A Highly Sensitive and Selective Fluoride Sensor Based on a Riboswitch-Regulated Transcription Coupled with CRISPR-Cas13a Tandem Reaction

Yuan Ma[a,b], Quanbing Mou[a], Peng Yan[a,c], Zhenglin Yang[d], Ying Xiong[a], Deyue Yan[b], Chuan Zhang[b]*, Xinyuan Zhu[b]*, and Yi Lu[a,d]*

[a] Department of Chemistry, University of Illinois at Urbana-Champaign, Urbana, Illinois 61801, United States.

[b] School of Chemistry and Chemical Engineering, Frontiers Science Center for Transformative Molecules, Shanghai Jiao Tong University, 800 Dongchuan Road, Shanghai 200240, China

[c] Key Laboratory of Biomedical Information Engineering of Ministry of Education, School of Life Science and Technology, Xi'an Jiaotong University, 710049, Xi'an, PR China

[d] Department of Biochemistry, University of Illinois at Urbana-Champaign, Urbana, Illinois 61801, United States.

*Corresponding author: yi-lu@illinois.edu, xyzhu@sjtu.edu.cn, chuanzhang@sjtu.edu.cn

1. Materials.

E. coli RNA Polymerase (Holoenzyme), Bovine Serum Albumin (BSA), adenosine 5′-triphosphate (ATP), cytidine 20 5′-triphosphate (CTP), guanosine 5′-triphosphate (GTP), uridine 5′-triphosphate (UTP), DNAse, RNAse, Proteinase K were purchased from NEW ENGLAND BioLabs Inc. (Ipswich, UK). CRISPR-Cas13a (Lbu) was purchased from Bio-lifesci Inc. China. Sodium fluoride, sodium chloride, magnesium chloride, ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA-Na_2), heparin, 2-mercaptoethanol, glycerol and Igepal CA-630 were purchased from Sigma-Aldrich, Inc. (St. Louis, USA). ApU (TriLink BioTechnologies), α-^{32}P-radiolabeled ATP (Perkin Elmer Inc.) were used as received. RNaseOUT™ recombinant ribonuclease inhibitor, yeast tRNA were purchased from Thermo Fisher Scientific Inc. All other reagents and solvents were obtained from the domestic suppliers and used as received.

Table S1. DNA sequences used in the experiments (5′ to 3′).

Oligonucleotide names	Oligonucleotide sequences
Template A	ATGCATCTAGATGCCAATGAATTCTTGACTATTTTACCTCTG GCGGTGATAATGGTTGCATGTAGTAAGGAGGTTGTATGGAA GACTAGGCGATGGTGTTCGCCATAAACGCTCTTCGGAGCTA ATGACACCTACCAGTATCACTACTGGTAGGTGTCTTTTTTTT GAGCAAGCTATTTAAAGAGGTGAGAAAATTGATTTATATTA TCGTTGGCA Grey: sequence containing λ_{PR} Promoter Blue: C-absent spacer Purple: Fluoride riboswitch Red: Cas13a targeting sequence
Template B	TGCCAACGATAATATAAATCAATTTTCTCACCTCTTTAAATA GCTTGCTCAAAAAAAAGACACCTACCAGTAGTGATACTGGT AGGTGTCATTAGCTCCGAAGAGCGTTTATGGCGAACACCAT CGCCTAGTCTTCCATACAACCTCCTTACTACATGCAACCATT ATCACCGCCAGAGGTAAAATAGTCAAGAATTCATTGGCATC TAGATGCAT
A1	ATGCATCTAGATGCCAATGAATTCTTGACTATTTTACCTCTG GCGGTGATAATGGTTGCATGTAGTAAGGAG
A2	GTTGTATGGAAGACTAGGCGATGGTGTTCGCCATAAACGCT CTTCGGAGCTAATGACACCTACCAGTATCAC
A3	TACTGGTAGGTGTCTTTTTTTTGAGCAAGCTATTTAAAGAGG TGAGAAAATTGATTTATATTATCGTTGGCA
B1	TGCCAACGATAATATAAATCAATTTTCTCACCTCTTTAAATA GCTTGCTCAAAAAAAAGACACCTACCAGTA
B2	GTGATACTGGTAGGTGTCATTAGCTCCGAAGAGCGTTTATG GCGAACACCATCGCCTAGTCTTCCATACAAC
B3	CTCCTTACTACATGCAACCATTATCACCGCCAGAGGTAAAA TAGTCAAGAATTCATTGGCATCTAGATGCAT
Ligation helper for A1, A2	AACACCATCGCCTAGTCTTCCATACAACCTCCTTACTACATG CAACCATTATCACC
Ligation helper for A2, A3	TTGCTCAAAAAAAAGACACCTACCAGTAGTGATACTGGTAG GTGTCATTAGCTCCG

Ligation helper for B1, B2	CGGAGCTAATGACACCTACCAGTATCACTACTGGTAGGTGT CTTTTTTTGAGCAA
Ligation helper for B2, B3	GGTGATAATGGTTGCATGTAGTAAGGAGGTTGTATGGAAGA CTAGGCGATGGTGTT
Spacer complementary	GTCTTCCATACAACCTCCTTACTACAT

Table S2. RNA sequences used in the experiments (5′ to 3′).

Oligonucleotides	Oligonucleotide sequences
crRNA	GACCACCCCAAAAAUGAAGGGGACUAAAACAGCCAACGAUA AUAUAAAUCA
Fluorescent reporter RNA	FAM-UUUUU-BHQ1
Model RNA	AAAUUGAUUUAUAUUAUCGUUGGCA

2. Measurements

Fluorescent spectra. The fluorescent spectra were performed on FluoroMAX-3P fluorometer (Horiba Jobin Yvon, Inc., Edison, NJ). The excitation wavelength was set as 485. The emission wavelength was set from 505 nm to 600 nm, with 1 nm step increment.

Portable fluorimeter. The portable fluorimeter (ANDalyze 1000) used in this work was purchased from ANDalyze, Inc. (Champaign, USA).

3. Methods

3.1 Radio-labeled *in vitro* transcription

Transcription reaction was carried out in two steps, an initiation step and a separate elongation step, using the same protocol adapted from previous published methods.[1]

The DNA template used for transcription contains a λ_{PR} promoter (grey color), a C-absent spacer (blue color), followed by the full-length riboswitch (purple color), and ended with Cas13a targeting sequence (red color), which can be detected by Cas13a to trigger cleavage of fluorescent reporter RNA.

In the initiation step, the initiation mixture containing 150 μM ApU, 2.5 μM of GTP and UTP, 1 μM ATP, α-^{32}P-radiolabeled ATP, DNA template (18.75 nM), 0.75 U E. coli RNA polymerase, 1 U RNAseOUT, in 1× transcription buffer (20 mM Tris–HCl, pH 8.0, 20 mM NaCl, 1 mM $MgCl_2$, 100 μM EDTA, 14 mM 2-mercaptoethanol, 5% glycerol) were prepared and then incubated at 30 °C for 10 min. During the initiation step, E. coli RNA polymerase elongation complexes were paused at the end of spacer due to the absence of CTP in the transcription initiate reaction mixture. As reported by literature,[1] the spacer may interfere with fluoride riboswitch folding. To prevent that, prior to elongation, DNA complementary to spacer (Spacer complementary) was added to transcription initiate mixture at 1 μM final concentration in 1× transcription buffer and then incubated at room temperature for 5 min to ensure hybridization and prohibit spacer to interfere fluoride riboswitch.

To initiate elongation, 5 μL paused transcription initiation solution was added to 2 μL of transcription chase solution, which contained different concentrations of NTP (10, 20, or 30 μM), 1 mg/mL heparin, and varying concentrations of fluoride in 1X transcription buffer. After incubation at 37 °C for 15 min, the reactions were quenched in stop solution (1× transcription buffer, 7 M urea, 30 mM EDTA, and trace bromophenol blue and xylene cyanol). Thereafter, the samples were analysed by 10% denaturing polyacrylamide gel electrophoresis (PAGE) gel and visualized by phosphorimager. The transcription readthrough was calculated by measuring the intensity of full-length bands (F) and terminated bands (T) from the autoradiogram, and calculated by F/(F + T).

3.2 Optimization of Cas13a reaction buffer

Cas13a-crRNA complexes were preassembled by incubating 400 nM Cas13a and 200 nM crRNA in different Cas13a reaction buffers as shown in Table S3. Then Cas13a-crRNA complexes were diluted to 50 nM Cas13a and 25 nM crRNA and mixed with fluorescent reporter RNA (200 nM), model RNA (10 nM) at 37 ℃ for 10 min.

Table S3. Different Cas13a reaction buffers.

Cas13a reaction buffers	components
Buffer 1	20 mM HEPES pH 6.8, 5 mM $MgCl_2$, 50 mM KCl, 10 μg/mL yeast tRNA, 10 μg/mL BSA, 5% glycerol, and 0.01% Igepal CA-630
Buffer 2	10 mM Tris-HCl, 10 mM NaCl, 1.5 mM $MgCl_2$
Buffer 3	Purchased from Bio-lifesci Inc.

3.2. Optimize Cas13a reaction time

Cas13a-crRNA complexes were preassembled by incubating 400 nM Cas13a and 200 nM crRNA in 1 × Cas13a reaction buffer 1 (20 mM HEPES pH 6.8, 5 mM $MgCl_2$, 50 mM KCl, 10 μg/mL yeast tRNA, 10 μg/mL BSA, 5% glycerol, and 0.01% Igepal CA-630) at 37 ℃ for 10 min. The transcription initial solution was prepared in a similar way as mentioned above, containing 2.5 uM GTP, UTP, and ATP. Then the detection assays were performed with a total volume of 10 μL, containing 1× cas13a reaction buffer, 1.25 μL Cas13a-crRNA complexes, 0.2 μL of 10 μM fluorescent reporter RNA, 4.142 μL transcription initial solution, 1.657 μL of transcription chase solution (30 μM NTP, varying concentrations of NaF in 1X transcription buffer). Then, the reaction mixtures were transferred into 384 well plate (Corning Costar) and sealed, then incubated at 37 ℃ for 60 min in fluorescence plate reader (BioTek® Synergy H1). The fluorescence measurements were taken every 10 min using 485 nm excitation and 525 nm emission. Background-corrected fluorescence values were calculated by subtracting fluorescence values obtained from reactions carried out in the absence of transcription chase solution.

3.3 Optimize Cas13a quenching method

To control the reaction time more precisely, different quenching methods for the tandem reaction were studied. Briefly, the tandem reaction was conducted in the same way as mentioned above. After 30 min of tandem reaction, the reaction solution was quenched at 65 °C or 85 °C for 10 min, respectively. Thereafter, the fluorescence intensity of each reaction solution was monitored by fluorescence plate reader at every 15 min.

3.3 Sensitivity, selectivity, spiked sample test

The Cas13a reaction mixtures were prepared in the same way as mentioned above.

For the sensitivity test, the Cas13a reaction mixture, transcription initiation solution, transcription chase solution containing varying concentrations of NaF were mixed and were incubated at 37 ℃ for 15 or 30 min. After that, the enzymatic reaction was stopped by heating at 65 ℃ for 10 min. Then the fluorescence intensity of the solution was detected by HORIBA Jobin Yvon FluoroMAX-3P using 485 nm as excitation and 505-600 nm as emission. As shown in Figure 2c, the results are fitted using this equation:

$y = -0.09589 \times \exp(-x/(3.25503 \times 10^{-4})) - 0.45834 \times \exp(-x/0.14855) - 0.45909 \times \exp(-x/0.1485) + 1.01246$, and the coefficient of determination (R^2) for fitting is 0.995.

For selectivity test, transcription chase solutions containing different anions were prepared, which contain 200 μM NaCl, 200 μM NaBr, 200 μM NaI, 200 μM Na_2CO_3, 200 μM $NaHCO_3$, 200 μM Na_2SO_4, 200 μM NaH_2PO_4, 200 μM Na_2HPO_4, 200 μM $NaNO_3$, 200 μM HCOONa, 200 μM CH_3COONa, 50 μM common anion mixture (NaCl, $NaNO_3$, Na_2CO_3, Na_2SO_4), or 18 μM anion mixture (NaCl, NaBr, NaI, Na_2CO_3, $NaHCO_3$, Na_2SO_4, NaH_2PO_4, Na_2HPO_4, $NaNO_3$, HCOONa, CH_3COONa). Other procedures were the same as sensitivity experiment.

For the anti-interference study, different samples containing 200 uM NaF were spiked with different common interferences, such as 100 μM NaCl, 100 μM KCl, 100 μM $MgCl_2$, 100 μM $CaCl_2$, 25 μM common cation mixture (NaCl, KCl, $MgCl_2$, $CaCl_2$), or common cation and anion mixture (12.5 μM Na_2CO_3, 25 μM KNO_3, 25 μM $MgCl_2$, 25 μM $CaSO_4$), and enzyme mixture (1 uL DNAse, RNAse and Proteinase K). To inactivate the enzyme interference, the samples were heat-pretreated at 95 ºC for 10 min to inactivate DNAse and protease. Other procedures were the same as sensitivity experiment.

To test the real sample spiked with fluoride, transcription chase solution with different concentrations of F^- were prepared by spiking different amounts of NaF. To test the real environmental sample, transcription chase solution were prepared with adding bottled water from supermarket, drinking water samples from tap water in Urbana (IL, US), lake water in Urbana (IL). Other procedures were the same as sensitivity experiment.

To test the sample with standard addition method, the samples were spiked with fluoride at additional final concentration of 0, 215.5, 431, 862, 1293 μM, respectively. Thereafter, all the spiked samples were analyzed by the tandem sensor. The calibration curve of relative fluorescence ratio verse spiked fluoride concentration is shown in Figure S14. The fluoride concentration of the real sample can be calculated based on extrapolate method.

The on-site and real-time detection with fluoride sensor was performed, using the similar protocol as the sensitivity test. The fluorescence intensity of the solution was detected by portable fluorimeter (ANDalyze 1000). As shown in Figure 4b, the results are fitted using this equation:

$y = -0.10998 \times \exp(-x/0.00675) - 0.56244 \times \exp(-x/0.18945) - 0.13058 \times \exp(-x/0.04884) + 0.87123$, and the coefficient of determination (R^2) for fitting is 0.996.

4. Figures

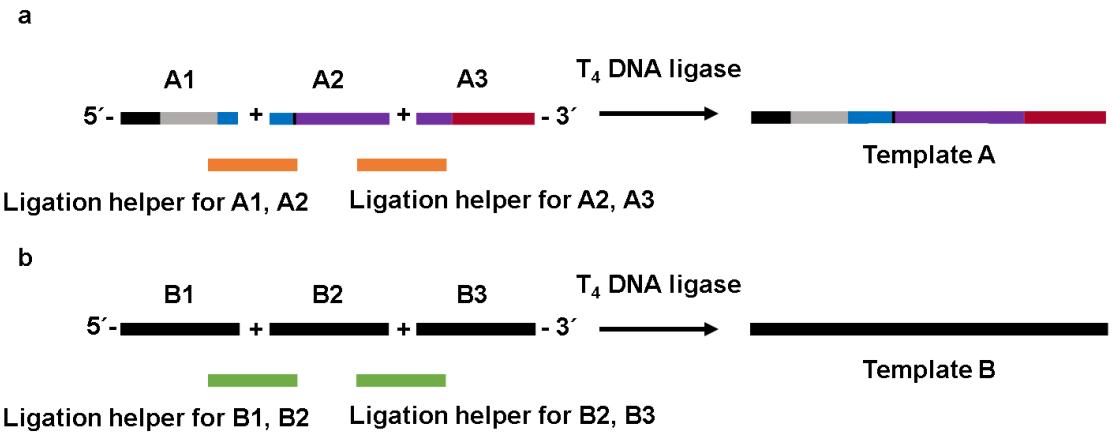


Figure S1. The ligation scheme for preparing template A (a) and template B (b).

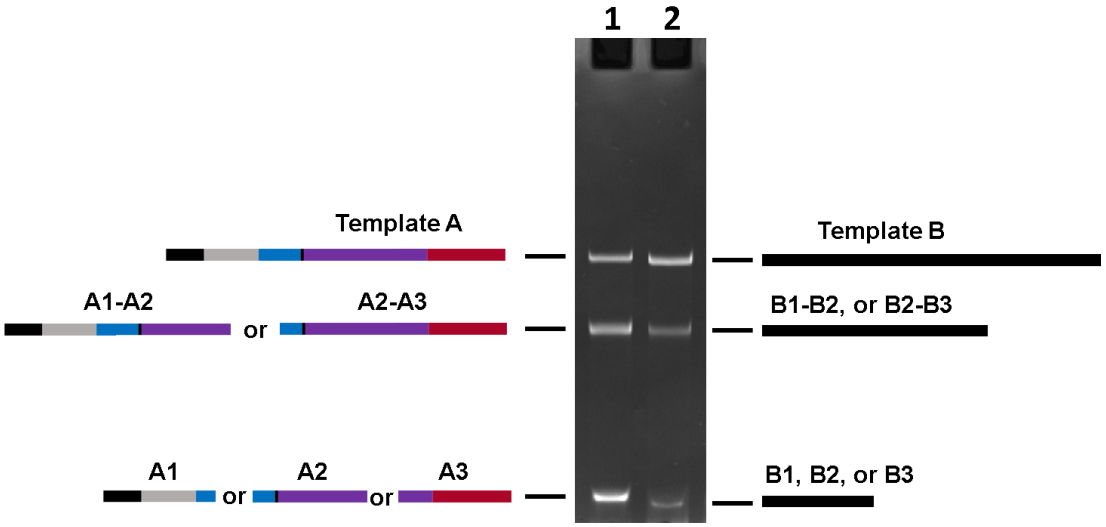


Figure S2. The 10% denaturing polyacrylamide gel electrophoresis (PAGE) gel analysis for ligation reaction: (1) Template A; (2) Template B.

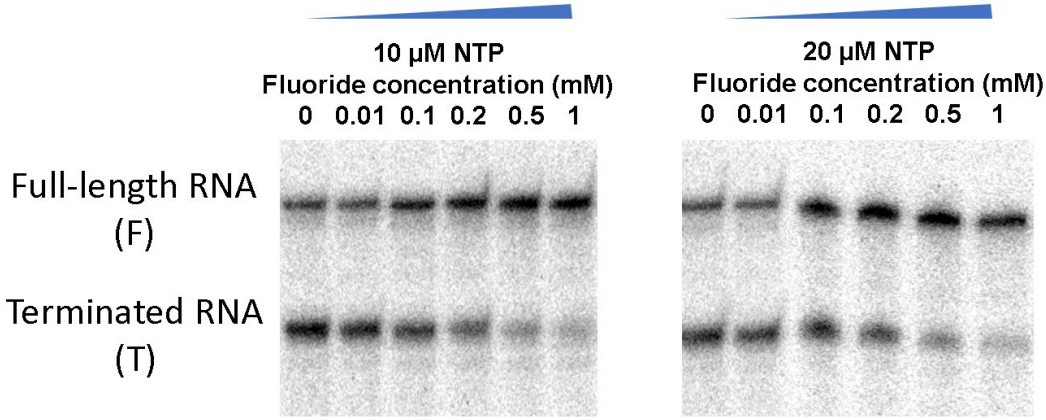


Figure S3. Fluoride-dependent transcription assay with 10, 20 μM NTP. Autoradiogram of a 10% PAGE denaturing gel separating the full-length (F) and terminated (T) RNA products

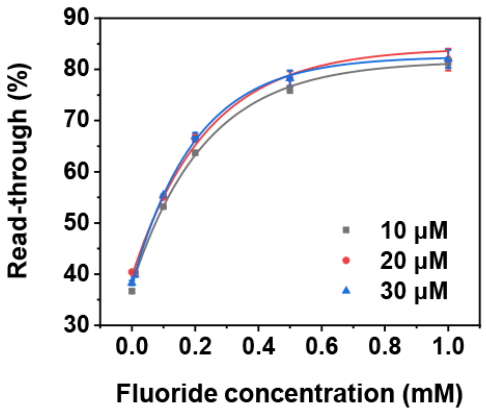


Figure S4. Fluoride-dependent transcription readthrough assay with 10, 20, 30 μM NTP, calculated by F/(F + T).

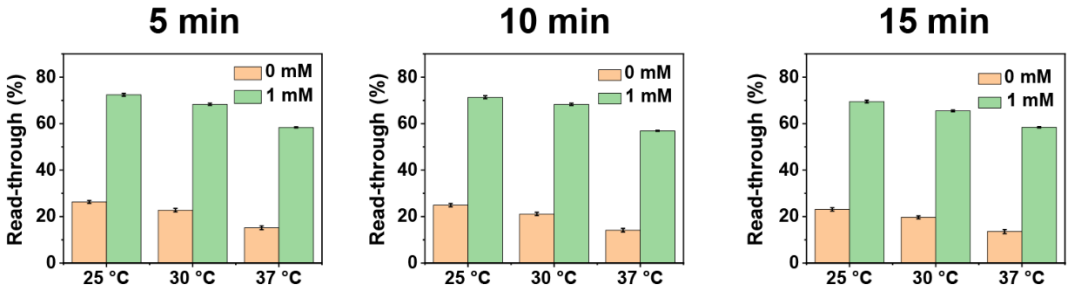


Figure S5. Readthrough of fluoride-dependent transcription assay with 0 or 1 mM fluoride at different time points with different reaction temperature, calculated by F/(F + T).

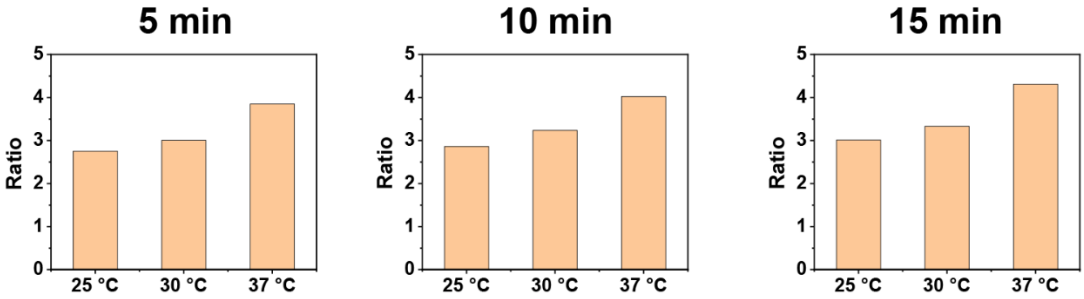


Figure S6. Readthrough ratio between 1 mM and 0 mM in fluoride-dependent transcription assay, calculated by F/(F + T).

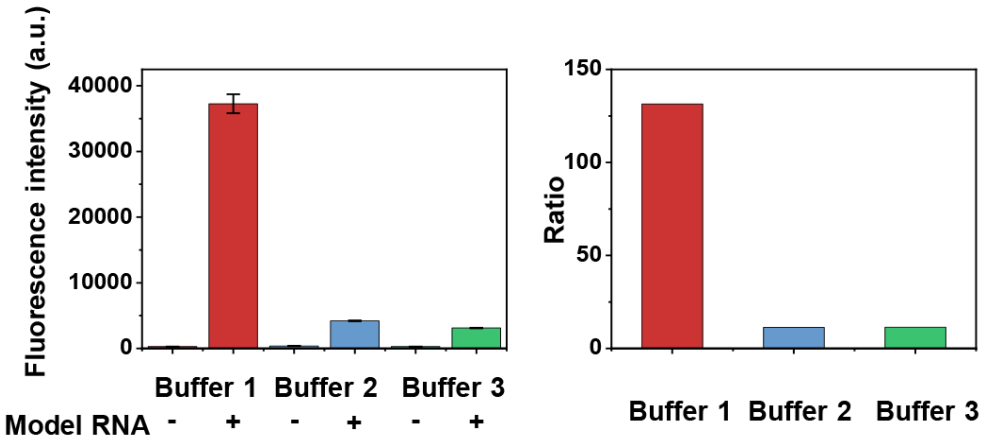


Figure S7. Optimization of Cas13a reaction buffer. (a) The fluorescence intensity of Cas13a reaction in different buffers with or without 10 nM model RNA. (b) The ratio of Cas13a reaction (10 nM model RNA/ 0 nM RNA) in different reaction buffers.

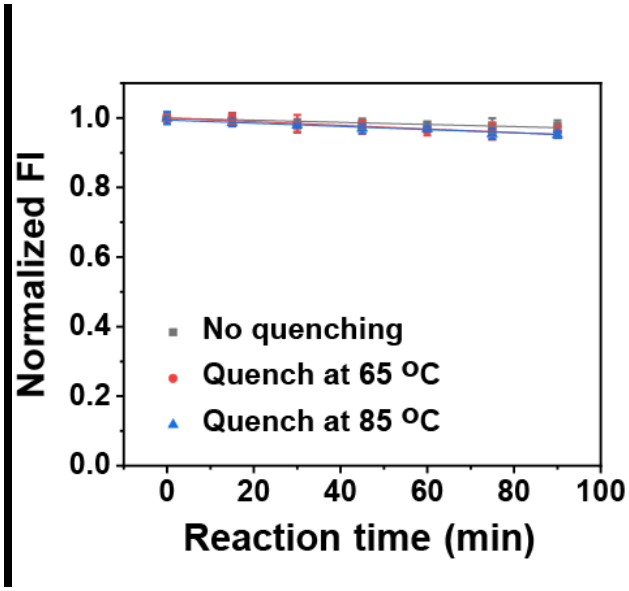


Figure S8. Optimization of quenching method for tandem reaction. After quenching at 65 °C or 85 °C for 10 min, normalized fluorescence intensity (FI) of each reaction solution was monitored by fluorescence plate reader at every 15 min.

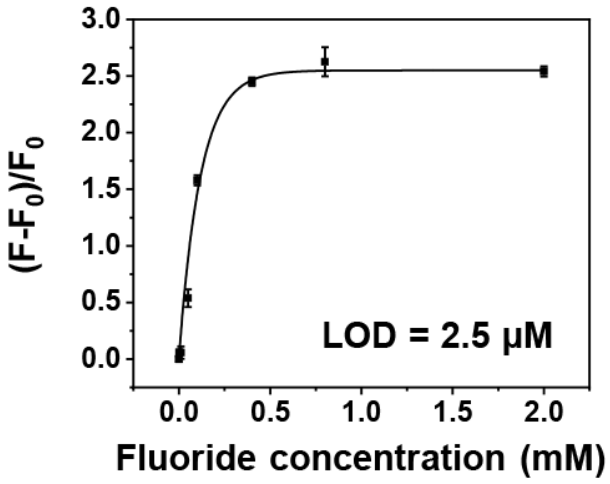


Figure S9. The $(F-F_0)/F_0$ of FRITCas13a sensor at different fluoride concentrations with 15 min detection time.

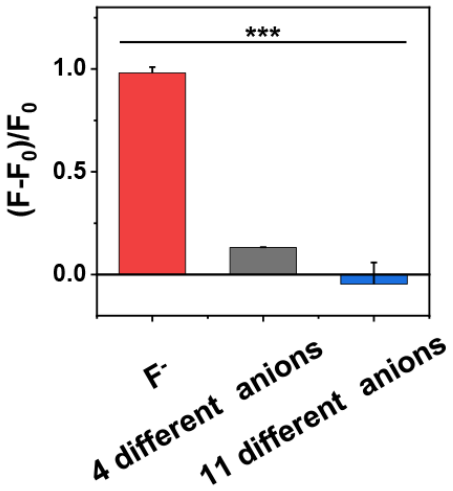
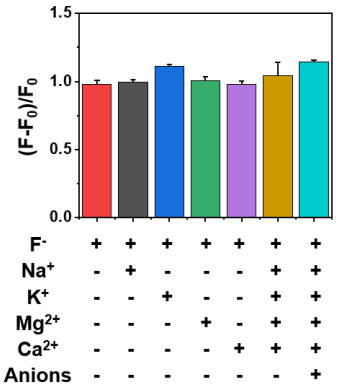


Figure S10. The selectivity of FRITCas13a tandem sensor against common anion mixtures. The $(F-F_0)/F_0$ value of FRITCas13a tandem sensor in the presence of F^-, 50 μM common anion mixture (NaCl, $NaNO_3$, Na_2CO_3, Na_2SO_4), or 18 μM anion mixture (NaCl, NaBr, NaI, Na_2CO_3, $NaHCO_3$, Na_2SO_4, NaH_2PO_4, Na_2HPO_4, $NaNO_3$, HCOONa, CH_3COONa).

a



b

Selectivity coefficient of different ions

Ion interferences	Selectivity coefficient (SC)
Na^+	1.02
K^+	1.14
Mg^{2+}	1.03
Ca^{2+}	1.00
Cation mixture (Na^+, K^+, Mg^{2+}, Ca^{2+})	1.06
Cation and anion mixture (Na^+, K^+, Mg^{2+}, Ca^{2+}, Cl^-, NO_3^-, CO_3^{2-}, SO_4^{2-})	1.17

Figure S11. The performance of the FRITCas13a tandem sensor in the presence of cations and other anions. (a) The $(F-F_0)/F_0$ value of FRITCas13a tandem sensor at fluoride concentration of 200 μM, in the presence of 100 μM NaCl, 100 μM KCl, 100 μM $MgCl_2$, 100 μM $CaCl_2$, 25 μM common cation mixture (NaCl, KCl, $MgCl_2$, $CaCl_2$), or common cation and anion mixture (12.5 μM Na_2CO_3, 25 μM KNO_3, 25 μM $MgCl_2$, 25 μM $CaSO_4$). (b) The selectivity coefficient of different ion interferences.

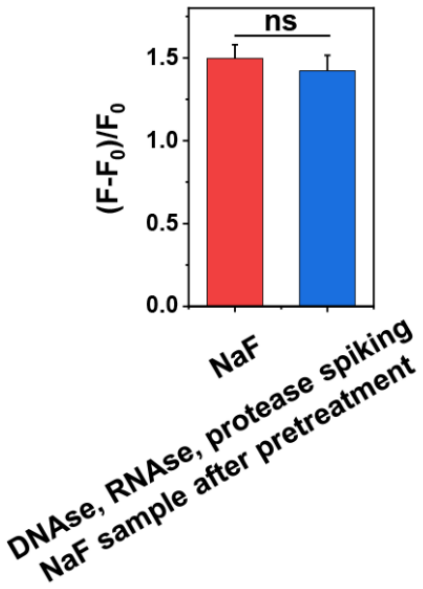


Figure S12. Effect of heat pretreatment and adding an RNAase inhibitor on the fluoride sensor spiked with DNAse I, RNAse A, and Protease K.

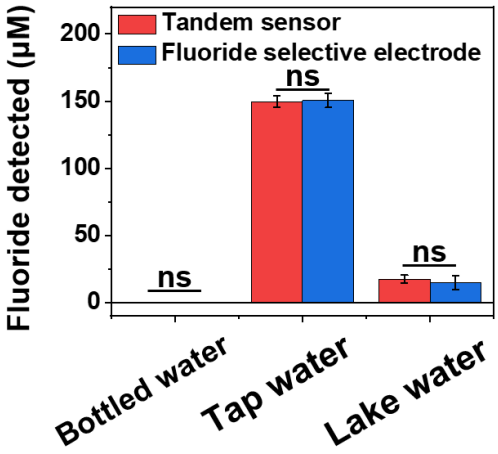


Figure S13. Fluoride concentrations of real environmental samples tested by the fluoride sensor in comparison with results obtained from standard test using fluoride selective electrode.

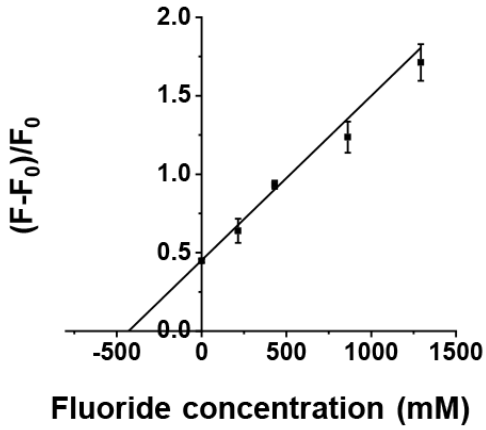


Figure S14. The calibration curve to determine the fluoride concentration through standard addition method. Based on extrapolation method, the fluoride concentration of spiked tap water sample was calculated to be 430.5 ± 11.7 μM (recovery yield of 95.7%), which demonstrate similar accuracy to the result obtained by fluoride selective electrode (463.9 ± 5.3 μM, recovery yield of 103.1%).

Reference

[1] B. Zhao, S. L. Guffy, B. Williams, Q. Zhang, *Nat. Chem. Biol.* **2017**, *13*, 968-974.