2, MeOH, 2-[(4-bromomethyl) phenyl]-4,4,5,5-tetramethy-1,3,2-dioxaborolane, 7-hydroxy-3H-phenoxazin-3-one sodium salt, Cs_2CO_3, DMF, and Na_2SO_4 were purchased from Aladdin (Shanghai, China). Opti-MEM was purchased from Invitrogen (MA, USA). Human cervical carcinoma cell line (HeLa), human breast adenocarcinoma cell line (MCF-7), and mouse endothelial cell line (L-02) were obtained from the cell bank of Central Laboratory at Xiangya Hospital (Changsha, China). Cell culture media was obtained from Thermo Scientific HyClone (MA, USA). Ultra-pure water (18.2 MΩ cm; Milli-Q, Millipore) was used for preparing all solutions. All chemicals are analytical grade unless otherwise indicated.

Instruments

The gel image was visualized by Tanon 4200SF gel imaging system (Tanon Science & Technology Co., Ltd., China). The fluorescence measurements were carried out at room temperature in a quartz cuvette on an FL-7000 spectrometer (Hitachi, Japan). Hydrodynamic size and zeta potential measurements were performed on a Malvern Zetasizer Nano (Malvern Instruments Ltd.). HPLC was performed on a RP-HPLC system (Waters, USA). UV were performed on Shimadzu UV-VIS Spectrophotometers (Shimadzu, Japan). All cell fluorescence images were acquired using an oil dipping objective (60×) on Nikon TI-E+A1 SI confocal laser scanning microscope (Japan). Flow cytometric analysis of cells was performed on a CytoFLEX™ flow cytometer (Beckman Counter, Inc., USA).

Preparation of LOX-based SNA and LOX X-SNA constructs

LOX SNAs were prepared using previously reported method using native LOX. LOX was reacted with 100-fold excess of a heterobifunctional crosslinker *N*-*ε*-malemidocaproyl-oxysuccinimide ester (EMCS) in 10 μM HEPES buffer (pH = 6.8-7.5) for 30 min.[1],[2] After purification by Sephadex G-15 (Sigma-Aldrich), modified LOXs were recovered in 10 μM HEPES buffer (pH = 6.5-8.5), and then the solution was added with 5'-terminus sulfhydryl-oligonucleotides for 30 min amine-thiol conjugation. The conjugates were subsequently purified with 10 rounds of ultrafiltration (Millipore Amicon, Ultra-0.5 mL Centrifugal Filter Units) via centrifugation at 10,000 *g* for 10 min.

For preparation of X-SNA construct, SNAs were incubated with pre-hybridized double strand DNA linker at 30 °C for 1 h in TAE buffer, then decreased from 30 °C to 4 °C over 60 min. The hybrid products were resuspended in PBS buffer.

Synthesis and characterization of fluorescence H_2O_2 indicator

DMF solution of 7-hydroxy-3H-phenoxazin-3-one, 2-[(4-bromomethyl) phenyl]-4,4,5,5-tetramethyl-1,3,2-dioxaborolane and Cs_2CO_3 was stirred at room temperature overnight then added with 100 ml H_2O. A red precipitate was isolated by centrifugation, and the solid was dissolved by CH_2Cl_2 and dried by Na_2SO_4. After removing the solvent under reduced pressure, the resulting products were purified using silica column chromatography (0.3% MeOH in CH_2Cl_2).

To obtain the p*K*a of dye, we did fluorometric titration experiments.[3] Briefly, the dye *K*a constant can be determined by a function of pH and measured fluorescence intensity using fluorometric titration. The steady-state fluorescence intensity *F* can be represented as a function of the proton concentration and *K*a, which is shown below:

$$F = \frac{F_{\min}[\mathrm{H}^+]^n + F_{\max} K\mathrm{a}}{K\mathrm{a} + [\mathrm{H}^+]^n}$$

In this function, F_{min} and F_{max} represent the fluorescence intensities at maximal and minimal H^+ concentrations respectively, and n is apparent stoichiometry of H^+ binding to the probe. We measured the fluorescence intensity at 585 nm of the dye solution at different

pH values and plotted the fluorometric titration results. By fitting the fluorescence titration data as a function of H^+ concentration using the above-mentioned equation, we can estimate the apparent constant of *K*a by using n as a free adjustable parameter. The p*K*a was calculated to be ca. 8.75.

Gel electrophoresis analysis

LOX SNA constructs were analyzed using 15% SDS-PAGE after 95 °C heat denaturation for 3 min. Gel was stained using Coomassie blue for protein imaging, and SYBR Green II for DNA imaging. Gel electrophoresis was performed at a constant voltage of 150 V for 1.5 h at room temperature. DNA image of the gel was visualized by Tanon 4200SF gel imaging system (Tanon Science & Technology Co., Ltd., China).

High performance liquid chromatography (HPLC)

Samples of 500 nM native LOX, SNA, and X-SNAs were diluted to 1 mL respectively and injected into a Waters RP-HPLC system equipped with a Waters Symmetry C18 column (3.5 μm, 4.6 × 150 mm). A gradient method was used, beginning with 95:5 vol/vol 0.05 M ammonium acetate (aq):MeCN, increasing to 65:35 vol/vol over 60 min (at a ramp of +0.5 vol% MeCN/min), with a flow rate of 1 mL/min.

Dynamic light scattering

Hydrodynamic sizes and Zeta results were performed on a Malvern Zetasizer Nano (Malvern Instruments Ltd.). Samples were diluted in PBS solution. The reported hydrodynamic diameters are based on intensity distributions.

Fluorescence measurements using spectrometer

All fluorescence measurements were performed on a FL-7000 spectrometer, using a quartz fluorescence cell with an optical path length of 1.0 cm. The excitation wavelength was fixed at 550 nm with a recording emission range from 565 nm to 650 nm. Error bars come from the average of three measurements with calculated standard deviations.

Atomic force microscopy (AFM)

AFM studies were performed using a Dimension Icon– Bruker instrument. The protein particle size measurements were performed using PeakForce Tapping mode at ambient conditions. Obtained images were analyzed by NanoScope Analysis v1.4 (Bruker).

Enzyme activity assay

All samples containing 1 μM LOX were mixed with PBS buffer (pH = 8) with 1 mM lactate and 10 μM FAD at room temperature for 1 h.

To evaluate the intracellular activity of native LOX and X-SNA, we used cell lysate to mimic the intracellular environment to perform the fluorescence assay due to the poor cell entry ability of native protein. Briefly, we first disrupted 10^5 HeLa cells in 1 mL 1× PBS using ultrasonic cell crusher. After removing cell fragments from PBS solution using centrifugation and filtration, the cell lysate was collected for activity assay. Native LOX and X-SNA with the same protein concentration were incubated with 10 μM FAD in cell lysate.

After 1h incubation to enable complete reaction, H_2O_2 indicator of 1 mM was then added into the solution and fluorescence emission was measured using FL-7000 spectrometer.

Modification of LOX with Cy5

To label native LOX with fluorophore Cy5, we used sulfo-Cy5 NHS ester to conjugate with primary amine groups on protein surface (Figure R5a). Briefly, native LOX proteins were reacted with 100-fold excess of sulfo-Cy5 NHS ester in 1× PBS at 4 °C for overnight to allow efficient modification. The product was purified by ultrafiltration using Amicon 10k at 10,000 g for multiple times. To calculate the number of Cy5 molecules per LOX protein, we measured the fluorescence emission and UV-vis absorption spectra of the conjugate (Figure R5b and R5b). The Cy5 concertation was calculated by the fluorescence emission intensity at 670 nm with excitation at 650 nm. The LOX concertation was calculated by the UV-vis absorption at 280 nm. The number of Cy5 molecules was calculated to be ca. 33 molecules per protein.

In vitro cell experiments

HeLa and L-02 cells were cultured in RPMI 1640 medium (Thermo Scientific Hyclone) supplemented with 12% fetal bovine serum

(Invitrogen). MCF-7 cells were grown in DMEM medium (Gibco) supplemented with 12% fetal bovine serum. Cells were cultured at 37 °C in a humidified atmosphere containing 5% CO_2 and seeded in glass Petri dish and grown overnight.

Cells were treated with medium containing inhibitor oxamate or external lactate for 4h following the reported procedure.[4] The treated cells were washed for three times with cold PBS and incubated with X-SNA samples of 5 nM protein concentrations in OptiMEM (serum-free) for up to 4 h. Then, cell samples were added 10 μM FAD for lactate oxidation reaction. Before confocal imaging, the cells were washed three times with PBS, and incubated with a fresh medium containing 20 mM H_2O_2 indicator at 37 °C for 30 min.

To study cellular uptake mechanism of X-SNAs, we first incubated HeLa cells with 5 nM protein at 4 °C or in the presence of NaN_3. Then, Hela cells were incubated at 37 °C for 30 min with culture medium containing different inhibitors including Me-*β*-CD (0.5 mM an inhibitor of lipid-raft-/caveolin mediated endocytosis), Nystatin (50 μg/mL caveolin-mediated endocytosis inhibitor), Amiloride (2 mM macropinocytosis-mediated endocytosis inhibitor), and CPZ (20 μg/mL clathrin-mediated endocytosis inhibitor), respectively, followed by incubation at 37 °C for 2 h with 5 nM protein.

To study the colocalization of X-SNA samples, cells were first treated with culture medium containing the X-SNA-1 of 5 nM protein concentration, and further incubated at 37 °C for 2 and 4 hours. CellLight Early Endosome-GFP was used to stain early endosomes and LysoTracker Green DND-26 was used to stain lysosomes. A 10 μL stock solution with 10^5 cells in 1 mL of culture medium was used in this experiment. Cells were washed 3 times with 1x PBS before imaging. The cells were then washed 3 times with 1x PBS before imagining. The samples were imaged under Nikon TI-E+A1 SI confocal laser scanning microscope (Japan) using 560 nm laser excitation for lactate and 620 nm laser excitation images for Cy5.

Inverted cell culture

For the inverted cell culture, we used the culture setup previously reported.[5] The schemes of both upright and inverted configurations are shown in Figure S11a. For inverted cell culture experiment, confocal dish with attached HeLa cells was hanging face down in the culture plate by gluing the back of the confocal dish to the culture plate cover using a plastic tip. Cells were grown in culture medium for overnight, and then were incubated with Cy3-labeled SNA and different sized X-SNA samples of 5 nM protein concentration for up to 4 h at 37 °C. The treated cells were washed for three times with cold 1× PBS buffer before confocal imaging.

Flow cytometry experiments

Hela cells of $1x10^6$ were seeded in a 30 mm dish and incubated for 24 h before experiments. After removing the culture medium, cells were incubated with SNA and X-SNA samples with 5 nM protein concentration in OptiMEM (serum-free). Then, 10 μM FAD was added into solution for lactate oxidation reaction. After 4h incubation at 37 °C in 5% CO_2 atmosphere, cells were washed three times with 1x PBS. The cells were resuspended in 400 μL of PBS for flow cytometric analysis on the BD FACSVerse™ flow cytometery.

MTT assay

HeLa cells were incubated with 80 nm LOX X-SNA-1 samples of different protein concentrations (0, 5, 10, 15, 20 nM) for 4h. Then the viability of X-SNA treated cells were evaluated using standard 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay.

Lactate concentration in cell lysate

Lactate concentration in cell lysate was determined based on the manufacturer's protocols (MAK064). Briefly, 10^7 cells were homogenized in 4 volumes of lactate assay buffer and centrifuged at 13,000 *g* for 10 min to remove insoluble material. A master reaction mixture containing 50 μL sample solution, 46 μL lactate assay buffer, 2 μL lactate enzyme mixture, and 2 μL lactate probe was added. Reaction solution was incubated at RT for 30 min. Sample absorbance was measured at 570 nm (A570) on a microplate reader. A total of 10 μL of 100 nmol/μL lactate standards was diluted with 990 μL lactate assay buffer to generate a 1 nmol/μL standard solution to establish the standard curve.

Supplementary Figures

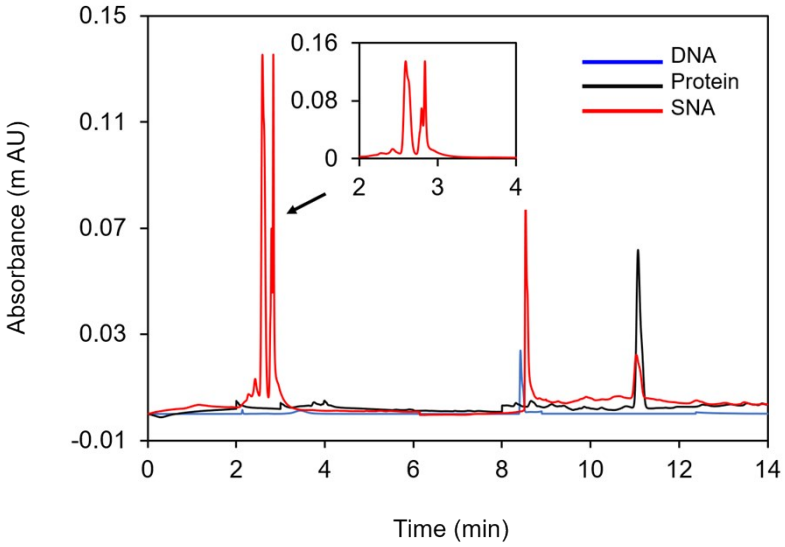
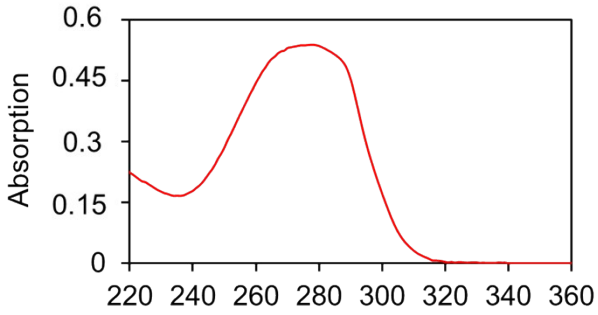


Figure S1. High-performance liquid chromatography characterizations of LOX SNA.

a



b

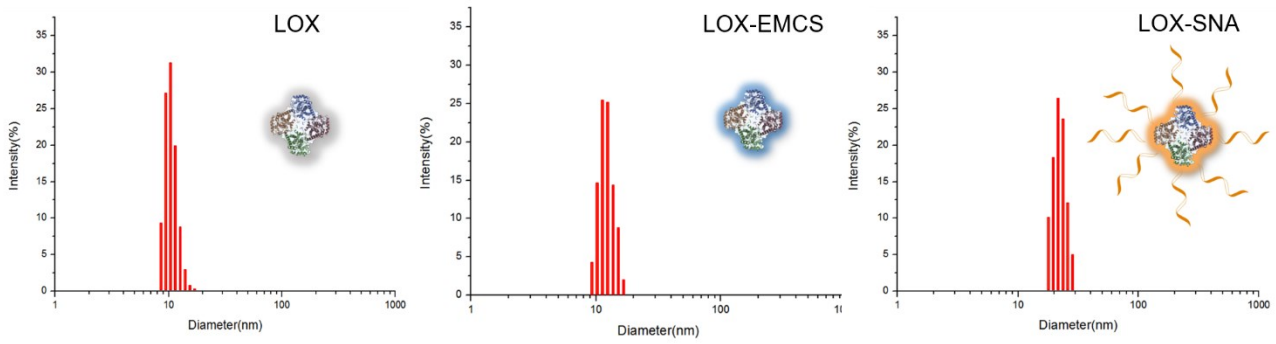
Name	A_{260}	A_{280}	Extinc. (260)	Extinc. (280)	Conc. (μM)
LOX	0.438	0.644	35040	51340	12.5
SH-DNA	0.341	0.294	338200	294000	1
LOX-SNA	0.45	0.54			

$$A_{260}(\text{LOX-SNA})=\varepsilon_{260}(\text{LOX})*b*\text{Conc.(LOX)}+\varepsilon_{260}(\text{DNA})*b*\text{Conc.(DNA)}$$

$$A_{280}(\text{LOX-SNA})=\varepsilon_{280}(\text{LOX})*b*\text{Conc.(LOX)}+\varepsilon_{280}(\text{DNA})*b*\text{Conc.(DNA)}$$

Figure S2. (a) UV-vis characterization of purified LOX SNA sample. (b) The concertation of SNA sample was calculated using Beer-Lambert law. DNA- functionalized protein concertation was calculated to be ~0.59 μM and DNA concertation was calculated to be ~7.1 μM, resulting in ~12 DNA per protein. By setting LOX as a sphere with the diameter of 12 nm, the DNA surfaces density was calculated to be $12 \times (1 \text{ mol} / \text{NA}) / 4\pi R^2 \approx 4 \text{ pmol/cm}^2$.

a



b

Protein	Native	EMCS-labeled	DNA-functionalized
LOX	11.5	12.1	40.6

Figure S3. (b) Dynamic light scattering characterizations of native LOX, ENCS-labeled LOX, and LOX SNAs. (b) The measured average hydrodynamic sizes of samples.

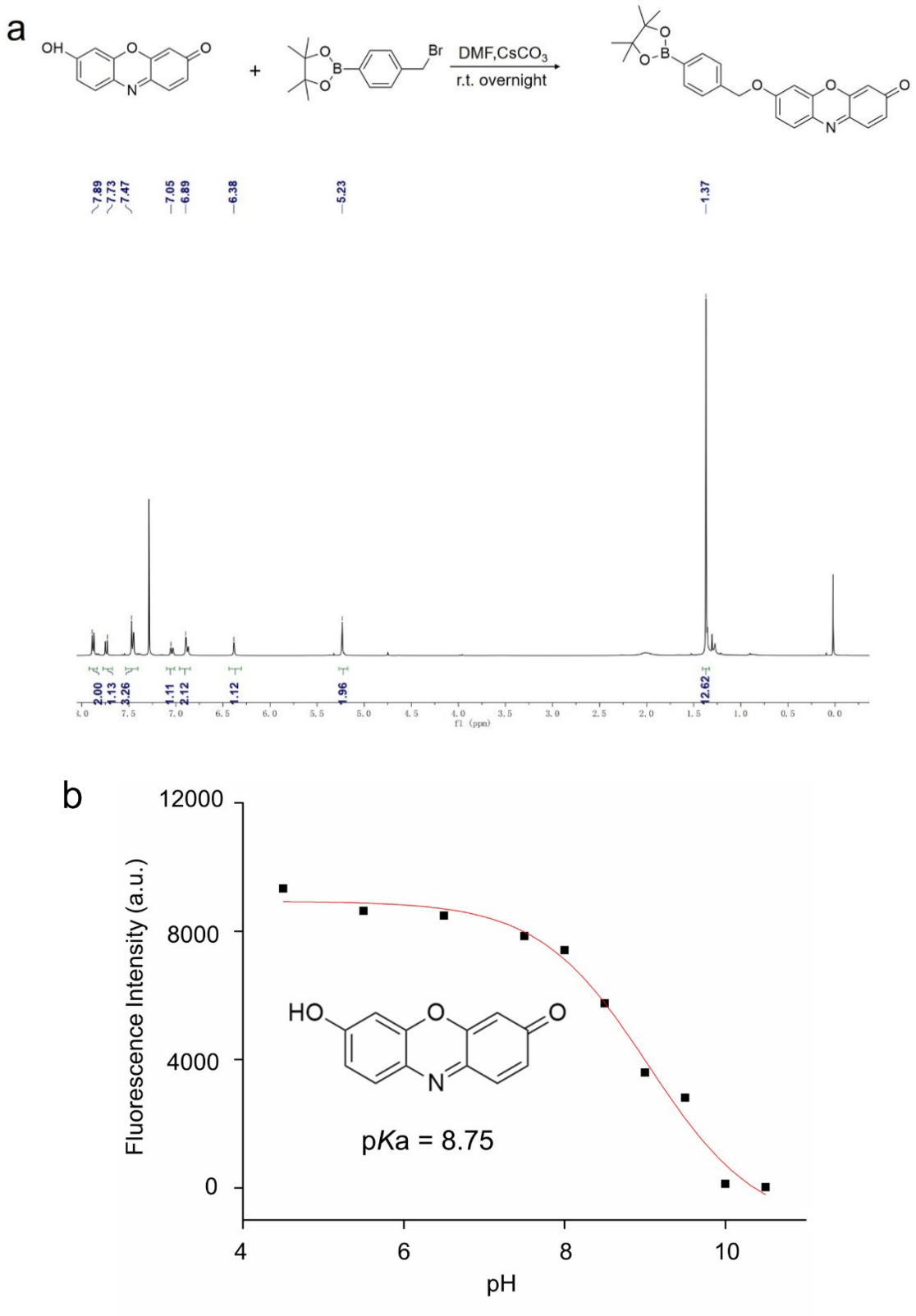


Figure S4. Characterization of H_2O_2-responsive fluorescent indicator.(a) Synthesis and ^1H NMR characterixzations of H_2O_2-responsive fluorescent indicator. ^1H NMR (400 MHz, $CDCl_3$): δ 7.85 (d, J = 7.5 Hz, 2H), 7.70 (d, J = 8.8 Hz, 1H), 7.42 (t, J= 8.7Hz, 3H), 7.00 (d, J = 8.8 Hz, 1H), 6.87 – 6.80 (m, 2H), 6.31 (s, 1H), 5.20 (s, 2H), 1.35 (s,12H). (b) Calibration curve showing the measured fluorescence intensity at 585 nm of the dye solution at different pH values.

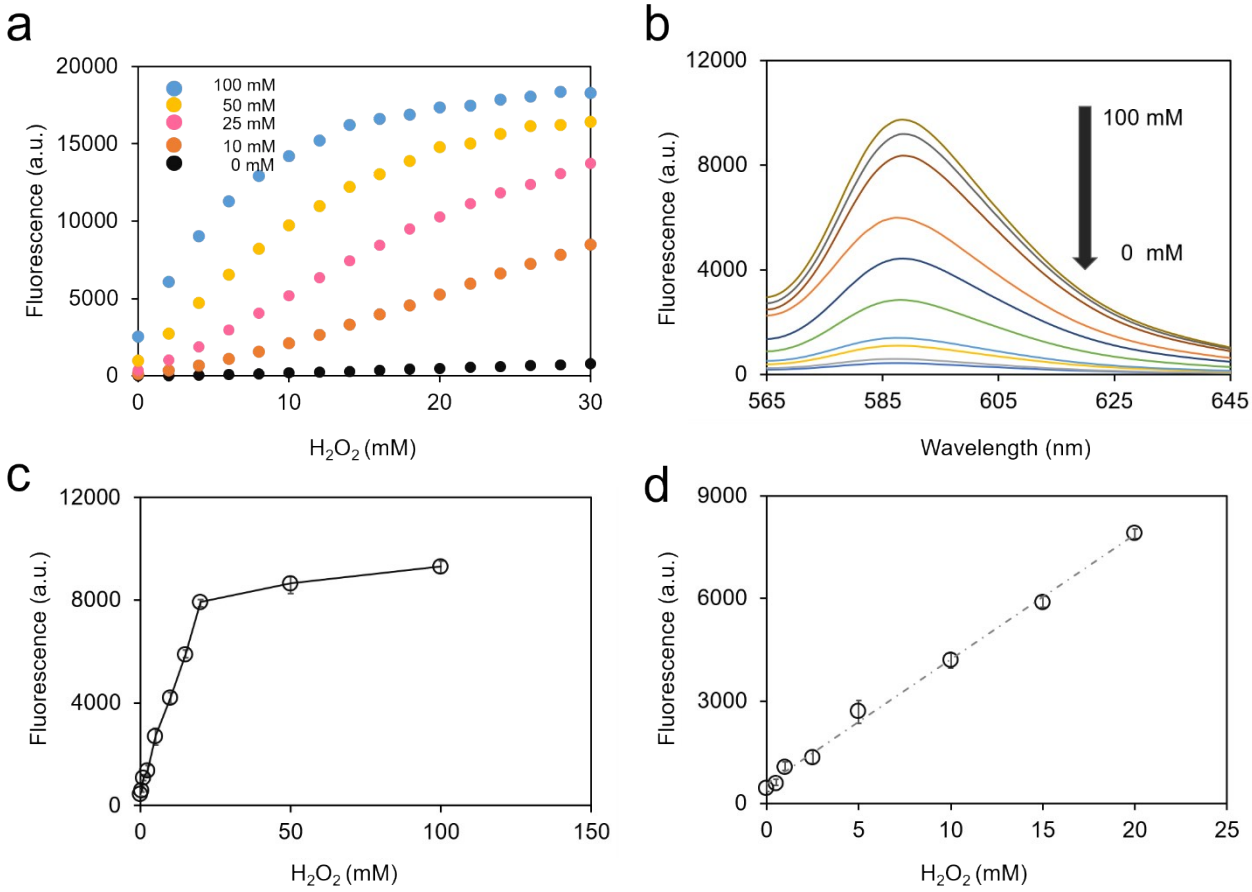


Figure S5. Optimization of the performance of H_2O_2-responsive indicator. (a) Calibration curves of indicators of different concentrations in response to different H_2O_2 concentrations. (b) Fluorescence spectra of indicators in response to different H_2O_2 concentrations. (c-d) Calibration curves correlating H_2O_2 concentrations with fluorescence intensity at high (c) and low H_2O_2 concentration range.

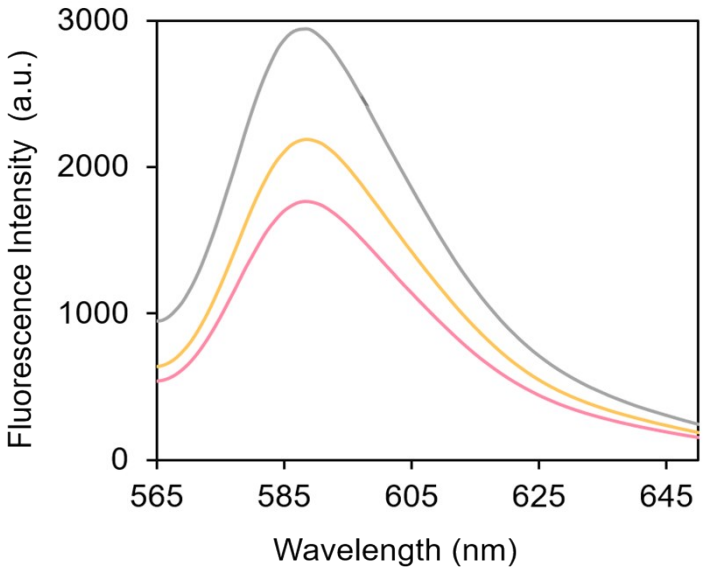


Figure S6. Fluorescence spectra of native LOX (gray), LOX-SNA (orange), and LOX X-SNA (pink) samples of 5 nM protein concentration. Sample solutions were incubated with 1mM lactate, 4 μM FAD for 30 min, and then added 1 mM fluorescence indicator.

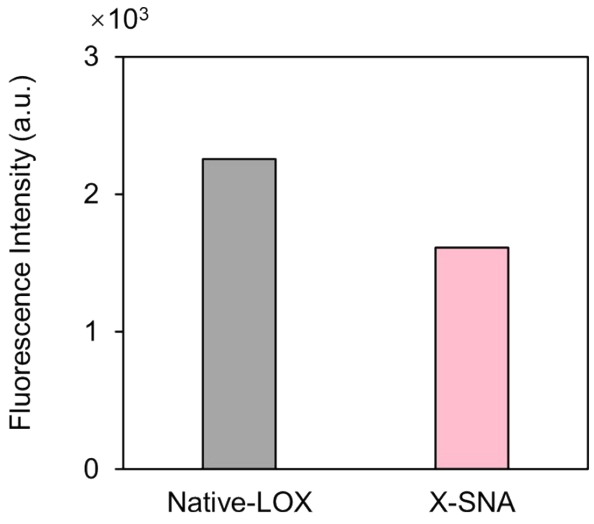


Figure S7. Histogram of fluorescence intensity showing the catalytic activity of native LOX and X-SNA in cell lysate using the fluorescence assay.

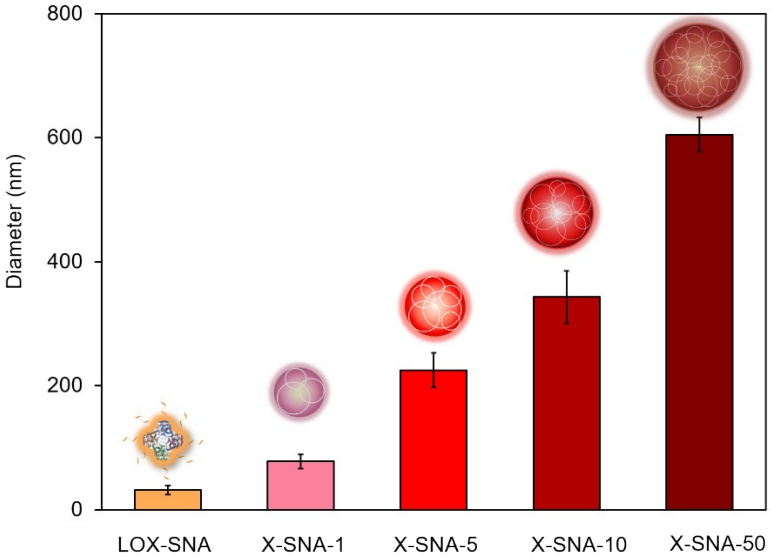


Figure S8. Size measurements of LOX SNA and four sized X-SNAs using from atomic force microscopy (AFM).

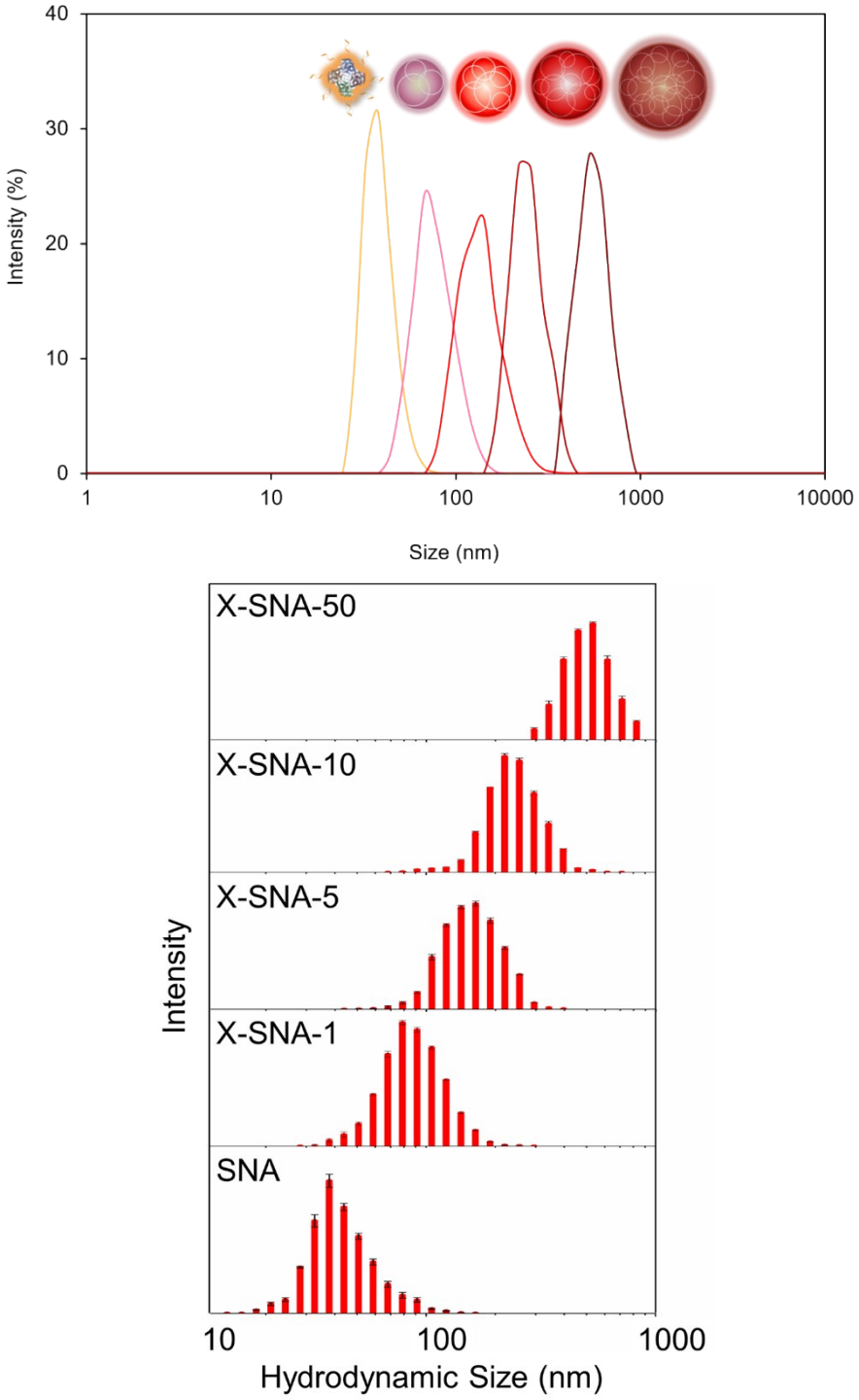
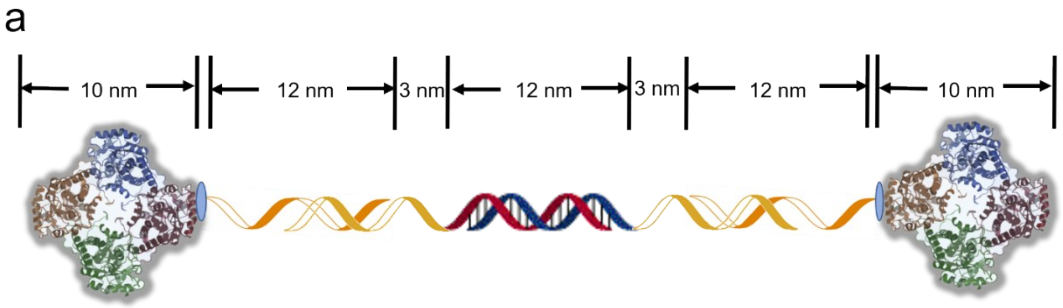
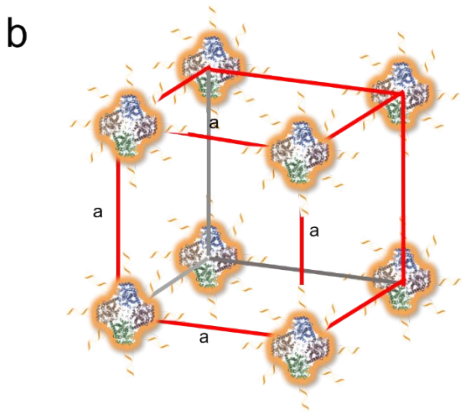
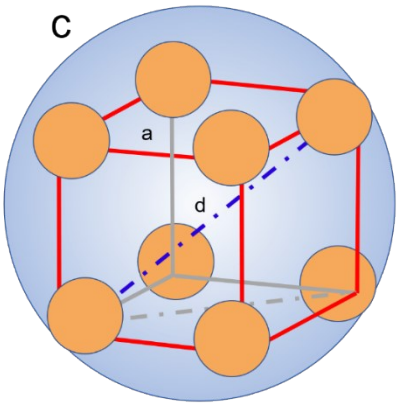


Figure S9. Size measurements of LOX SNA and four sized X-SNAs using from dynamic light scattering (DLS). Error bars come from standard deviation of three independent measurements.





a=52 nm
$v=a^3$ =140608 nm^3



$V=\frac{4}{3}\pi r^3$

d

	d (nm)	r (nm)	V/v (unit)	Protein (unit)
X-SNA-1	78	39	1.77	10
X-SNA-2	139.2	69.6	10.09	43
X-SNA-3	245.5	122.75	55.09	147
X-SNA-4	506.7	253.35	484.35	1143

Figure S10. (a) Scheme showing two neighboring LOX SNAs were crosslinked by dsDNA. (b) and (c) Primitive cubic packing model of the SNAs in X-SNA construct for the calculation of number of proteins per X-SNA particle. (d) Table showing the number of proteins per particle in four different sized X-SNAs.

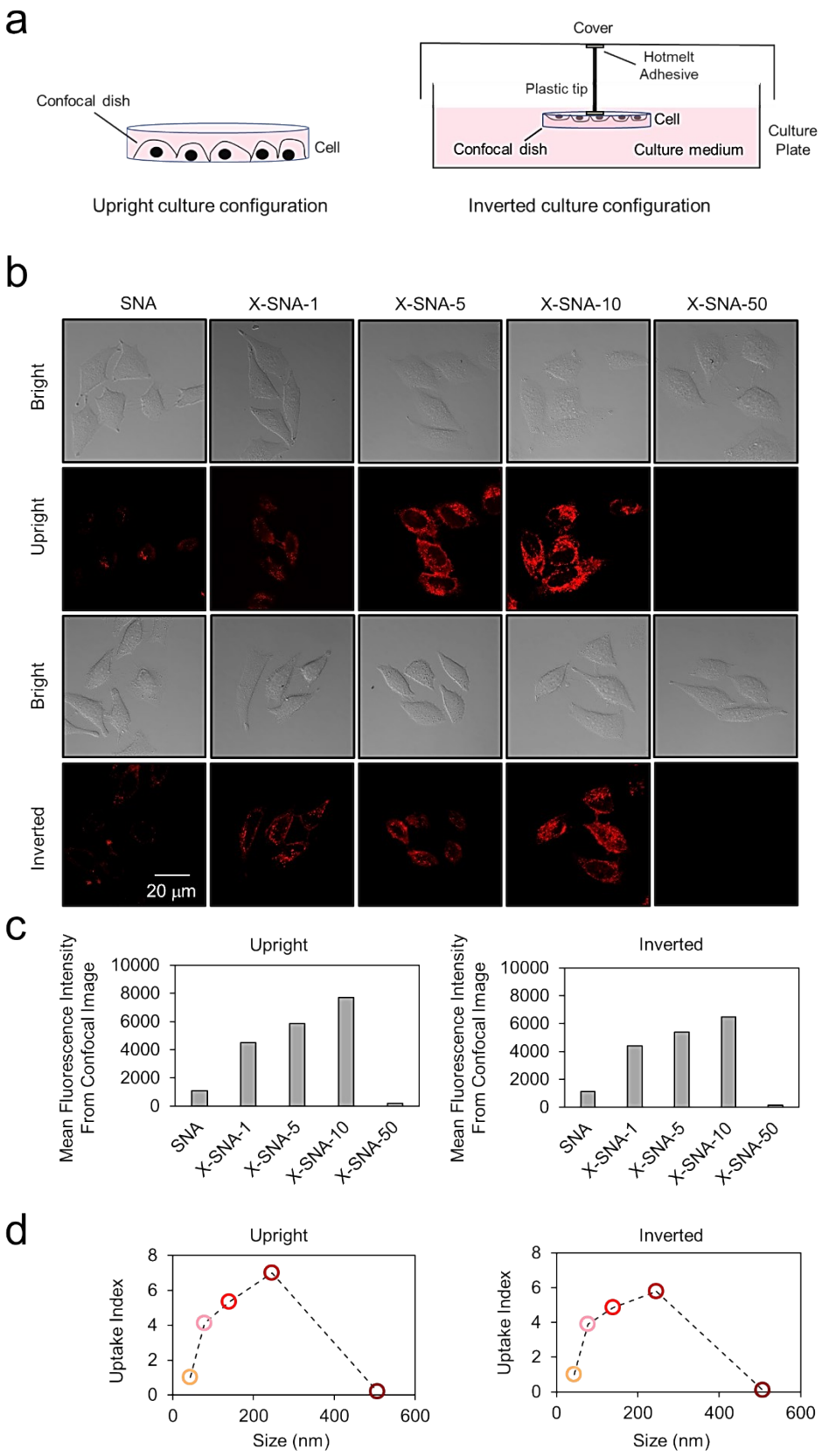


Figure S11. (a) Schematic of upright and inverted culture configurations to seed cells. (b) Confocal images of HeLa cells treated with LOX X-SNAs and SNA in upright and inverted cell culture configurations. (c) Histograms showing mean fluorescence intensity of HeLa cells treated with X-SNAs and SNA in upright (left) and inverted (right) culture configurations. (d) Calibration curves showing the size-dependent cellular uptake of X-SNAs and SNA in upright (left) and inverted (right) culture configurations.

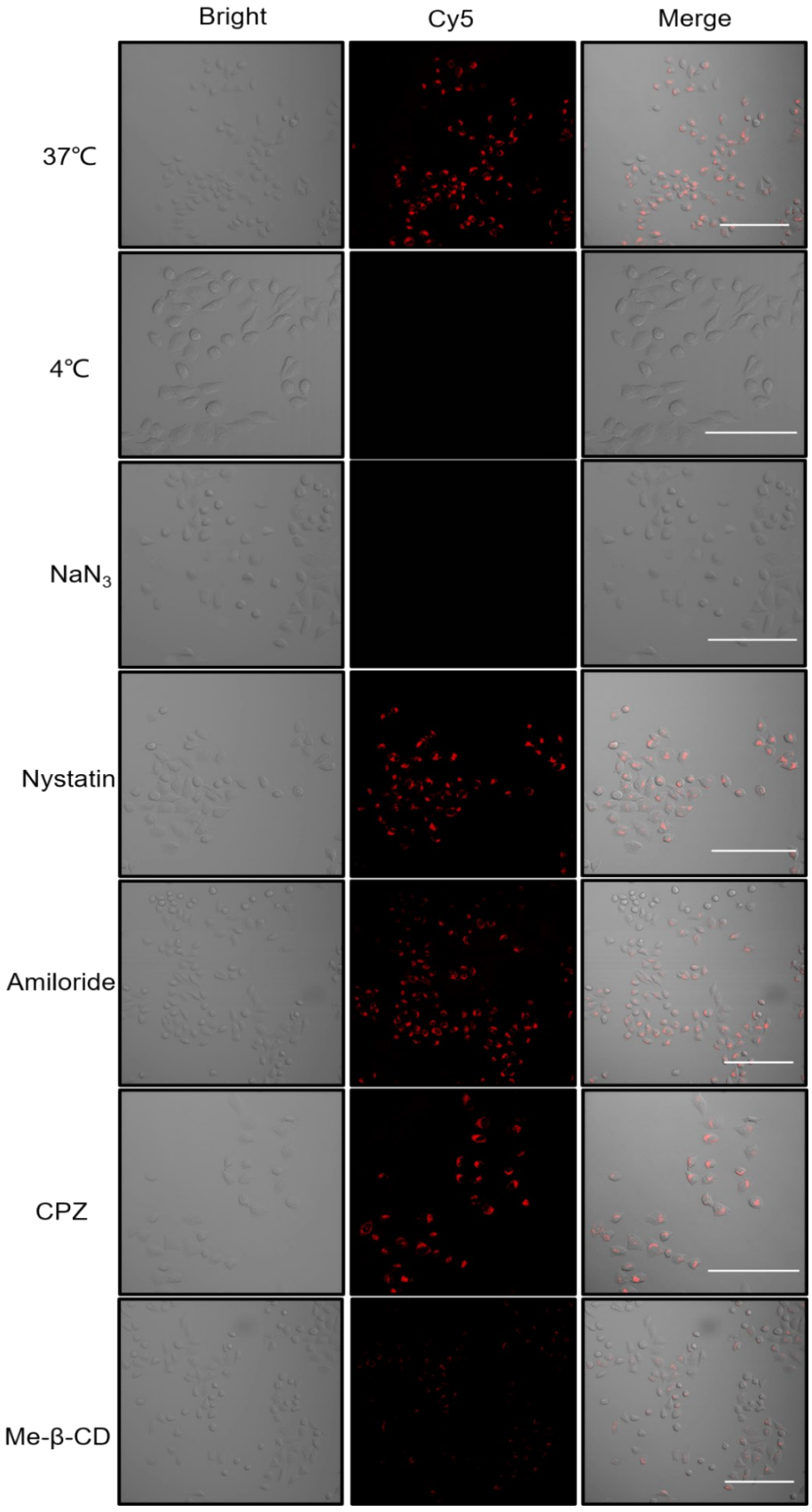


Figure S12. Confocal images of X-SNA treated HeLa cells under different conditions and with different inhibitors for cellular uptake mechanism study. Scale bar = 100 μm.

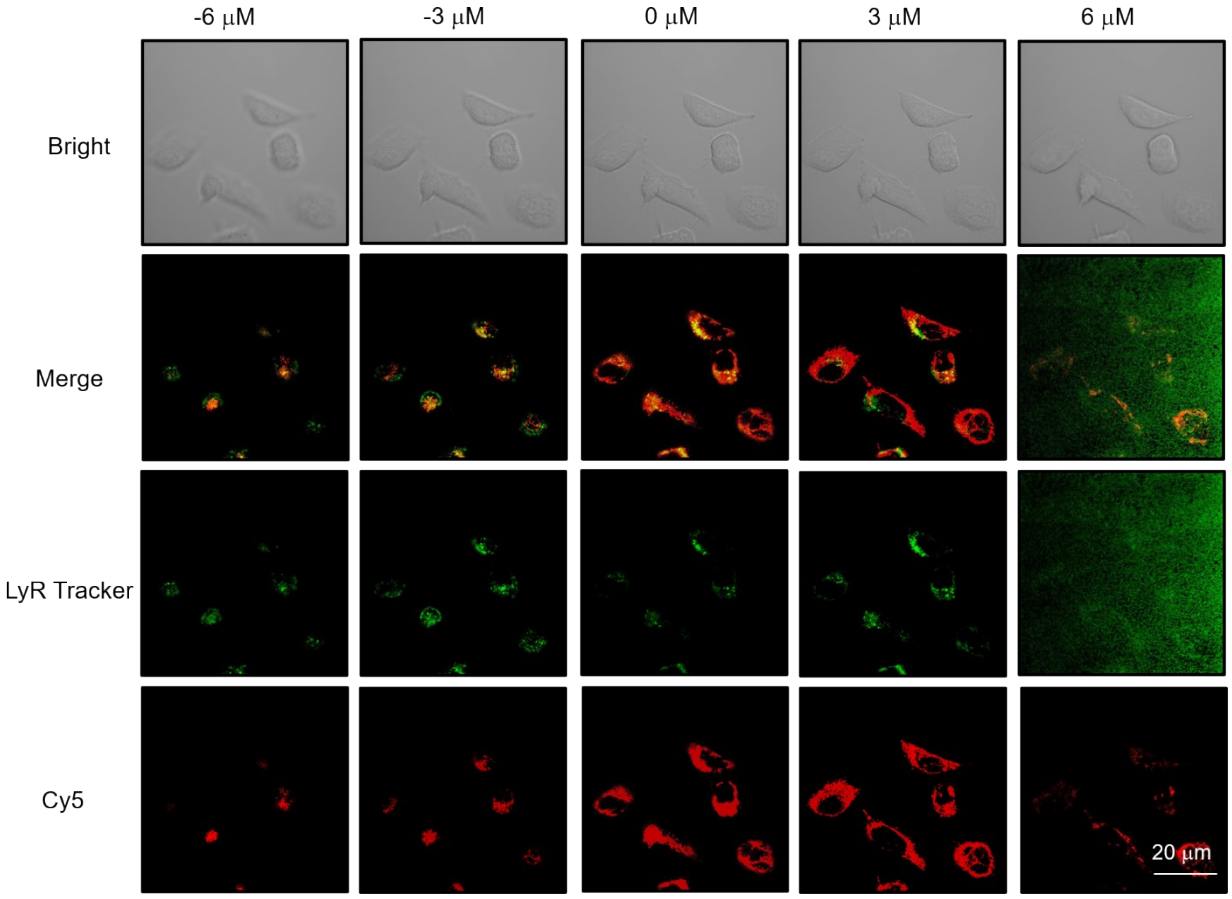


Figure S13. Confocal z-stack images of HeLa cells treated with X-SNAs and lysotracker for lysosomes staining.

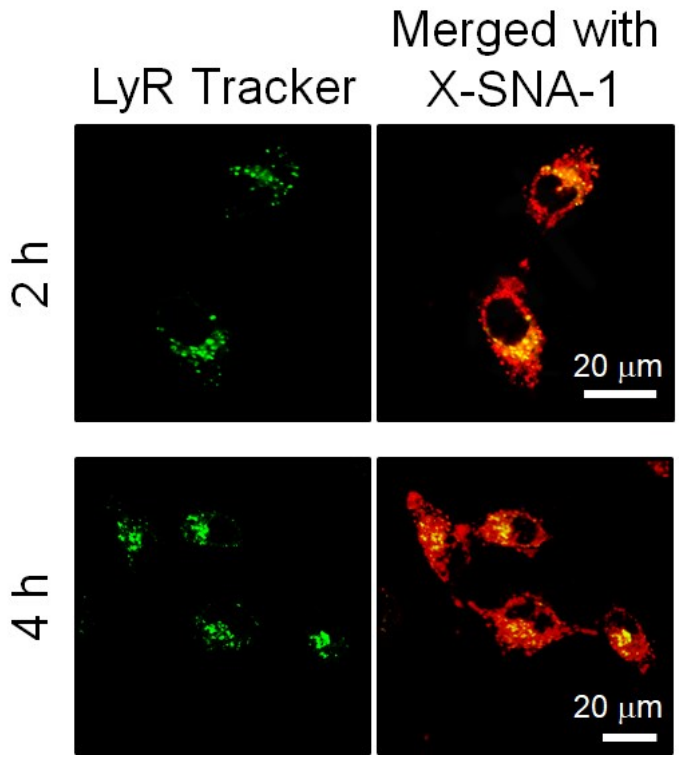


Figure S14. Confocal images showing colocalization of X-SNA-1 and lysosomes in HeLa cells after 2- and 4-hour incubation.

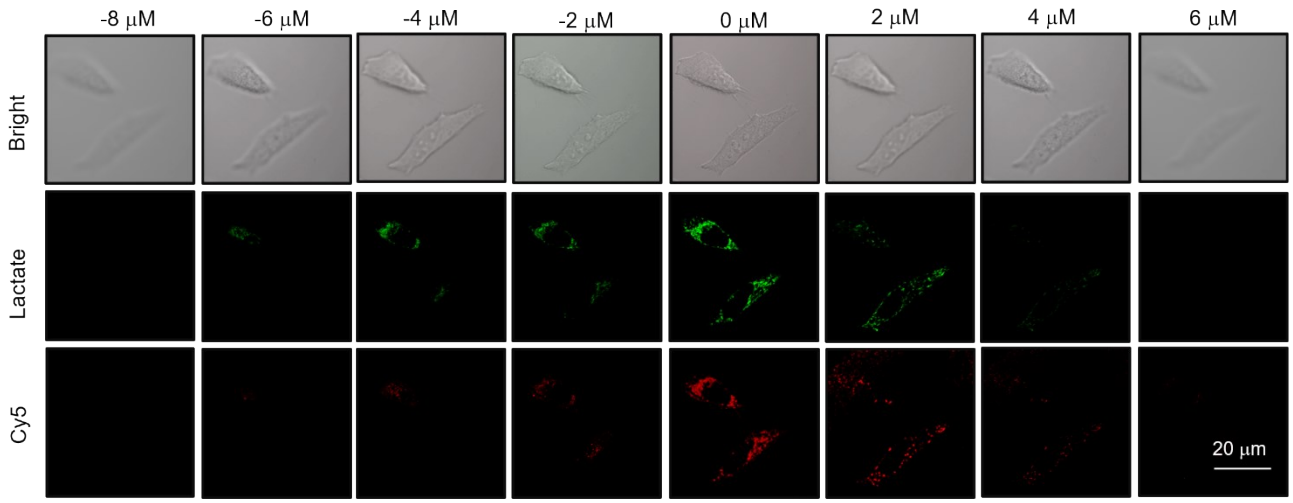


Figure S15. Confocal images of X-SNA and H_2O_2-responsive fluorescent indicator treated HeLa cells. Green fluorescence indicated the presence of intracellular lactate and red fluorescence indicated the X-SNA.

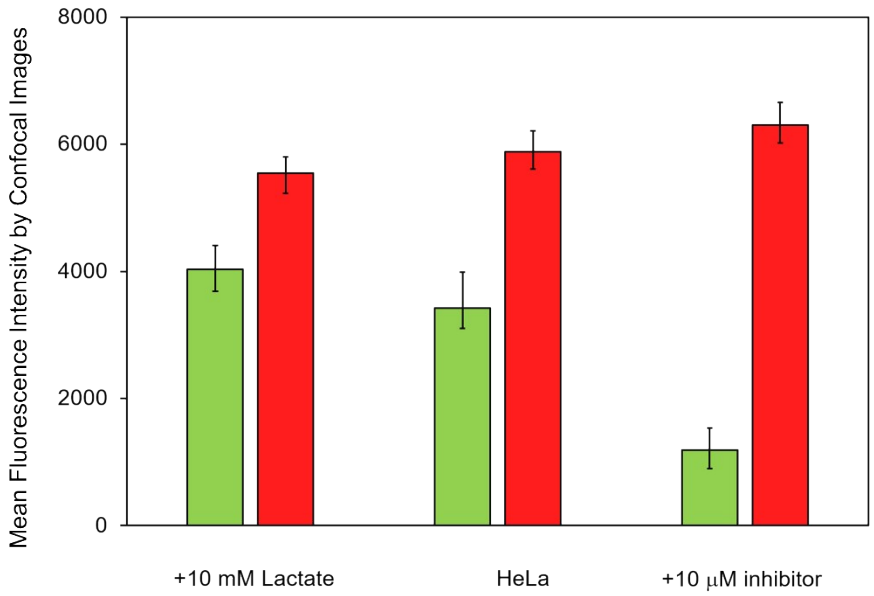


Figure S16. Histogram showing mean fluorescence of HeLa cells treated with lactate inhibitor or external lactate measured by confocal microscopy images. (Red: Cy5 on X-SNA; Green: lactate indicator)

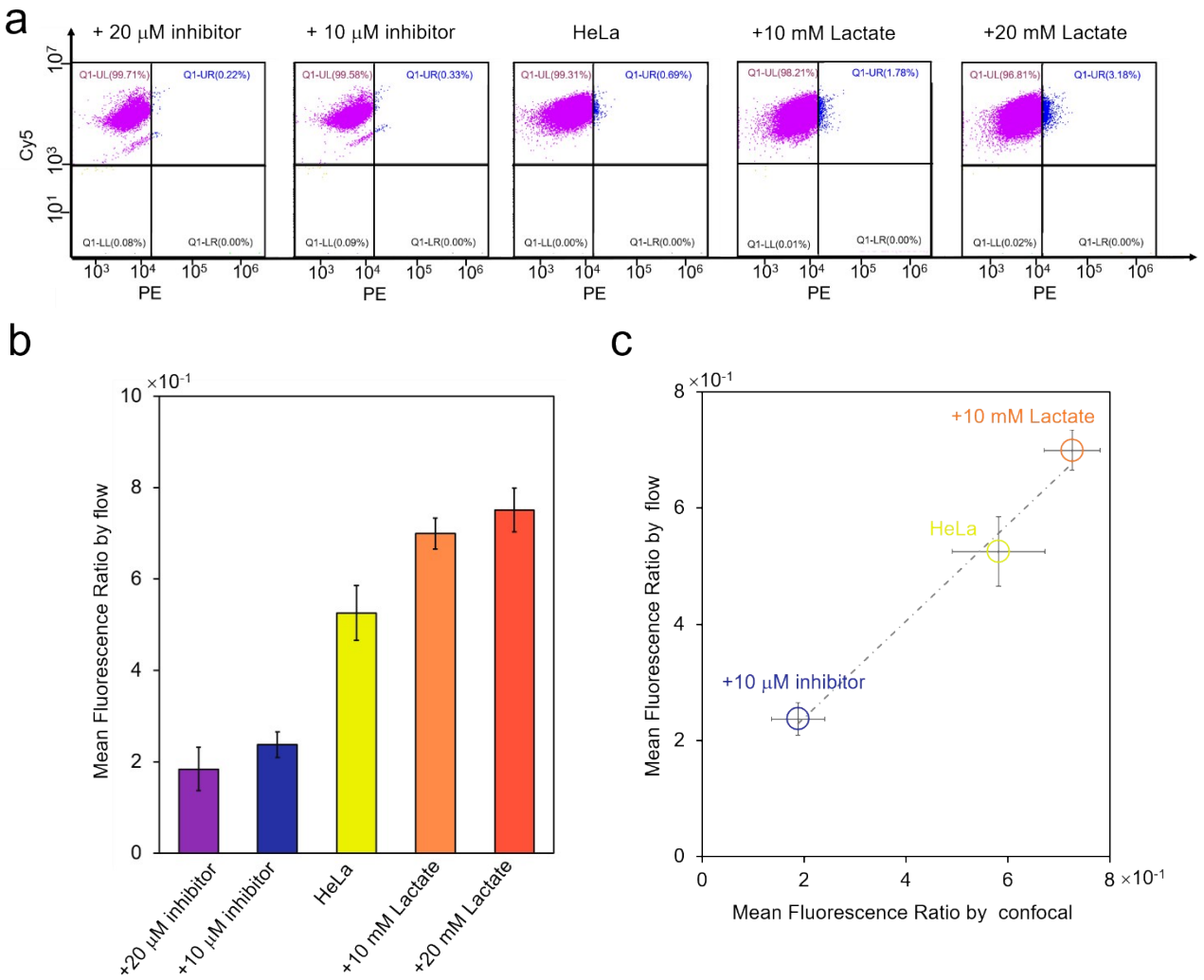


Figure S17. (a) Contour plots of flow cytometry measurements of HeLa cells treated with lactate inhibitor or with extra added lactate. X-axis represents lactate reporter fluorescence and Y-axis represents Cy5 on X-SNA. The values of Y-axis remained almost unchanged, while the values of X-axis increased with the increase of lactate concentration. (b) Histogram of the mean fluorescence ratio of HeLa cells treated with lactate inhibitor or with extra added lactate. (c) Correlation of mean fluorescence ratio measured by confocal images and by flow cytometry.

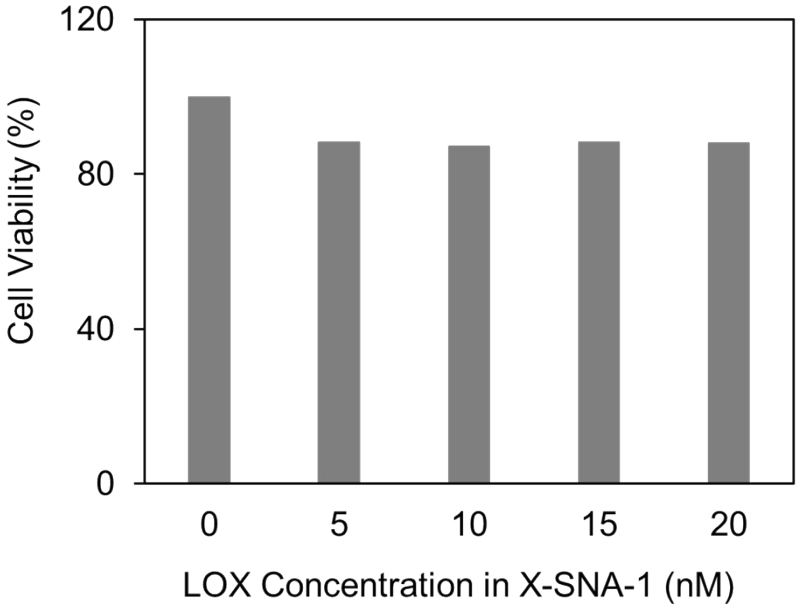


Figure S18. MTT cell viability studies of HeLa cells treated with 80 nm X-SNA-1 of different protein concentrations.

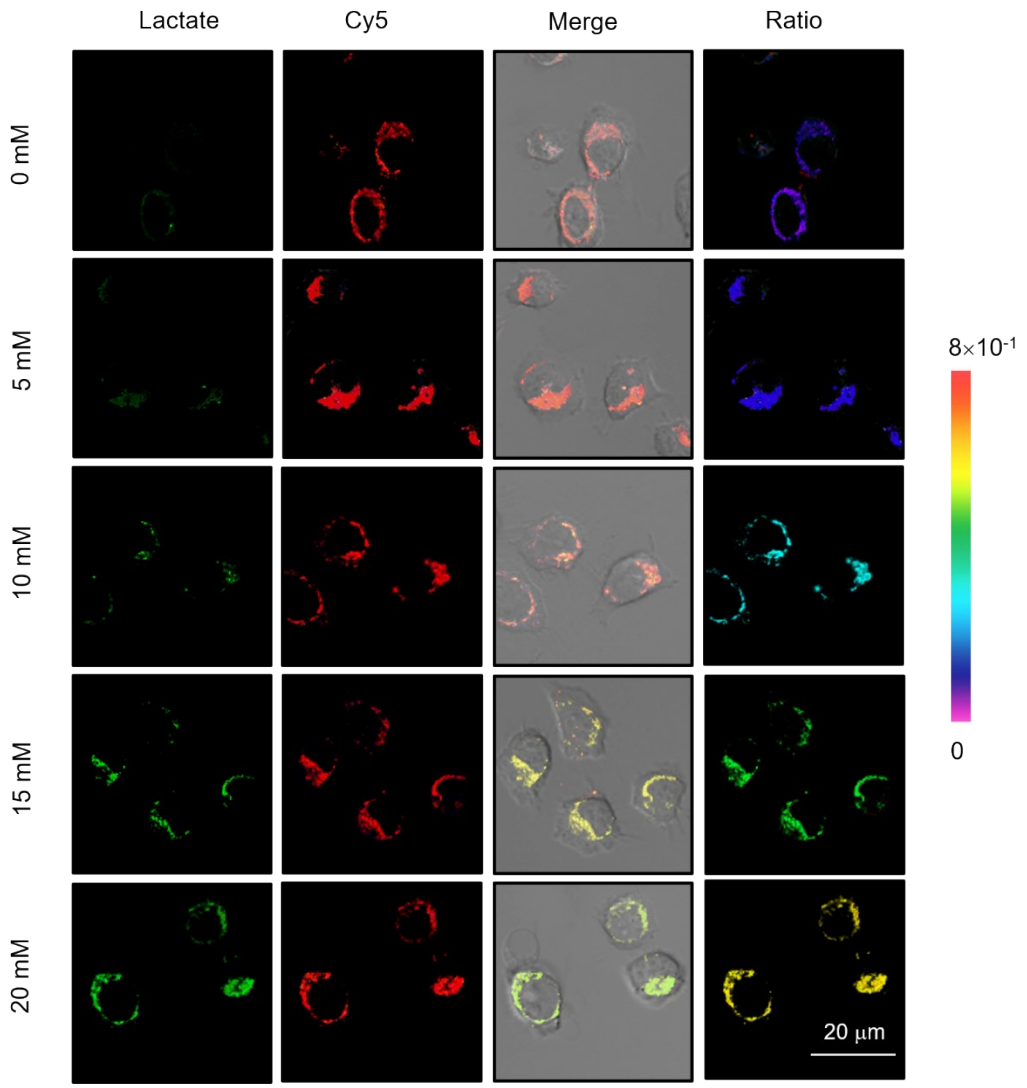


Figure S19. Confocal images of L-02 cells treated with external lactate of different concentrations ranging from 5 to 20 mM.

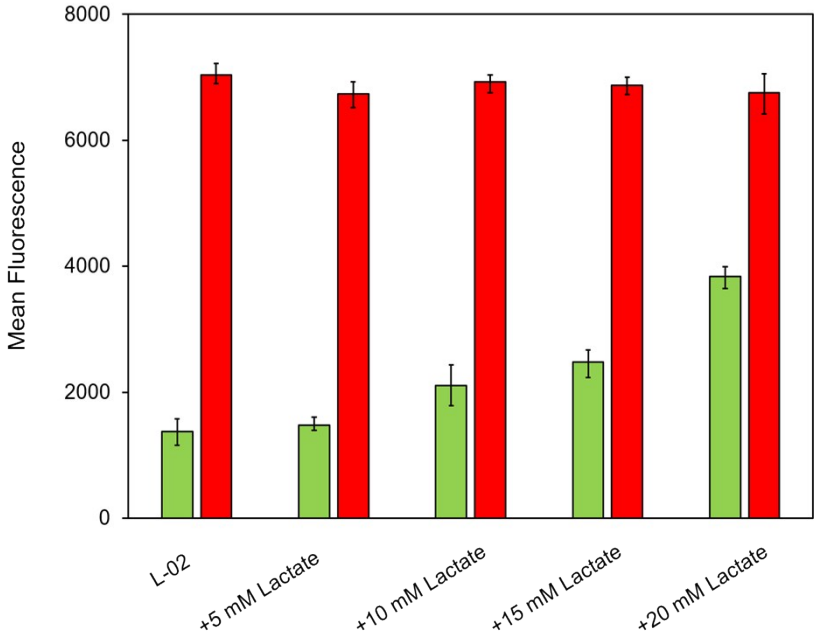


Figure S20. Histogram of mean fluorescence of L-02 cells treated with external lactate of different concentrations ranging from 5 to 20 mM (Red: Cy5 on X-SNA; Green: lactate indicator).

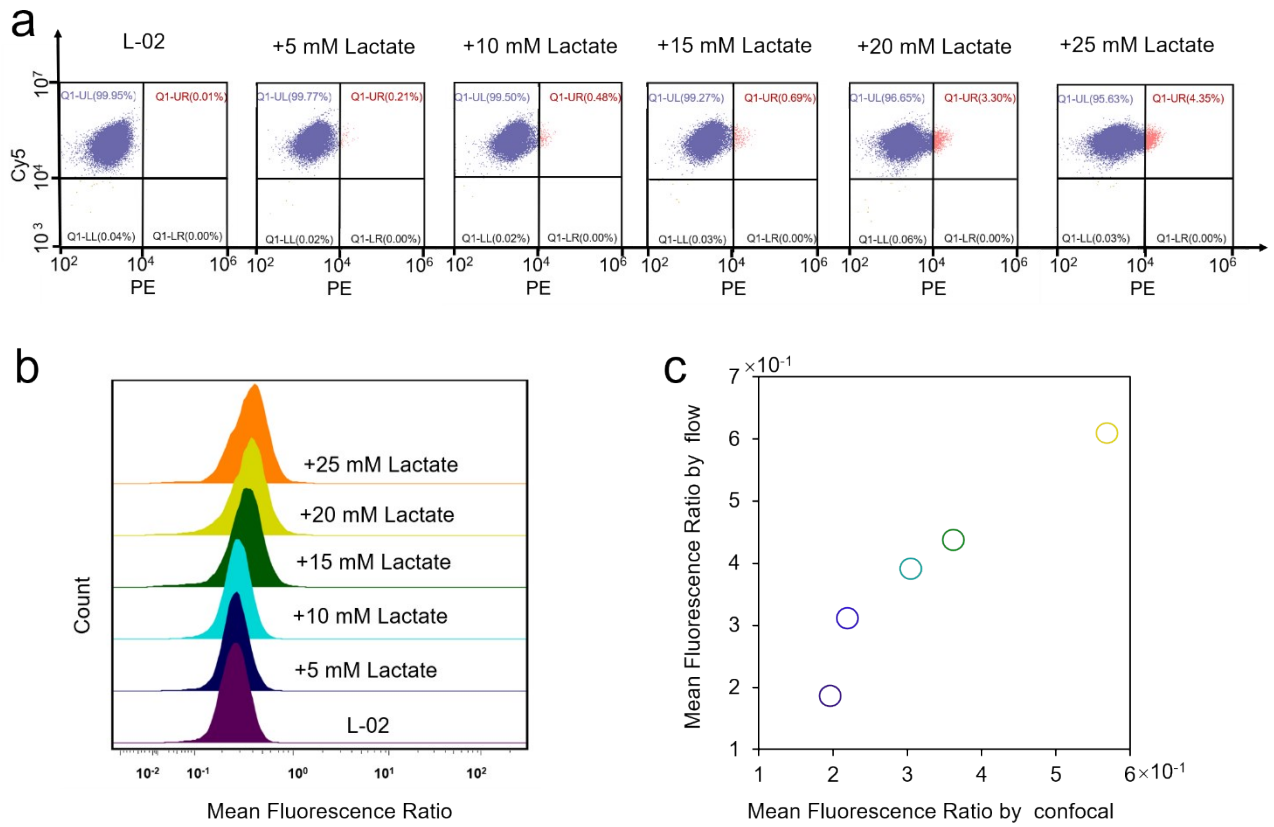


Figure S21. (a) Contour plots of flow cytometry measurements of L-02 cells treated with external lactate of different concentrations ranging from 5 to 20 mM. X-axis represents lactate reporter fluorescence and Y-axis represents Cy5 on X-SNA. The values of Y-axis remained almost unchanged, while the values of X-axis increased with the increase of lactate concentration. (b) Mean fluorescence ratio of L-02 cells treated with external lactate of different concentrations. (c) Correlation of mean fluorescence ratio measured by confocal images and by flow cytometry.

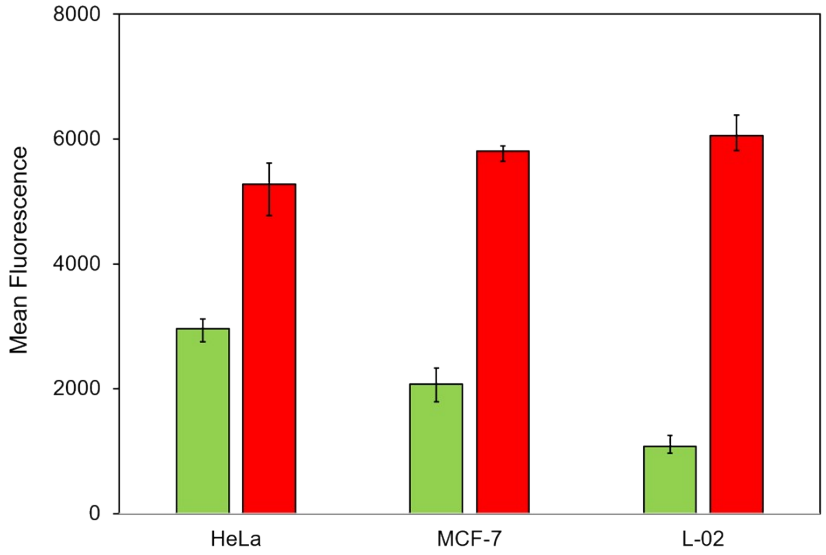


Figure S22. Histogram of mean fluorescence intensity of HeLa cells, MCF-7 cells, and L-02 cells measured by confocal images (Red: Cy5 on X-SNA; Green: lactate indicator).

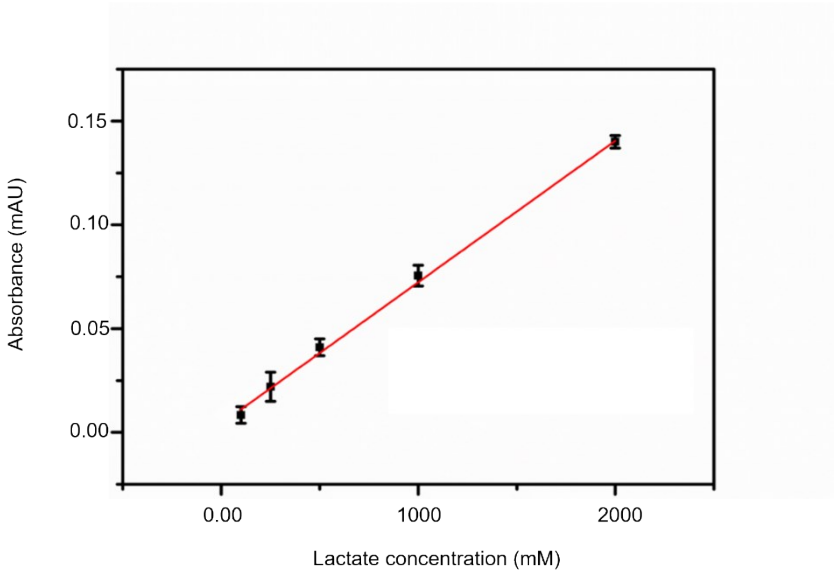


Figure S23. Standard curve showing UV absorbances versus lactate concentration using commercial lactate kit.

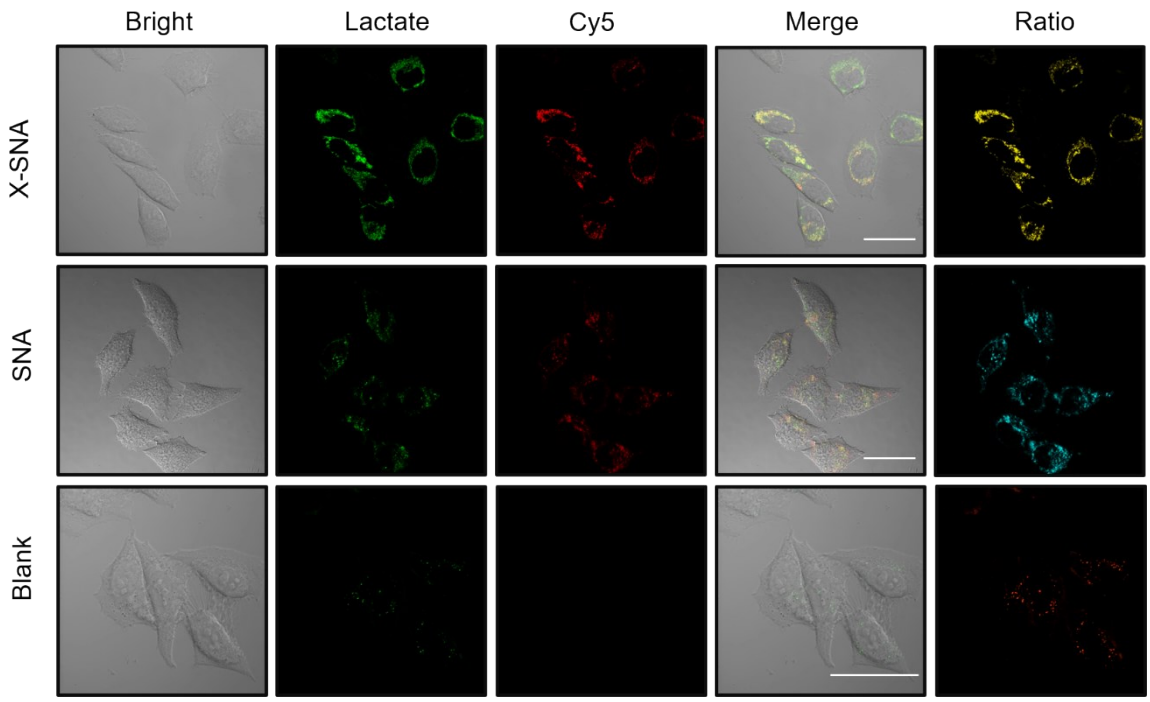


Figure S24. Confocal images of HeLa cells treated with LOX-based X-SNA and SNA constructs for intracellular lactate detection. Scale bar = 20 μm.

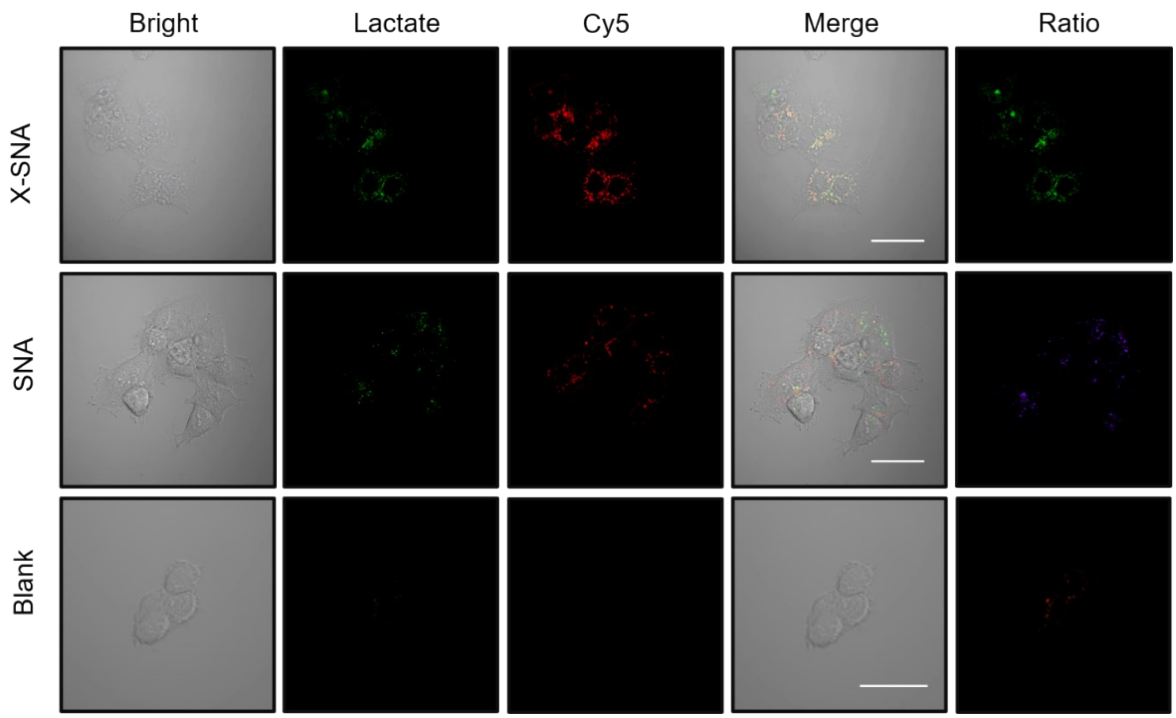


Figure S25. Confocal images of MCF-7 cells treated with LOX-based X-SNA and SNA constructs for intracellular lactate detection. Scale bar = 20 μm.

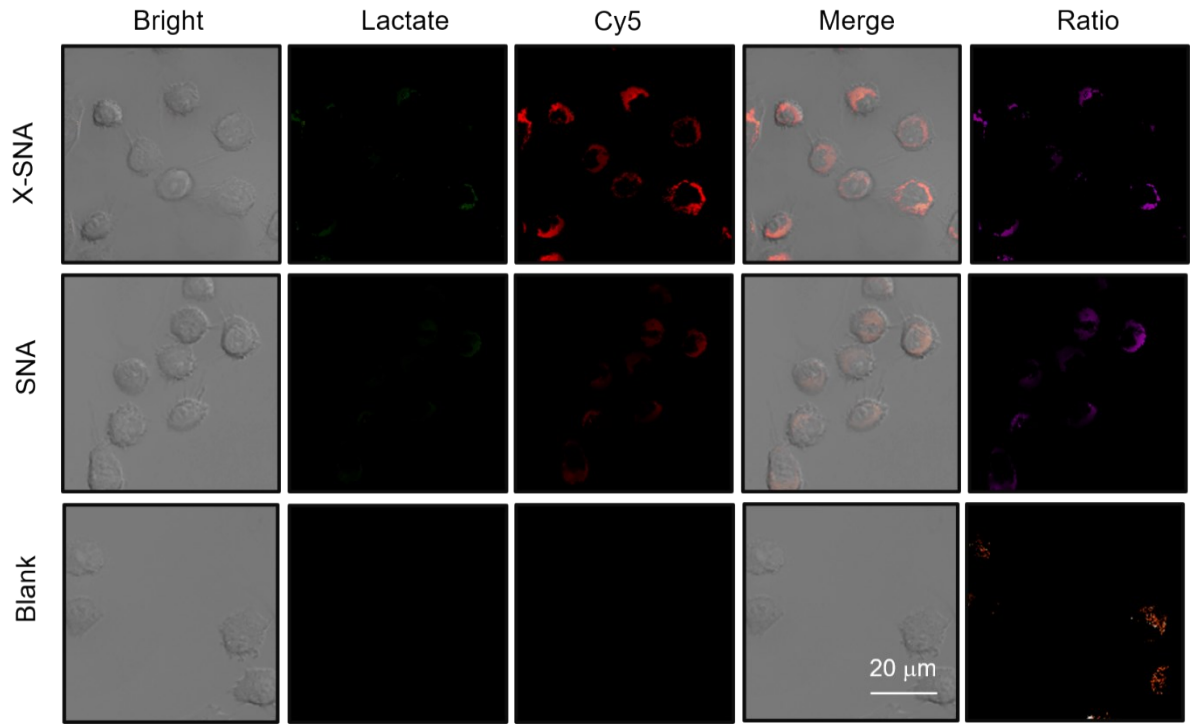


Figure S26. Confocal images of L-02 cells treated with LOX-based X-SNA and SNA constructs for intracellular lactate detection. Scale bar = 20 μm.

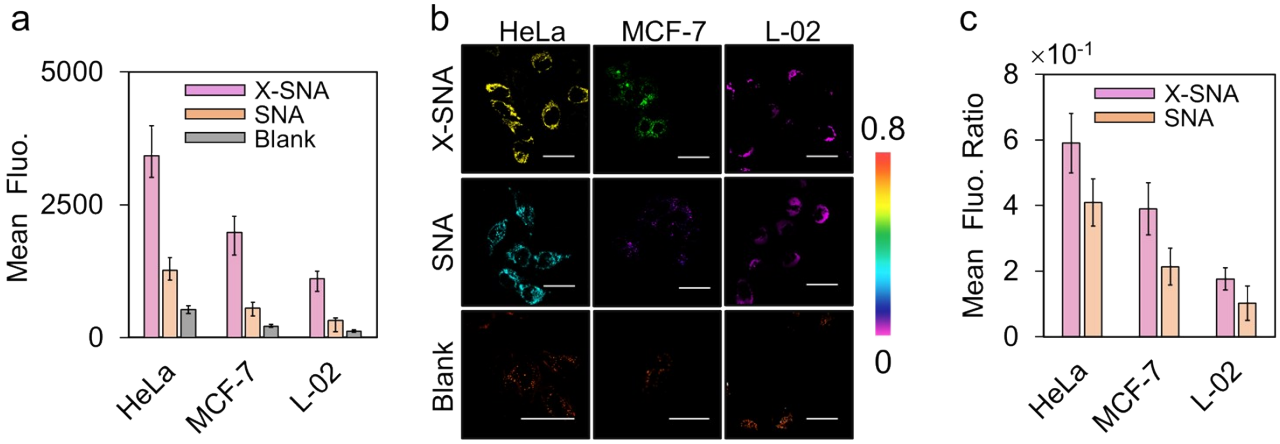


Figure S27. (a) Histogram of mean green fluorescence intensity of HeLa cells, MCF-7 cells, and L-02 cells from confocal images (Purple: cells treated with X-SNA; Organe: cells treated with SNA; Grey: non-treated cells). (b) Confocal ratiometric color images of cells treated with X-SNA, SNA, or simple cell medium. (b) Histogram of mean fluorescence ratio of HeLa cells, MCF-7 cells, and L-02 cells from confocal ratiometric color images, comparing the ratiometric lactate detection signals from X-SNA with that from SNA.

Table S1. Sequences of the oligonucleotides used in this work

Name	Sequences (5'-3')
Thiol-modified	HS-dT-TTTTTGGTGGTGGTGGTGGTGGTGGTGGTGGT
Linker-1	TTCCTTTTCCTTTTCCTTTTCCTTTTCCTTTTCCTTTTTTTTTTTTTACCACCACCACCACC ACCACCACCACCACC
Linker-2	AAGGAAAAGGAAAAGGAAAAGGAAAAGGAAAAGGAAbACCACCACCACCACCAC CACCACCACCACC
Cy5Linker-1	TTCCTTTTCCTTTTCCTTTTCCTTTTCCTTTTCCTTTTTT-dT-Cy5- TTTTTACCACCACCACCACCACCACCACCACC

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