Integrating Lanthanide Coordination Polymer Ratiometric fluorescence biosensor with concatenated DNA circuit for Locus-Specific N^6-methyladenosine Quantification

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EXPERIMENTAL SECTION

Chemicals and materials

All oligonucleotide sequences (**Table S1**) were synthesized and purified by HPLC at Shanghai Sangon Biotechnology Co. Ltd. (Shanghai, China). Recombinant RNase inhibitor, RNAase Free water and tri-(2-formyl ethyl) phosphine hydrochloride (TCEP, 99%) were purchased from Shanghai Sangon Biotechnology Co. Ltd. (Shanghai, China). 5×MazF reaction buffer (0.05% Tween 20), MazF were purchased from Takara Biotechnology Co., LTD. (Dalian, Liaoning, China). Streptavidin magnetic beads (MBs, ~ 1.0 μm, 20 mg/mL) were obtained from Biomag Biotechnology Co., Ltd. (Wuxi, China). Guanine monophosphoric acid (GMP), Luminol, $Tb(NO_3)_3 \cdot 6H_2O$ were purchased from Sigma-Aldrich. 3-Mercaptopropionic acid (3-MPA, 98%) and sodium sulfide (Na_2S, 90%) were purchased from Shanghai Maclin Biochemical Technology Co., Ltd. (Shanghai, China). Copper oxide nanoparticles (CuO, 50 nm in diameter) was purchased from Aladdin Biochemical Technology Co., Ltd. (Shanghai, China). The FTO was purchased from Active Motif (Carlsbad, CA, USA). Human embryonic kidney cell line (HEK-293T cells) was obtained from the Cell Bank of Chinese Academy of Sciences (Shanghai, China). The UNIQ-10 column total RNA purification kit was bought from Sangon Biotechnology Co. Ltd (Shanghai, China). All chemicals used are analytical reagents, obtained from commercial sources, and used directly without additional purification.

Tabel S1 Sequence of oligonucleotides[a]

Name	Sequence(5'-3')
m^6A-mRNA	5'-UUGUGCAAGGm^6ACAGCAACAAC-3'
A-mRNA	5'-UUGUGCAAGGACAGCAACAAC-3'
Probe 1	5'-GTT GTT GCT GT-Azide-3'
Probe 2	5'-DBCO-CCTTGCACAA-3'

H1	5'-TTTTTGTGCAAGGACAGCAACAACGGTGTGTGTGGGTTGTTGCTGTCCTTCTTTCCCTCTC-3'
H2	5'-CAACAACCCACACACACCGTTGTTGCTGTCCTTGCACAA-Biotin-3'
H3	5'-HS-CGACATGTTCTCTTTCCCTCTCTTCGGCTTGAGAGGGAAAGAAGGACAG-3'
H4	5'-AAGCCGAAGAGAGGGAAAGCTGTCCTTCTTTCCCTCTCCGACATGTTCA-SH-3'
Single-base mismatch mRNA	5'-UUGUACAAGGACAGCAACAAC-3'
5mC-mRNA	5'-UUGUGCAAGGA**C**AGCAACAAC-3'

[a]In m^6A-mRNA, the marked base "A" in red represents m^6A. In single-base mismatched mRNAs, the underlined base "A" represents single-base mismatch A in m^6A-mRNAs. In 5mC-mRNA, the blue marked base "C" indicates 5-methylcytosine (5mC).

Preparation of Luminal-Tb-GMP LnCPs probe

The preparation of luminol-Tb-GMP LnCPs was achieved by simple modifications of the reaction conditions reported in the previous work. Briefly, luminol (10 mM, 40 μL) was firstly added to GMP (100 mM, 90 μL) followed by incubating at room temperature for 30 min. Subsequently, an aqueous solution of $Tb(NO_3)_3 \cdot 6H_2O$ (100 mM) was added to the above mixture, forming a white flocculent suspension. Then, the obtained flocculation suspension was purified through a series of centrifugation and rinse cycles in deionized wate. Finally, the obtained luminal-Tb-GMP LnCPs was dispersed in 1 mL water for further use.

Mazf-mediated cleavage and Click chemistry-mediated LDR

The MazF-mediated cleavage reaction was conducted in a solution (10 μL) containing 1× MazF reaction buffer (0.01% Tween-20, 40 mM $Na_3(PO_4)_2$, and pH 7.5), 5 U of MazF, 20 U of RNase inhibitor, and different concentration of m^6A-mRNA at 37 °C for 1 h, followed by denaturation for 10 min at 95 °C. After cleavage reaction, 600 nM DNA probe a, 600 nM DNA probe b, 2 μL of PBS (pH 8.0), 30 mM NaCl was added into the reaction mixture (30 μL) to produce the probes ab ligation product. The click chemistry-mediated LDR was carried out with 50 thermal cycles at 85 °C for 30 s and 25 °C for 150 s.

Assembly of DNA nanostructure and LnCPs ratiometric sensor for m^6A assay

H1 (0.5 μM, 30 μL), H2-MB suspension (30 μL), H3 (0.5 μM, 30 μL), H4 (0.5 μM, 30 μL), and 30 μL of the above reaction mixture were added into 200 μL of 10 mM MOPS buffer (100 mM $NaNO_3$, 2.5 mM $Mg(NO_3)_2$, pH 7.4). This reaction solution was reacted at 37 °C for 3 h to complete the CHA and HCR amplification reaction, followed by washing three times with buffer solution and separation by a strong magnet. The obtained MB-H2-H1-$(H3\text{-}H4)_n$ DNA nanostructure was resuspended with MOPS buffer (400 μL). Then, 10 μL of TCEP was added to the MB-H2-H1-(H3-H4)n DNA nanostructure solution for 30 min to reduce disulfide bonds. Whereafter, MB-H2-H1-$(CuO\ NP\text{-}H3\text{-}H4\text{-}CuO\ NP)_n$ nanostructure complex was prepared by adding CuO NPs (50 μL, 40 μg/mL) colloid to the MB-H2-H1-$(H3\text{-}H4)_n$ DNA nanostructure solution followed by the incubation at room temperature for 12 h and and 2 M NaCl solution was cautiously dropwise added at a final concentration of 0.1 M for salt-stabilizing. After magnetic separation, the product of MB-H2-H1-$(CuO\ NP\text{-}H3\text{-}H4\text{-}CuO\ NP)_n$ complex was added to 20 μL 3 mM HNO_3 solution for 10 min to change CuO to Cu^{2+}. Subsequently, the dissolved Cu^{2+} was mixed with 20 μL luminolTb-GMP LnCPs stock suspension and 60 μL Tris buffer (10 mM, pH 9.0) with a total volume of 100 μL, followed by incubation for 50 min at 37 °C. Finally, the fluorescence spectra were measured with the excitation of 290 nm. The luminol fluorescence signal at 430 nm was

maintained, while the Tb^{3+} fluorescence signals at 488, 547, 586, and 630 nm decreased with different concentrations of m^6A-RNA. The signal intensity ratio of luminol and Tb^{3+} (F_{430}/F_{546}) was calculated for the quantitative determination of m^6A-RNA.

Cell culture and RNA extraction from cell and tissues

Human embryonic kidney cell line (HEK-293T cells) was cultured in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin at 37 °C in a humidified atmosphere containing 5% CO_2. At the exponential phase of cell growth, cellular RNA was extracted by using SanPrep column RNA Extraction Kit (Sangon Biotechnology Co. Ltd., China), and the concentration of extracted total RNA was quantified by using a Thermo NanoDrop 2000 spectrophotometer. Human lung tumor tissues and matched adjacent nontumor tissues were taken from Liaocheng Third People's Hospital. For paraffin-embedded tissue samples, total RNA was extracted using a miRNeasy FFPE kit (Qiagen, Germany). Dynabeads® mRNA Purification Kit (Thermo Scientific, Wilmington, USA) was employed to purify mRNA from the extracted total RNA, and fragmentation of mRNA can be performed by NEBNext® Magnesium RNA Fragmentation Module (New England Biolabs, Maryland, USA). The samples experiments were conducted in the same way as the m^6A-RNA detection procedure.

Detection of m ^{6}A-mRNA in mixtures and in Real samples

To investigate the ability of this proposed LnCPs ratiometric biosensor to detect m^6A-mRNA methylation levels in mixtures, we prepared a series of mixtures by mixing m^6A-mRNA and control mRNA at different ratios. The total concentration of m^6A-mRNA and control mRNA in the mixture was 10 pM, and the mixture contained 0.001%, 0.01%, 0.1%, 1%, 10%, and 100% m^6A-mRNA, respectively.

To investigate the feasibility of the proposed method for real sample analysis, 10% serum was spiked with 10 μL of a mixture containing different concentrations of m^6A-mRNA, 20 U ribonucrenase inhibitors, 5 U MazF, and 1×MazF reaction buffer (0.01% Tween 20, 40 mM $Na_3(PO_4)_2$, pH 7.5). The reaction was carried out at 37 °C for 1 h,

followed by denaturation for 10 min at 95 °C. After cleavage reaction, subsequent reactions and measurements follow the same procedure described above.

Clinical serum and tissue samples from healthy people and people with lung cancer were obtained from Liaocheng Third People's Hospital. For tissue samples, total RNA was extracted using a miRNeasy FFPE kit (Qiagen, Germany). Dynabeads®mRNA Purification Kit (Thermo Scientific, Wilmington, USA) was employed to purify mRNA from the extracted total RNA, and the resulting mRNA was fragmented by using a NEBNext® Magnesium RNA Fragmentation Module (New England Biolabs, Maryland, USA). The serum and tissue samples experiments were conducted in the same way as the HNE activity detection procedure. The collection of all samples was approved by the Ethical Committee of the Third People' s Hospital of Liaocheng. Informed consent was obtained for any experimentation with human subjects.

Results and discussion

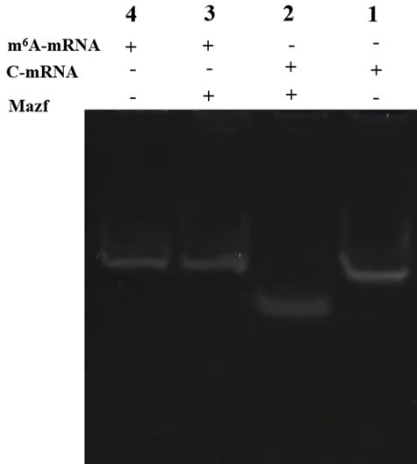
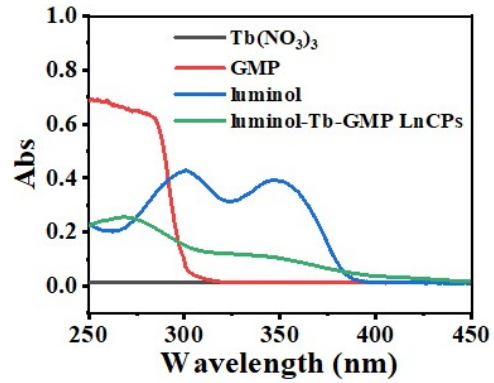
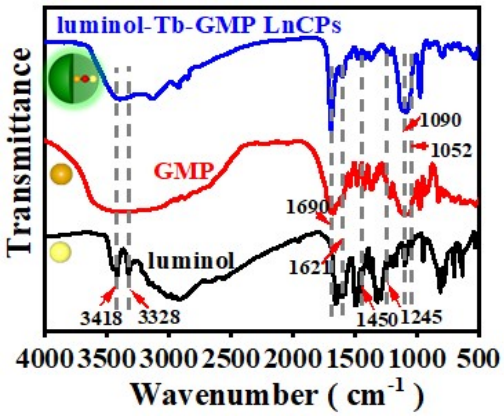


Fig. S1 PAGE characterization of the MazF-mediated cleavage.



(A)



(B)

Fig. S2 Characterization of luminal-Tb-GMP LnCPs: (A) UV-vis spectra, (B) FTIR spectra.

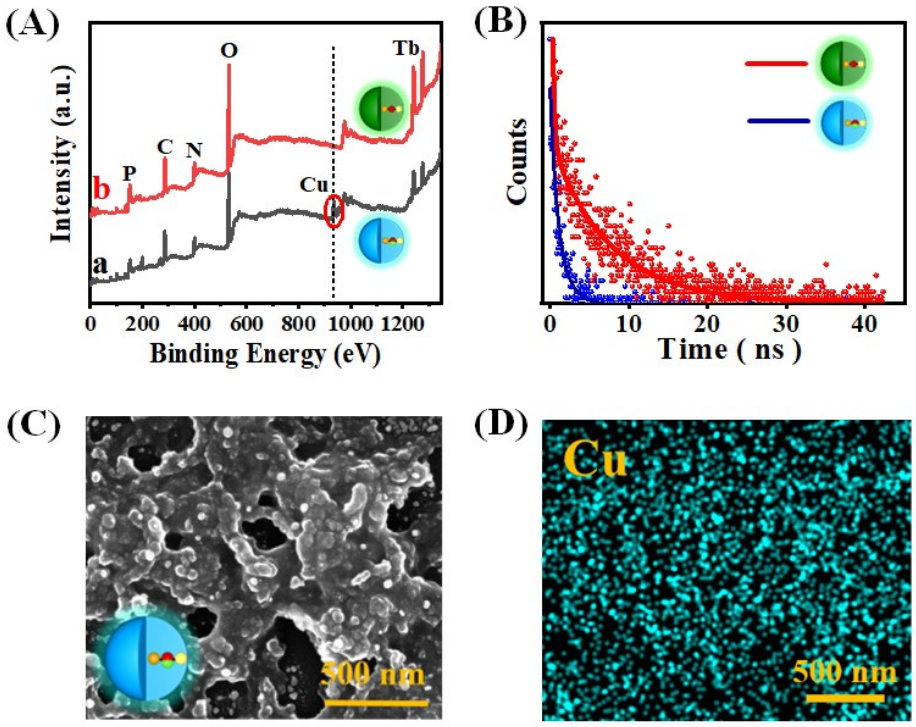
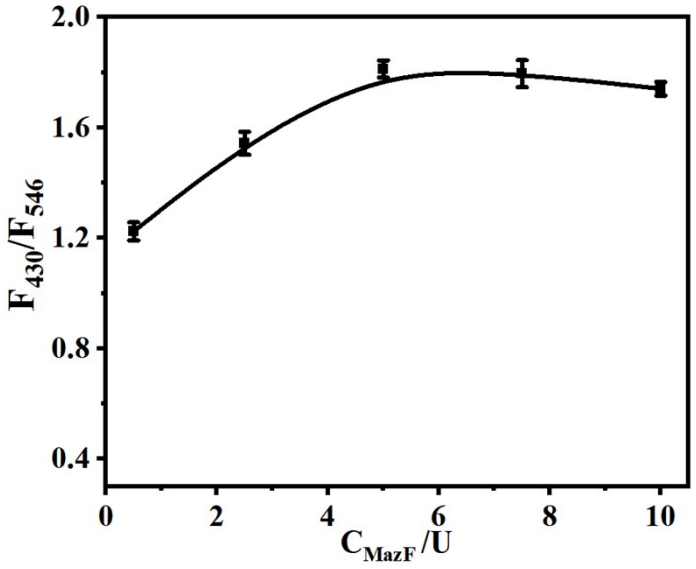
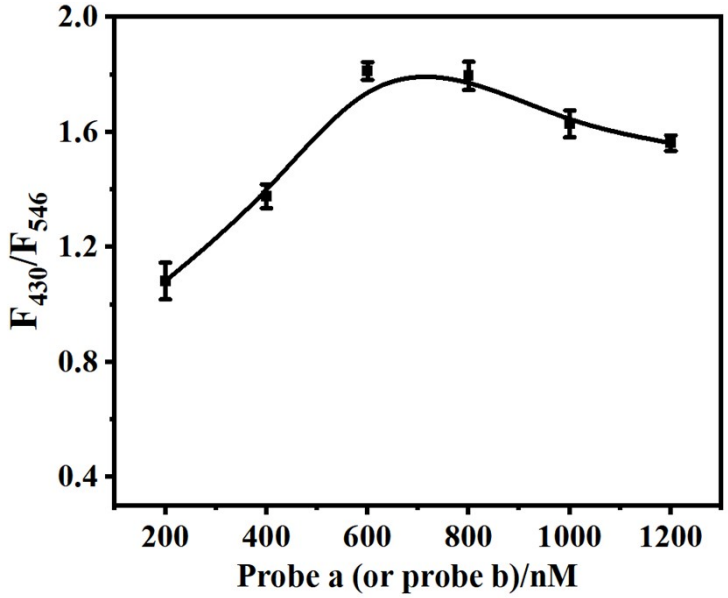


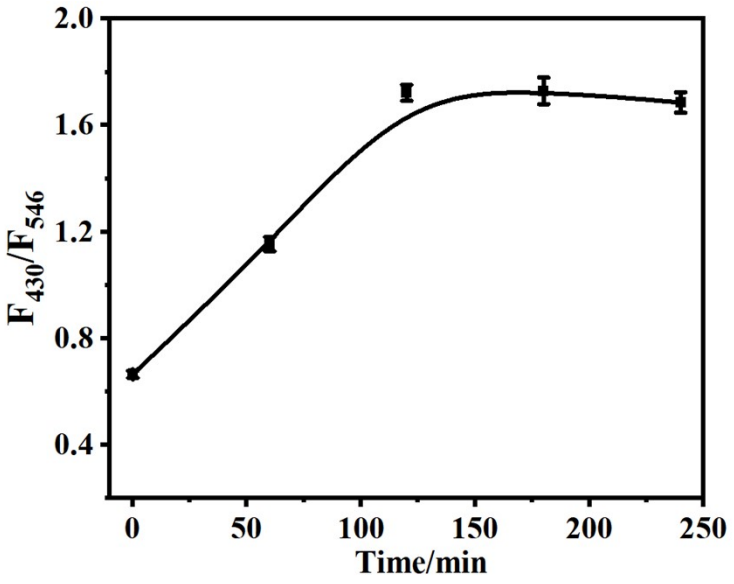
Fig. S3 (A) the XPS of luminol-Tb-GMP LnCPs in presence of m^{6}A-mRNA (gray) and absence of m^{6}A-mRNA (red), (B) the fluorescence lifetime of luminol-Tb-GMP LnCPs in presence of m^{6}A-mRNA (blue) and absence of m^{6}A-mRNA (red), (C) the SEM of luminol-Tb-GMP LnCPs in presence of m^{6}A-mRNA, (D) the mapping corresponding to figure C.



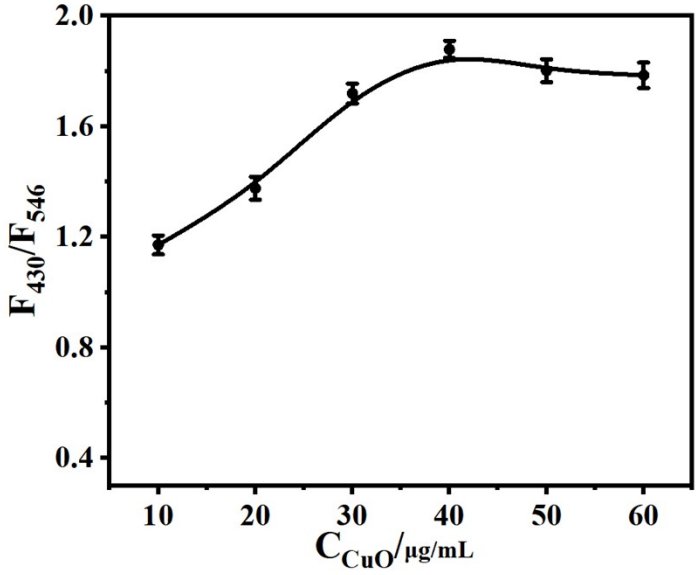
(A)


2.0
1.6
1.2
0.8
0.4
F_{430}/F_{546}
200
400
600
800
1000
1200
Probe a (or probe b)/nM

(B)


2.0
1.6
1.2
0.8
0.4
F_{430}/F_{546}
0
50
100
150
200
250
Time/min

(C)



(D)

Fig. S4 Optimization of experimental condition: (**A**) the concentration of MazF, (**B**) the concentration of probe a (or probe b), (**C**) the concatenated DNA circuit reaction time, (**D**) the concentration of CuO NPs.

Table S2. Comparison of the LnCPs Ratiometric biosensor with other reported N^6-methyladenosine detection strategy.

No.	Strategy	Methods	LOD	References
1	Click Chemistry Mediated Assembly of Quantum Dot Nanosensor	Fluorescence	1.42×10^{-15} M	Chin. J. Chem., 2024,42, 499
2	Single QD-based fluorescence resonance energy transfer nanosensor	FRET	4.7×10^{-16} M	Anal. Chem., 2023, 95, 17945
3	Dual-signal amplified photoelectrochemical biosensor	PEC	1.67×10^{-12} M	Biosens. Biolectron. 2018, 108, 89.
4	MazF-mediated TdT -assisted polymerization on-bead fluorescence	Fluorescence	2.24×10^{-17} M	Anal. Chem., 2023, 95, 5454.
5	MazF-rolling circle amplification	MALDI-TOF MS	100 fM	Anal. Chim. Acta, 2024, 1303, 342532.
6	Rolling circle extension-assisted loop-mediated isothermal amplification	Real-time PCR and visible detection	100 a mol	Nucleic Acids Res. 2023, 51, e51
7	Double blocking gap-filling-ligation coupled with cascade isothermal amplification	Fluorescence	1 fM	Chem. Commun. 2023,59, 10769
8	Gold nanoparticles modified glassy carbon electrode-based Electrochemical immunosensor	Electrochemical	2.57 pM	Sens. Actuators B Chem. 2015, 221, 1
9	Tri-Double Resolution Strategy	electrochemilum inescence	0.03 pM	ACS Sensors, 2023, 8, 2771
10	Click chemistry-Powered concatenated DNA circuit-based LnCPs Ratiometric biosensor	Fluorescence	28 aM	This work

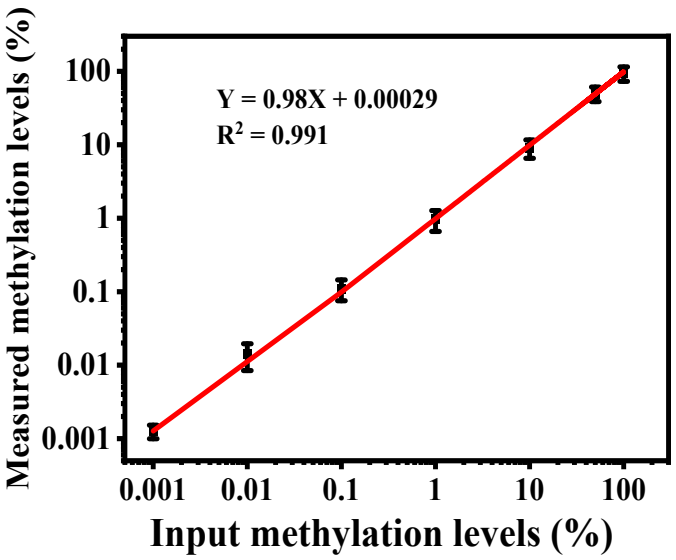


Fig. S5 Linear relationship between the measured and input methylation levels. The total concentration of m^6A-mRNA and C-mRNA is 10 pM.

Table S3 Determination of m^6A-mRNA added in diluted human blood serum (n = 3) with the proposed LnCPs biosensor.

Sample	Add/m^6-mRNA	Found/m^6-mRNA	Recovery (%)	RSD (%)
1	1.0×10^{-12}	9.89×10^{-13}	98.97	3.2
2	1.0×10^{-13}	1.05×10^{-13}	105.2	4.2
3	1.0×10^{-14}	9.97×10^{-15}	99.71	3.9
4	1.0×10^{-15}	1.03×10^{-15}	102.4	5.4

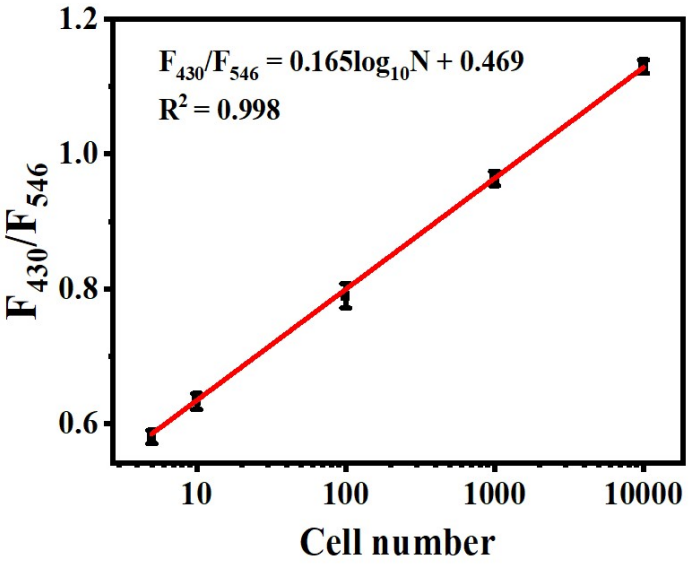


Fig. S6 Ratiometric values of fluorescence intensity (F_{430}/F_{546}) for the analysis of different HEK-293T cell numbers from 5 to 10000 cells.

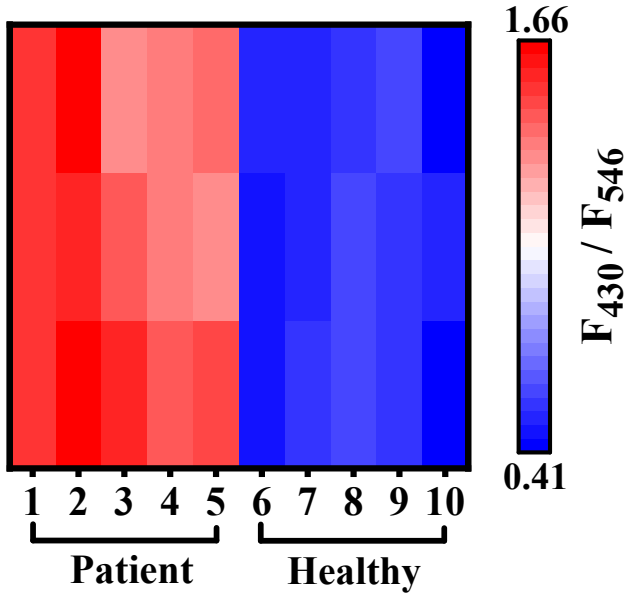


Fig. S7 The Heat map of fluorescence intensity ratiometric values (F_{430}/F_{546}) in response to different lung tissues.

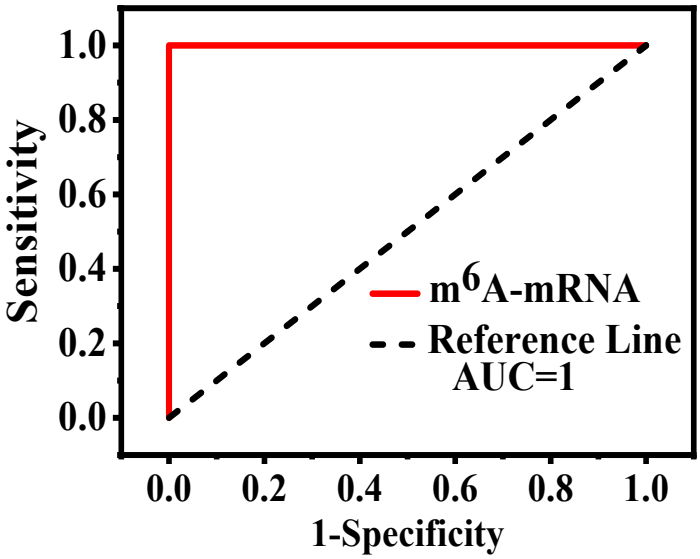
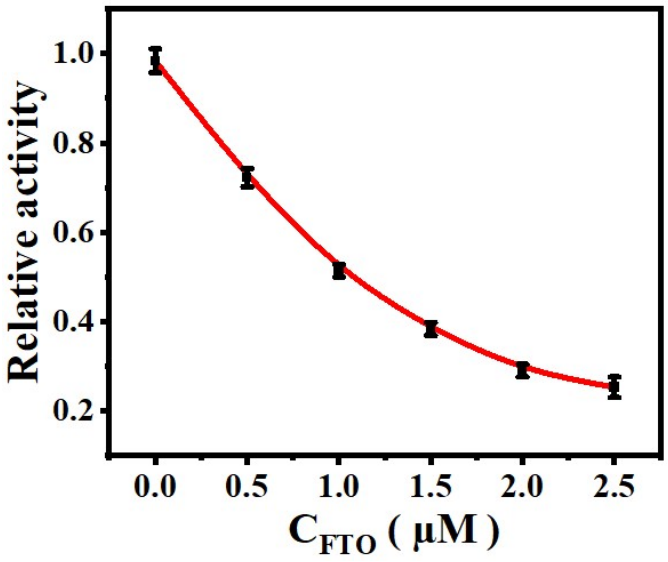
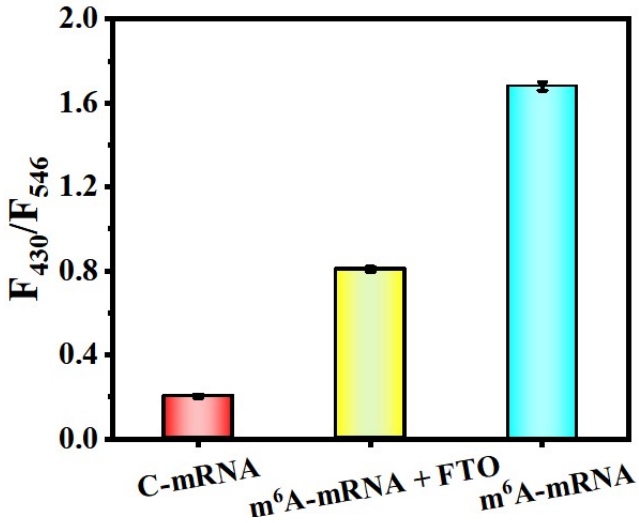


Fig. S8 ROC analysis to evaluate the accuracy of this the proposed LnCPs biosensor in discriminating lung cancer patients from healthy individuals.



(A)



(B)

Fig. S9 (A) The relative activity of m^6A-mRNA with different concentration N^6 -methyladenosine (m^6A) demethylase FTO. (**B**) Comparison of the fluorescence intensity ratiometric values (F_{430}/F_{546}) produced by C-mRNA, m^6A-mRNA+FTO, and m^6A-mRNA.