

Supplementary Information

A versatile method for designing biosensors via regulatory domains of allosteric enzymes

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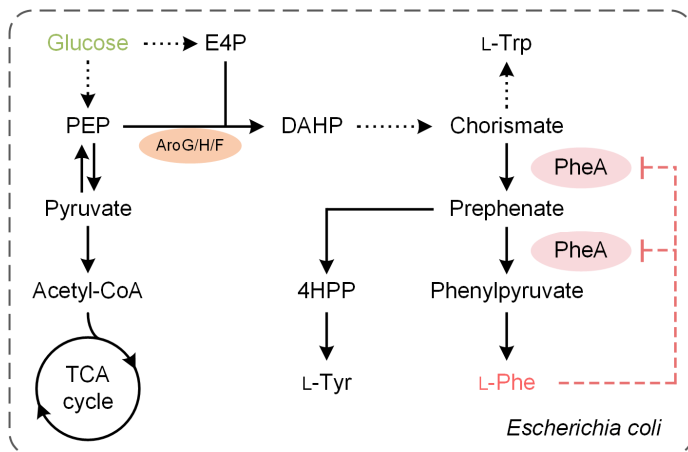
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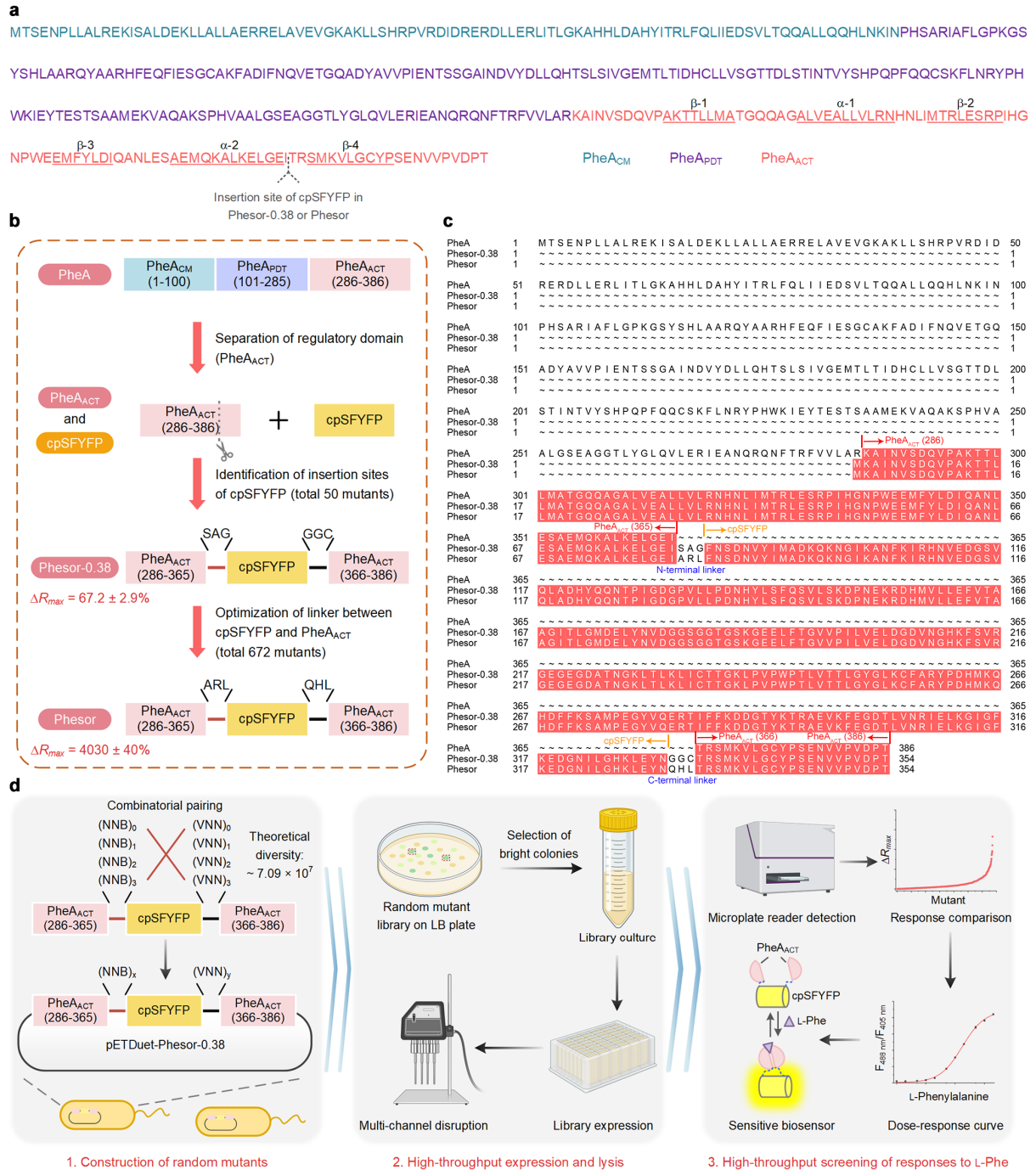
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Supplementary Figures and Tables:



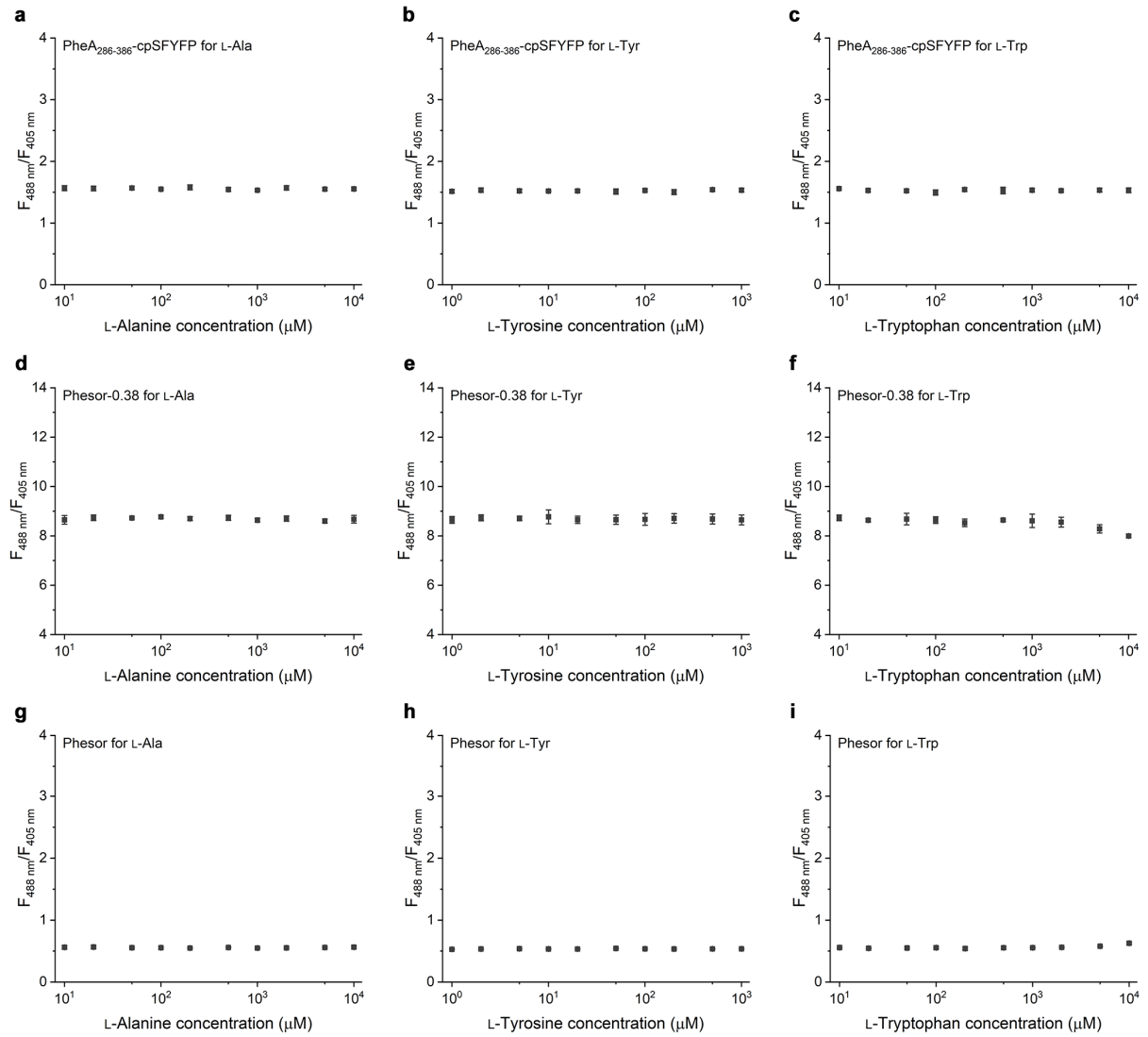
Supplementary Figure 1 Schematic representation of the biosynthetic pathway of L-phenylalanine in *Escherichia coli*. PEP, phosphoenolpyruvate; E4P, erythrose 4-phosphate; DAHP, 3-deoxy-D-arabino-heptulosonate 7-phosphate; 4HPP, 4-hydroxyphenylpyruvate; AroG/H/F, three DAHP synthase isoenzymes; PheA, chorismate mutase.



Supplementary Figure 2 Workflow for the development of L-phenylalanine biosensor

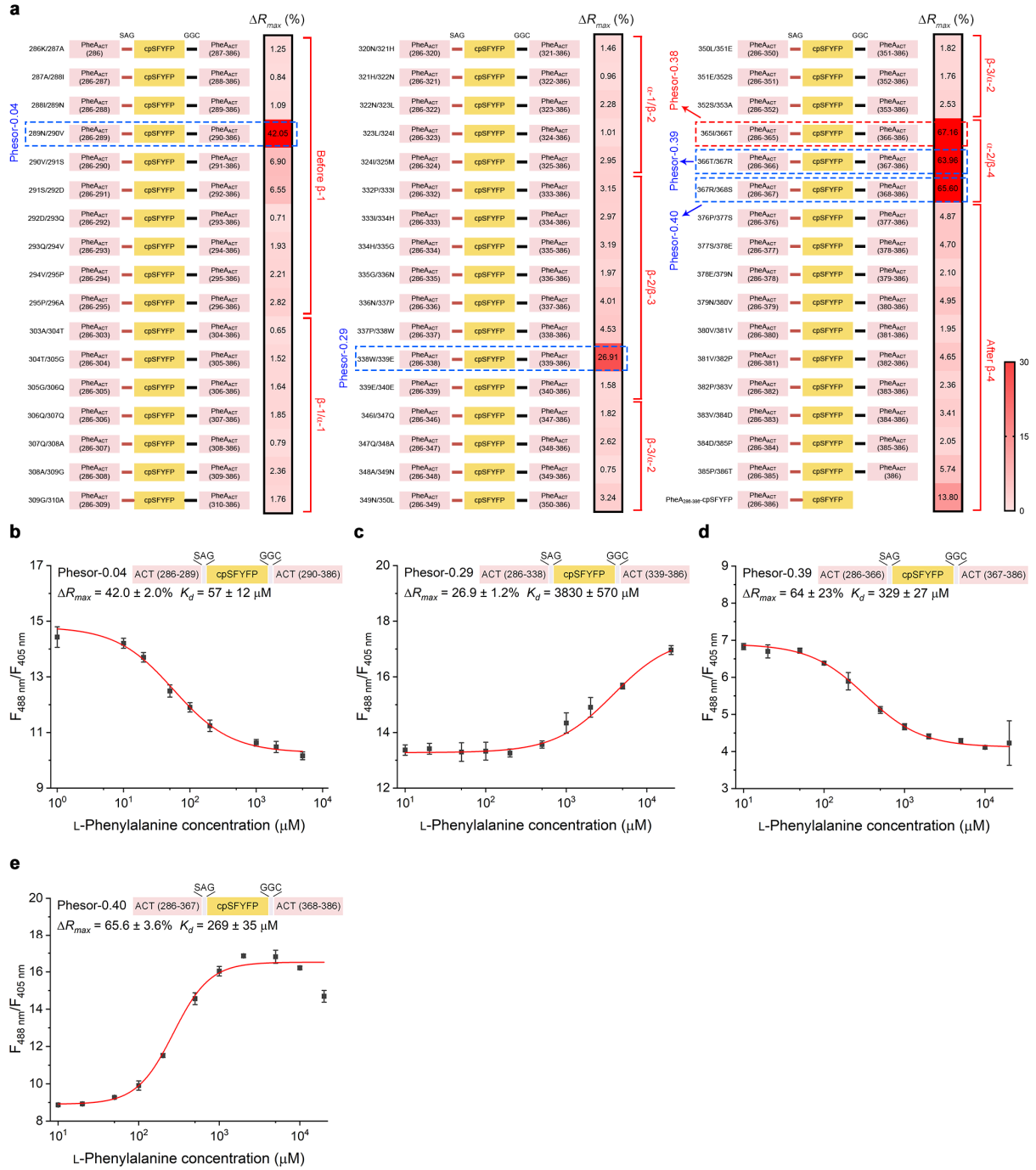
Phesor. **(a)** Schematic representations of the amino acid sequence and the predicted secondary structure of PheA. The chorismate mutase (PheA_{CM}) domain, prephenate dehydratase (PheA_{PDT}) domain, and ACT (PheA_{ACT}) domain of PheA were shown in cyan, purple, and red, respectively. The predicted α -helices and β -sheets of the PheA_{ACT} domain

were indicated with underlines and the corresponding designations were listed above the sequence. **(b)** Schematic representation of the construction and optimization of Phesor. The final L-phenylalanine biosensor, Phesor, was constructed by inserting cpSFYFP between the 286K-365I and 366T-386T fragments of PheA, with an N-terminal linker of Ala-Arg-Leu and a C-terminal linker of Gln-His-Leu. **(c)** Sequence alignment of PheA, Phesor-0.38, and Phesor. The PheA_{ACT} region, the cpSFYFP region, and the N- and C-terminal linkers were indicated. **(d)** Schematic representation of the optimization workflow for the L-phenylalanine biosensor through random linker mutagenesis and high-throughput screening. The linker regions were randomized using NNB or VNN codons and combinatorially assembled at both termini (0, 1, 2, or 3 random amino acids), yielding a mutant library with a theoretical diversity of $\sim 7.09 \times 10^7$. Bright colonies were batch-picked, induced for expression, and lysed. Mutants with significantly enhanced sensitivity were then selected by high-throughput analysis of the fluorescence response to L-phenylalanine using a microplate reader. Created in BioRender. Kang, Z. (2026) <https://BioRender.com/vzanjnw>.



Supplementary Figure 3 Specificity analysis of different L-phenylalanine biosensors. **(a-c)**

Dose-response curves of PheA_{ACT}-cpSFYFP (PheA₂₈₆₋₃₈₆-cpSFYFP) for increasing concentrations of L-alanine **(a)**, L-tyrosine **(b)**, and L-tryptophan **(c)**. **(d-f)** Dose-response curves of Phesor-0.38 for increasing concentrations of L-alanine **(d)**, L-tyrosine **(e)**, and L-tryptophan **(f)**. **(g-i)** Dose-response curves of Phesor for increasing concentrations of L-alanine **(g)**, L-tyrosine **(h)**, and L-tryptophan **(i)**. All data shown are means $\pm$ standard deviations (s.d.) ($n = 4$ independent experiments). Source data are provided as a Source Data file.



Supplementary Figure 4 Construction of Phesor-0.38 by insertion site screening. **(a)**

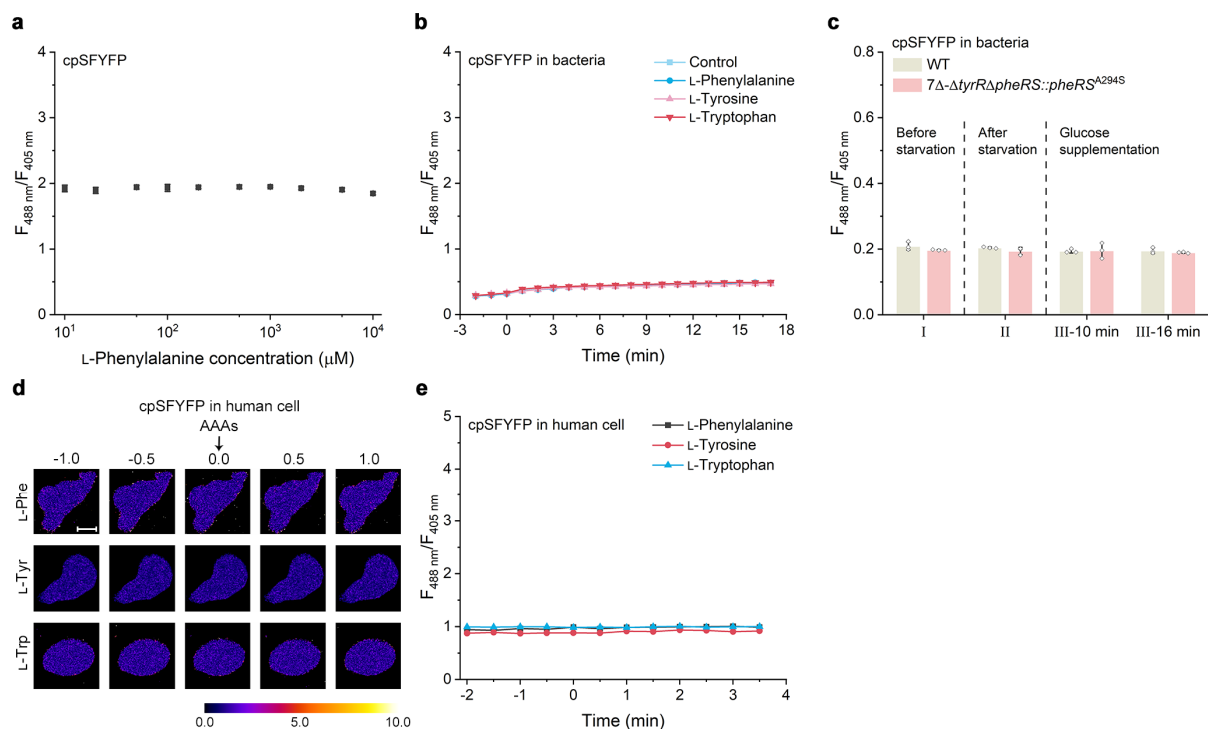
Schematic representation of the construction of the L-phenylalanine biosensor Phesor-0.38

based on insertion site screening. The ΔR_{max} of different biosensor variants in response to L-phenylalanine were presented in a heatmap, where the color depth represented the magnitude of ΔR_{max} (%). The structure of Phesor-0.38 was shown in the red dotted box, and the

structures of other L-phenylalanine biosensor insertion variants (including Phesor-0.04, Phesor-0.29, Phesor-0.39, and Phesor-0.40) were shown in the blue dotted boxes. **(b-e)** Dose-response curves of Phesor-0.04 **(b)**, Phesor-0.29 **(c)**, Phesor-0.39 **(d)**, and Phesor-0.40 **(e)** for increasing concentrations of L-phenylalanine. The dose-response curve of Phesor-0.38 for increasing concentrations of L-phenylalanine was indicated in Fig. 2a. All data shown are means $\pm$ s.d. (n = 3 independent experiments for **a, e**; n = 4 independent experiments for **b-d**). Source data are provided as a Source Data file.

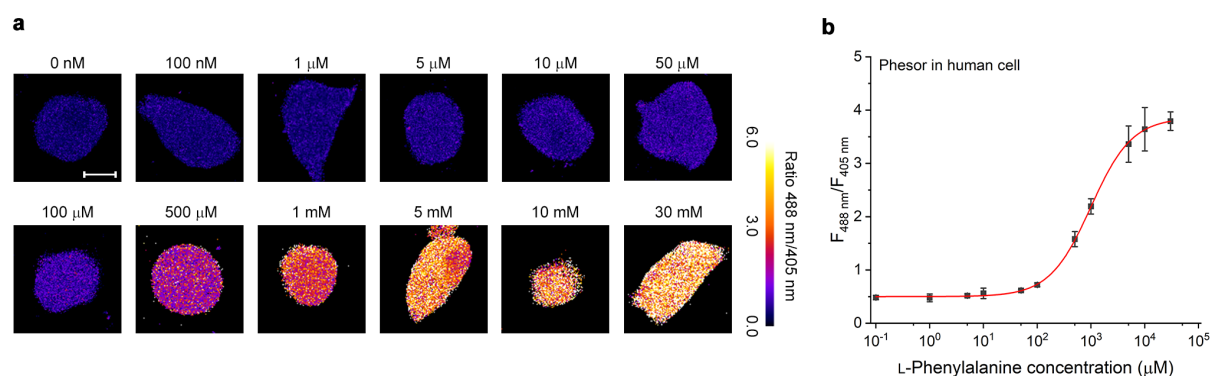
filtration chromatography. Red curve, chromatogram of purified Phesor; black line, standard curve for protein molecular mass standards. **(c, d)** Fluorescence excitation **(c)** and emission **(d)** spectra of Phesor in the absence or presence of 1 mM L-phenylalanine. **(e)** Analysis of the binding properties of Phesor to L-phenylalanine by TSA. The ΔT_m values indicated the changes in T_m values compared to the control (with 0 mM L-phenylalanine). **(f)** Analysis of the pK_a value of Phesor in the absence or presence of 1 mM L-phenylalanine. **(g-i)** Fluorescence responses of Phesor to increasing concentrations of L-phenylalanine at varying biosensor concentrations (0.5, 1, and 3 μM). The data show the ratiometric readout ($F_{488 \text{ nm}}/F_{405 \text{ nm}}$) **(g)**, alongside the corresponding single-channel fluorescence intensities, $F_{488 \text{ nm}}$ **(h)** and $F_{405 \text{ nm}}$ **(i)**. **(j)** Specificity analysis of Phesor. The fluorescence ratios of Phesor were determined in the presence of 10 mM indicated metabolites (except L-tyrosine, which was determined at a concentration of 1 mM due to solubility limitations). PPA, phenylpyruvate; 2-PA, 2-phenylacetamide; *N*-ac-L-Phe, *N*-acetyl-L-phenylalanine; D-PLA, D-phenyllactate. **(k)** Kinetic analysis of the fluorescence response of Phesor to L-phenylalanine addition. Indicated concentrations of L-phenylalanine were added at time point zero. **(l)** Kinetic analysis of the fluorescence response of Phesor to L-phenylalanine depletion. Indicated concentrations of L-phenylalanine were added prior to testing, and 25 mg mL⁻¹ aromatic aldehyde synthase from *Petroselinum crispum* (AAS) together with 50 μM pyridoxal 5'-phosphate (PLP) were added at time point zero. **(m)** pH-stability analysis of Phesor. Dose-response curves of Phesor for increasing concentrations of L-phenylalanine were determined at the indicated pH values. **(n)** pH-correction of the fluorescence ratio of Phesor by cpSFYFP. Data were normalized to the ratio at pH 7.4. All data shown are means $\pm$ s.d. ($n = 3$ independent experiments for **c-e, g-l**,

n; n = 4 independent experiments for **f**; n = 3 independent experiments for the pH 6.6, 7.0, 7.4, 7.8, and 8.2 groups in **m**; n = 4 independent experiments for the pH 5.0, 5.5, 6.0, 8.6, 9.0, 9.5, and 10.0 groups in **m**). Source data are provided as a Source Data file.

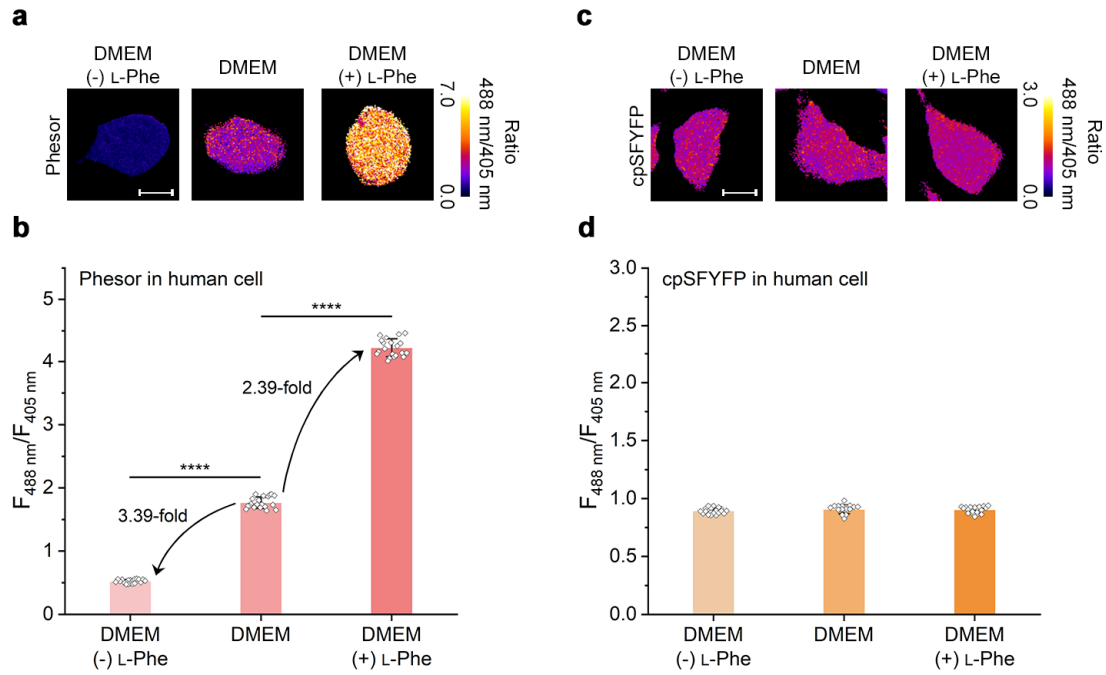


Supplementary Figure 6 Characterization of the control biosensor cpSFYFP for L-phenylalanine detection. **(a)** Dose-response curve of cpSFYFP for increasing concentrations of L-phenylalanine. **(b)** Time courses of the fluorescence ratio changes of cpSFYFP expressed in permeabilized *E. coli* BL21(DE3) in response to exogenous 500 μ M L-phenylalanine, L-tyrosine, or L-tryptophan. **(c)** Fluorescence ratios of *E. coli* BL21(DE3) WT and *E. coli* BL21(DE3) $7\Delta\text{-}\Delta\text{tyrR}\Delta\text{pheRS}::\text{pheRS}^{\text{A294S}}$ expressing cpSFYFP before carbon starvation (I), 8 h after carbon starvation (II), and 10 min and 20 min after glucose supplementation (III). **(d, e)** Sequential ratiometric images **(d)** and fluorescence ratios **(e)** of permeabilized HEK293FT cells expressing cpSFYFP before and after the addition of 5 mM L-phenylalanine, L-tyrosine, or L-tryptophan. Scale bar, 10 μ m. The micrographs are representative of three experiments that show similar results. Data shown in panel **a-c** are means $\pm$ s.d. ($n = 4$ independent experiments for **a**; $n = 3$ independent experiments for **b, c**).

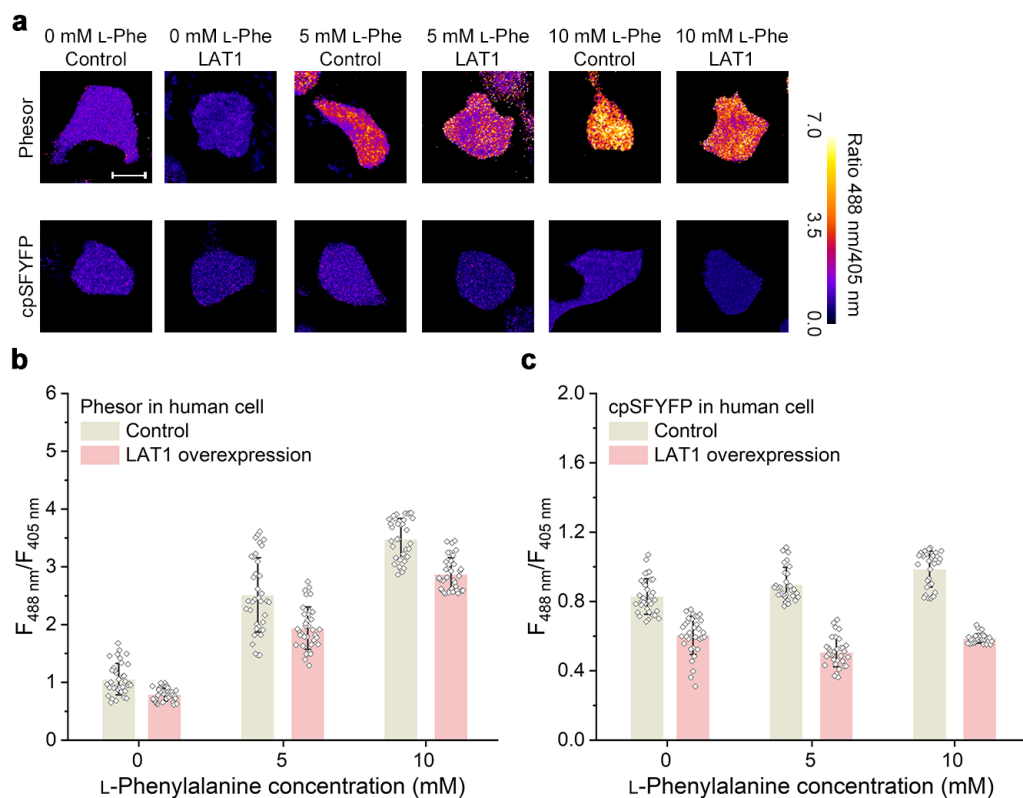
Source data are provided as a Source Data file.



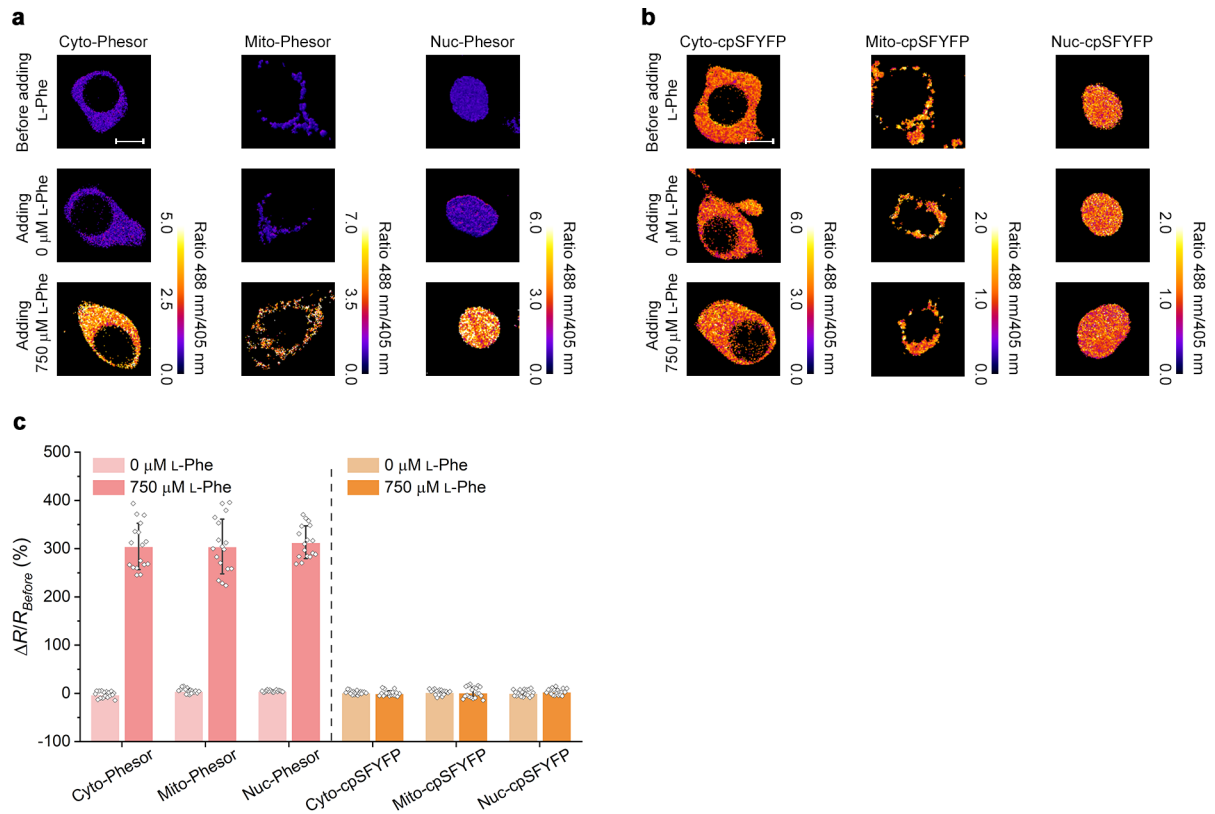
Supplementary Figure 8 Characterization of Phesor in HEK293FT cells. **(a)** Ratiometric images of single HEK293FT cells expressing Phesor in response to exogenous increasing concentrations of L-phenylalanine addition. Scale bar, 10 μ m. The micrographs are representative of three experiments that show similar results. **(b)** Dose-response curve of Phesor expressed in HEK293FT cells for increasing concentrations of L-phenylalanine. The HEK293FT cells were permeabilized with 80 μ M digitonin. All data shown are means $\pm$ s.d. (n = 25 cells). Source data are provided as a Source Data file.



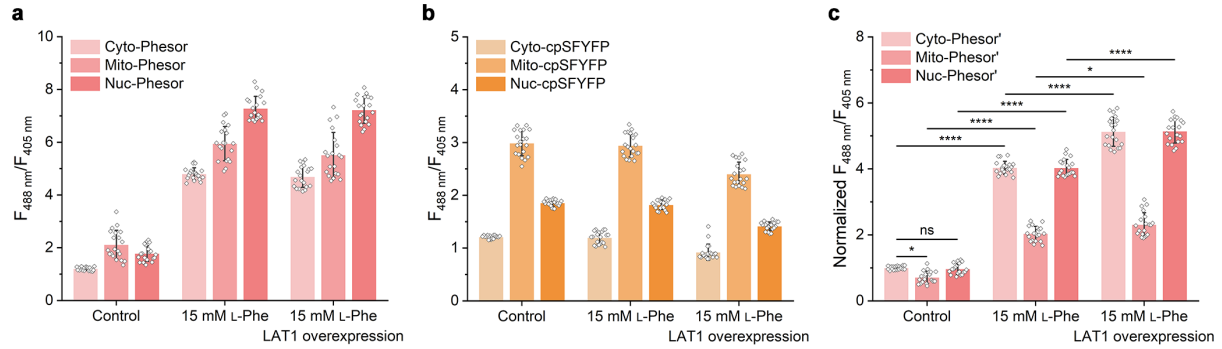
Supplementary Figure 9 Fluorescence responses of Phesor in HEK293FT cells to extreme L-phenylalanine metabolic perturbations. **(a, b)** Ratiometric images **(a)** and fluorescence ratios **(b)** of HEK293FT cells expressing Phesor cultured in L-phenylalanine-free DMEM, standard DMEM, and DMEM supplemented with 10 mM L-phenylalanine. Scale bar, 10 μm . The micrographs are representative of three experiments that show similar results. **(c, d)** Ratiometric images **(c)** and fluorescence ratios **(d)** of HEK293FT cells expressing cpSFYFP cultured in L-phenylalanine-free DMEM, standard DMEM, and DMEM supplemented with 10 mM L-phenylalanine. Scale bar, 10 μm . The micrographs are representative of three experiments that show similar results. All data shown are means $\pm$ s.d. ($n = 20$ cells). ****, $P < 0.0001$; one-way ANOVA with Tukey's multiple comparisons test. Exact P values are reported in the Source Data file. Source data are provided as a Source Data file.



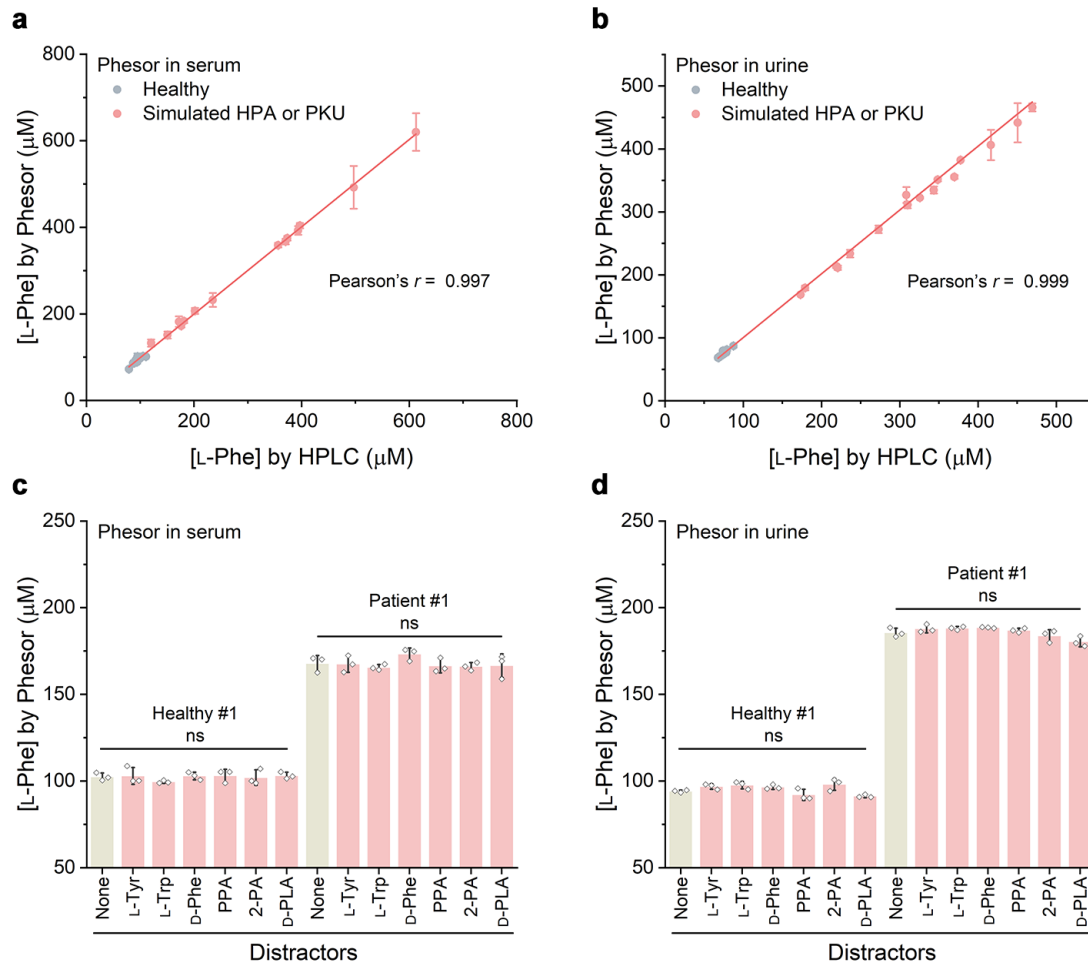
Supplementary Figure 10 Analysis of the function of LAT1 in the uptake of L-phenylalanine in HEK293FT cells using Phesor. **(a)** Ratiometric images of single HEK293FT cells expressing Phesor or cpSFYFP in response to L-phenylalanine addition and LAT1 overexpression. Cells were treated with 0, 5, or 10 mM L-phenylalanine with or without LAT1 overexpression, and the fluorescence ratios were determined after 20 h. Scale bar, 10 μ m. The micrographs are representative of three experiments that show similar results. **(b, c)** Fluorescence ratios of HEK293FT cells expressing Phesor **(b)** or cpSFYFP **(c)** in response to L-phenylalanine addition and LAT1 overexpression. LAT1 overexpression may lead to potential intracellular pH fluctuations, which affected the fluorescence ratio of cpSFYFP. cpSFYFP-corrected fluorescence ratios of Phesor were shown in Fig. 2i. All data shown are means $\pm$ s.d. (n = 36 cells for **b**; n = 33 cells for **c**). Source data are provided as a Source Data file.



Supplementary Figure 11 Characterization of different subcellularly localized Phesor in HEK293FT cells. **(a, b)** Ratiometric images of single HEK293FT cells expressing Cyto-Phesor, Mito-Phesor, and Nuc-Phesor **(a)**, or Cyto-cpSFYFP, Mito-cpSFYFP, and Nuc-cpSFYFP **(b)** before and after the addition of 0 or 750 μ M L-phenylalanine. Scale bar, 10 μ m. The micrographs are representative of three experiments that show similar results. **(c)** Fluorescence responses of different subcellularly localized Phesor or cpSFYFP to exogenous 750 μ M L-phenylalanine addition. The HEK293FT cells were permeabilized with 80 μ M digitonin. Data shown in panel **c** are means $\pm$ s.d. ($n = 17$ cells for different subcellularly localized Phesor; $n = 19$ cells for different subcellularly localized cpSFYFP). Source data are provided as a Source Data file.

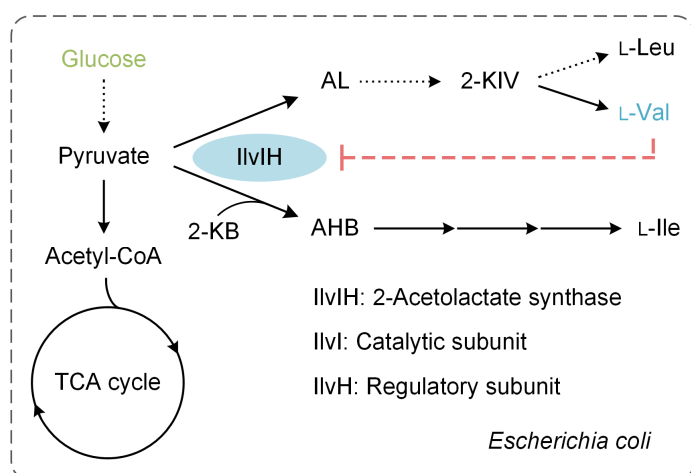


Supplementary Figure 12 Analysis of subcellular distribution of L-phenylalanine using different subcellularly localized Phesor. **(a, b)** Fluorescence ratios of HEK293FT cells expressing different subcellularly localized Phesor **(a)** or cpSFYFP **(b)** in response to 15 mM L-phenylalanine addition and LAT1 overexpression. Cells were treated with 0 or 15 mM L-phenylalanine with or without LAT1 overexpression, and the fluorescence ratios were determined after 20 h. The pH values varied between different subcellular compartments, which affected the fluorescence ratio of cpSFYFP. The fluorescence ratios of different subcellularly localized Phesor required correction using cpSFYFP. **(c)** cpSFYFP-corrected fluorescence ratios of HEK293FT cells expressing different subcellularly localized Phesor. All data shown are means $\pm$ s.d. ($n = 20$ cells). *, $P < 0.05$; ****, $P < 0.0001$; ns, no significant difference ($P \geq 0.05$); one-way ANOVA with Tukey's multiple comparisons test. Exact P values are reported in the Source Data file. Source data are provided as a Source Data file.

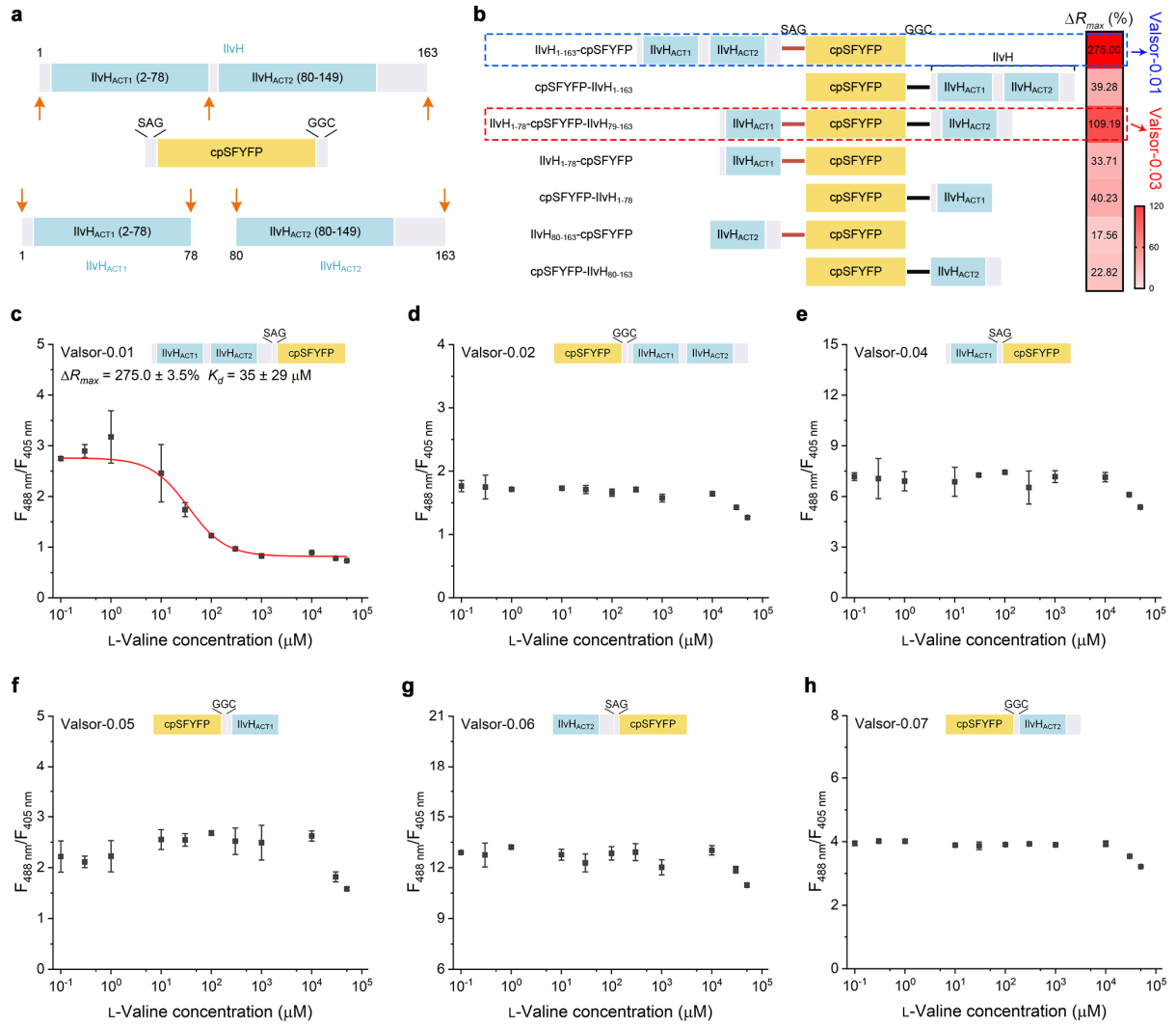


Supplementary Figure 13 Analysis of the accuracy and anti-interference ability of Phesor for the quantitative detection of L-phenylalanine in serum and urine. **(a, b)** Correlation analysis between Phesor and HPLC for quantitative detection of L-phenylalanine in serum **(a)** and urine **(b)** samples from healthy adults and simulated patients with hyperphenylalaninemia (HPA) or phenylketonuria (PKU). Quantitative results measured by Phesor were plotted against those obtained by HPLC. r , correlation coefficient. **(c, d)** Comparison of the quantitative results of Phesor for L-phenylalanine in serum **(c)** and urine **(d)** from healthy individuals and simulated patients with phenylketonuria in the absence or presence of the indicated L-phenylalanine analogs. The concentration of distractors used was set at 1 mM depending on their physiological and pathological concentrations in serum and urine. All data

shown are means $\pm$ s.d. ($n = 14$ healthy individuals and $n = 14$ simulated patients for **a**; $n = 9$ healthy individuals and $n = 16$ simulated patients for **b**; $n = 3$ independent experiments for **c**-**d**). ns, no significant difference ($P \geq 0.05$); one-way ANOVA with Tukey's multiple comparisons test. Exact P values are reported in the Source Data file. Source data are provided as a Source Data file.



Supplementary Figure 14 Schematic representation of the biosynthetic pathway of L-valine in *E. coli*. AL, 2-acetolactate; 2-KIV, 2-ketoisovalerate; 2-KB, 2-ketobutyrate; AHB, 2-aceto-2-hydroxybutyrate.

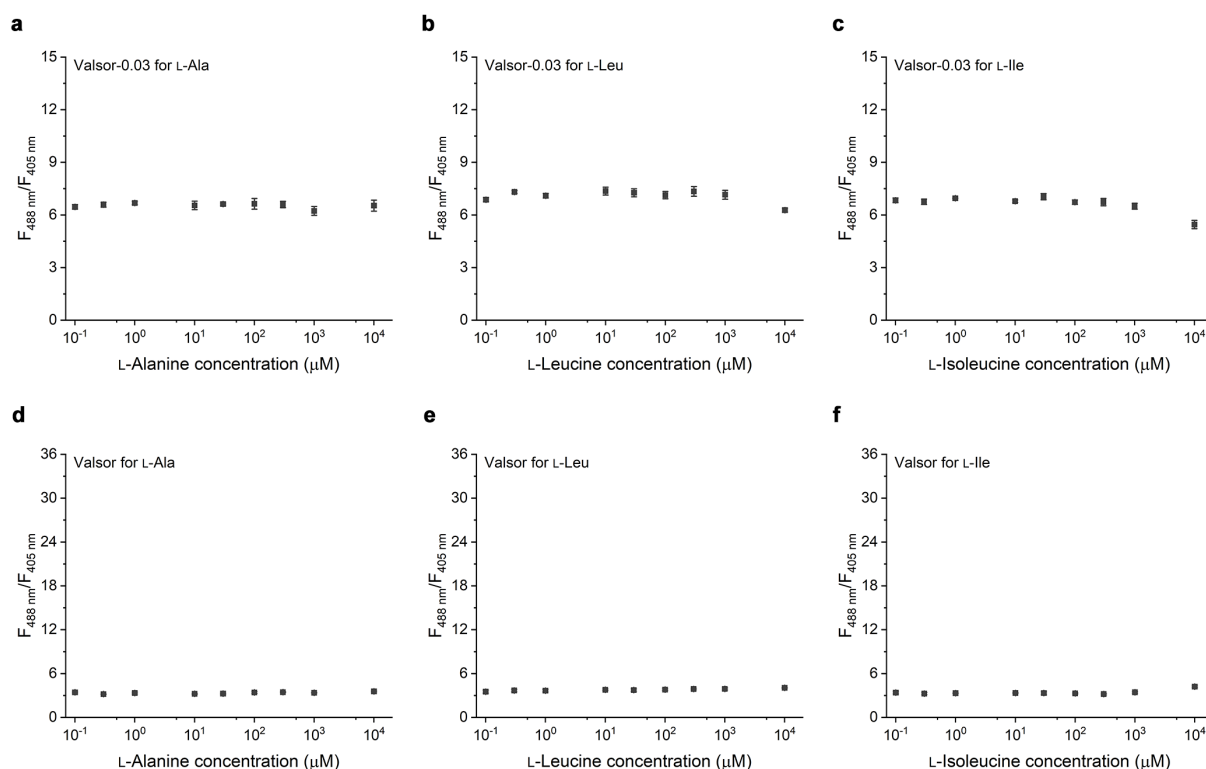


Supplementary Figure 16 Construction of Valsor-0.03 by insertion site screening. **(a)**

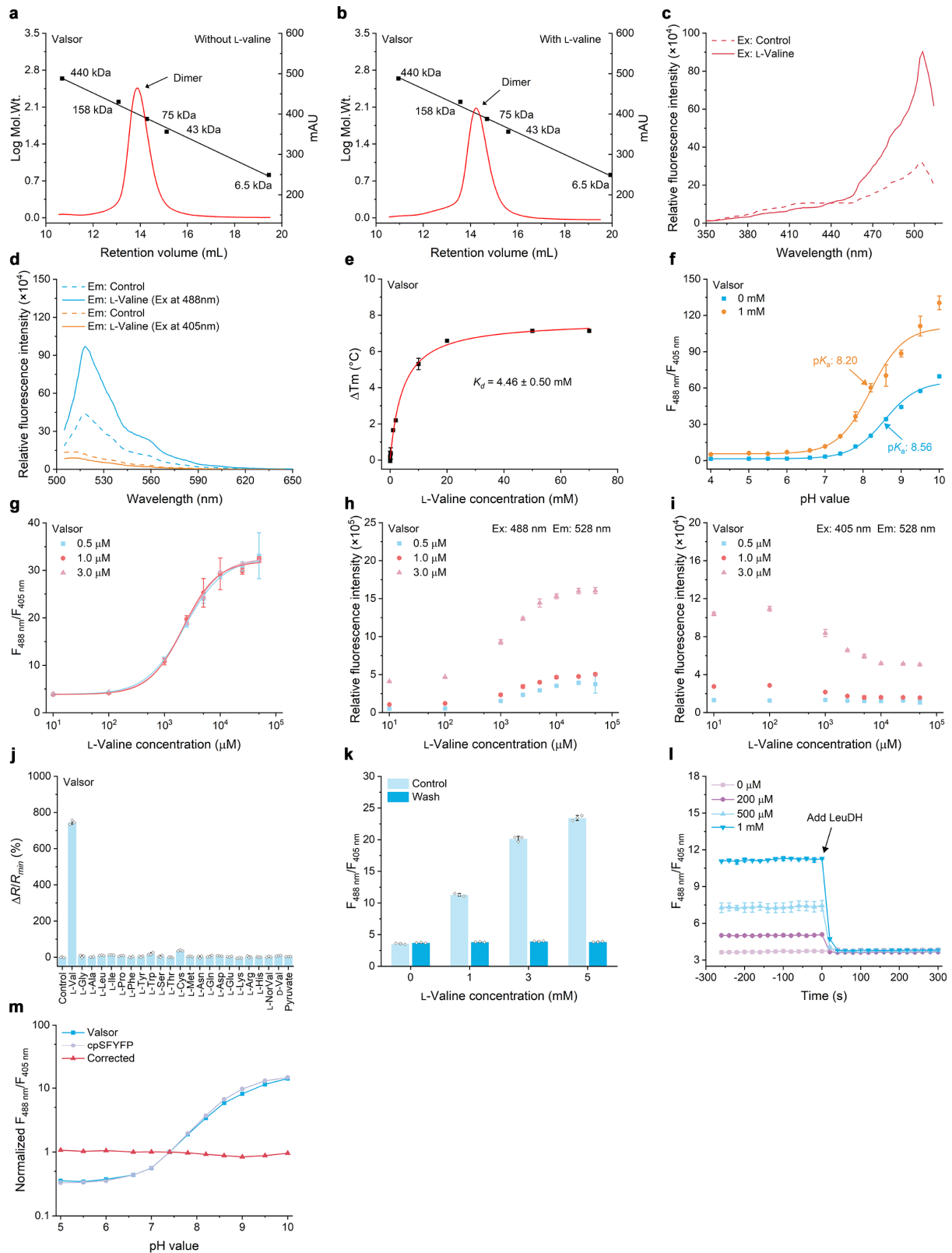
Schematic representation of the construction of the initial seven L-valine biosensor insertion variants. **(b)** Schematic representation of the construction of the L-valine biosensor Valsor-0.03 based on insertion site screening. The ΔR_{max} of different biosensor variants in response to L-valine were presented in a heatmap, where the color depth represented the magnitude of ΔR_{max} (%). The structure of Valsor-0.03 was shown in the red dotted box, and the structure of another L-valine biosensor insertion variant, Valsor-0.01, was shown in the blue dotted box. **(c-h)** Dose-response curves of Valsor-0.01 (c), Valsor-0.02 (d), Valsor-0.04 (e), Valsor-0.05 (f), Valsor-0.06 (g), and Valsor-0.07 (h) for increasing concentrations of L-valine. The dose-

response curve of Valsor-0.03 for increasing concentrations of L-valine was indicated in Fig.

3b. All data shown are means $\pm$ s.d. (n = 3 independent experiments). Source data are provided as a Source Data file.

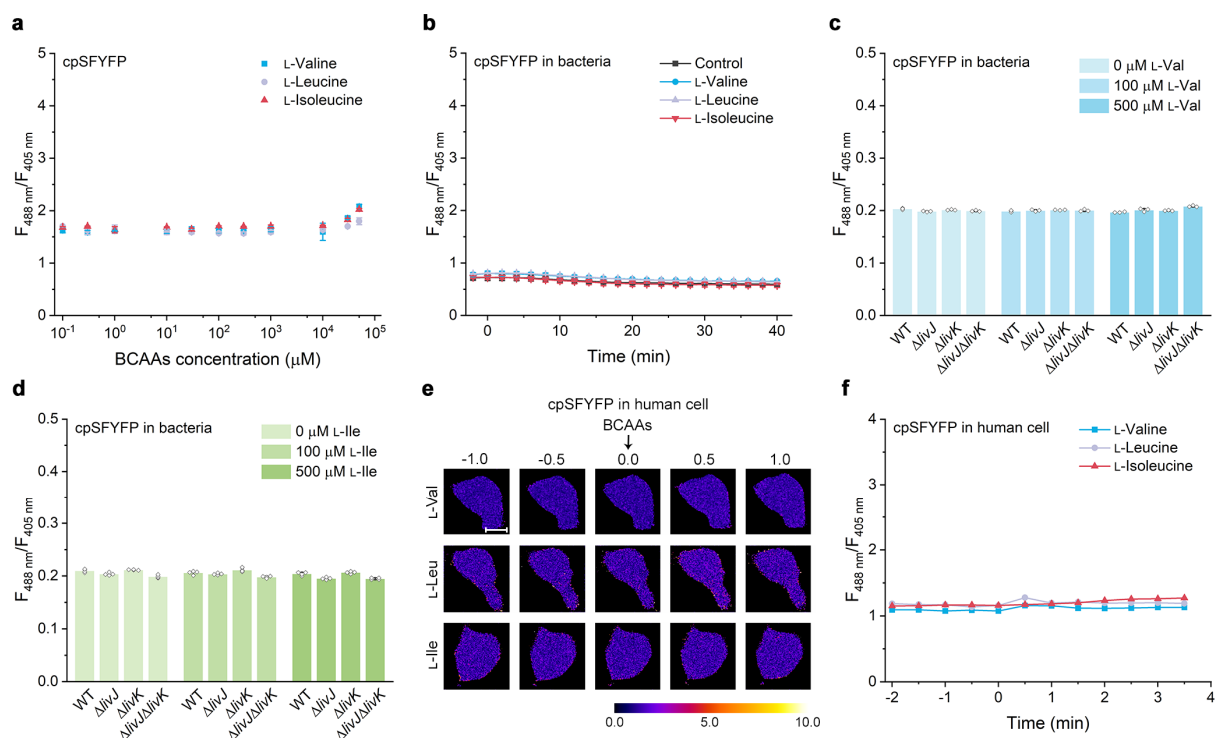


Supplementary Figure 17 Specificity analysis of different L-valine biosensors. **(a-c)** Dose-response curves of Valsor-0.03 for increasing concentrations of L-alanine **(a)**, L-leucine **(b)**, and L-isoleucine **(c)**. **(d-f)** Dose-response curves of Valsor for increasing concentrations of L-alanine **(d)**, L-leucine **(e)**, and L-isoleucine **(f)**. All data shown are means $\pm$ s.d. ($n = 3$ independent experiments). Source data are provided as a Source Data file.



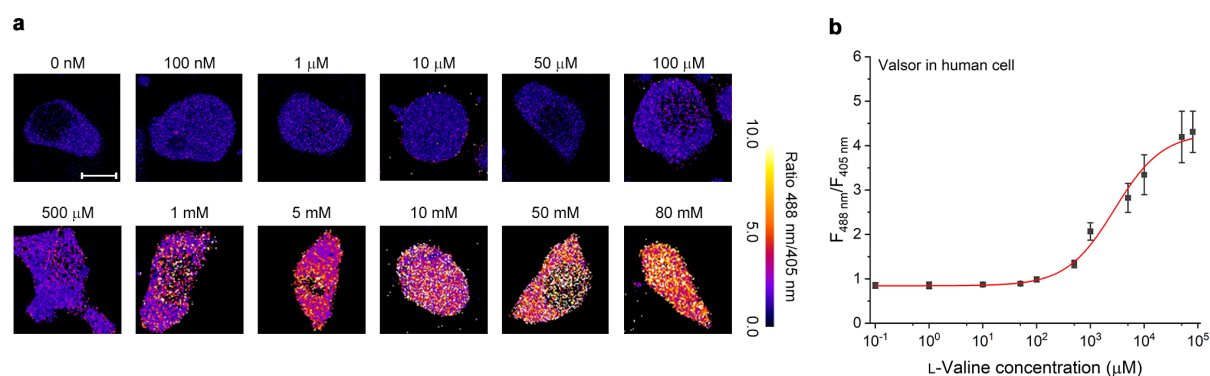
Supplementary Figure 18 Characterization of Valsor *in vitro*. **(a, b)** Analysis of the oligomeric state of Valsor in the absence **(a)** or presence **(b)** of 1 mM L-valine by gel

filtration chromatography. Red curve, chromatogram of purified Valsor; black line, standard curve for protein molecular mass standards. **(c, d)** Fluorescence excitation **(c)** and emission **(d)** spectra of Valsor in the absence or presence of 1 mM L-valine. **(e)** Analysis of the binding properties of Valsor to L-valine by TSA. The ΔT_m values indicated the changes in T_m values compared to the control (with 0 mM L-valine). **(f)** Analysis of the pK_a value of Valsor in the absence or presence of 1 mM L-valine. **(g-i)** Fluorescence responses of Valsor to increasing concentrations of L-valine at varying biosensor concentrations (0.5, 1, and 3 μ M). The data show the ratiometric readout ($F_{488\text{ nm}}/F_{405\text{ nm}}$) **(g)**, alongside the corresponding single-channel fluorescence intensities, $F_{488\text{ nm}}$ **(h)** and $F_{405\text{ nm}}$ **(i)**. **(j)** Specificity analysis of Valsor. The fluorescence ratios of Valsor were determined in the presence of 10 mM indicated metabolites (except L-tyrosine, which was determined at a concentration of 1 mM due to solubility limitations). **(k)** Reversibility analysis of Valsor. The fluorescence ratios of Valsor after L-valine addition and subsequent removal were recorded. **(l)** Kinetic analysis of the fluorescence response of Valsor to L-valine depletion. Indicated concentrations of L-valine were added prior to testing, and 30 mg mL⁻¹ L-leucine dehydrogenase from *Bacillus subtilis* 168 (LeuDH) together with 3 mM NAD⁺ were added at time point zero. **(m)** pH-correction of the fluorescence ratio of Valsor by cpSFYFP. Data were normalized to the ratio at pH 7.4. All data shown are means $\pm$ s.d. (n = 3 independent experiments for **c-e, g-m**; n = 4 independent experiments for **f**). Source data are provided as a Source Data file.

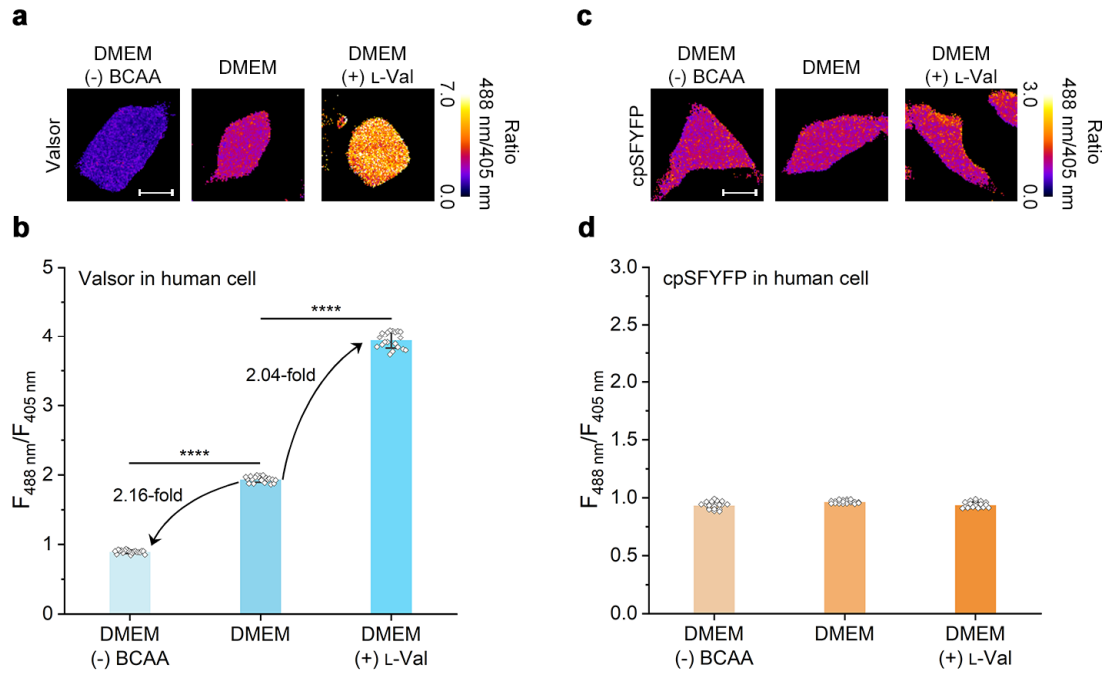


Supplementary Figure 19 Characterization of the control biosensor cpSFYFP for L-valine and L-isoleucine detection. **(a)** Dose-response curves of cpSFYFP for increasing concentrations of L-valine, L-leucine, and L-isoleucine. **(b)** Time courses of the fluorescence ratio changes of cpSFYFP expressed in permeabilized *E. coli* BL21(DE3) in response to exogenous 500 μ M L-valine, L-leucine, or L-isoleucine. **(c)** Fluorescence ratios of cpSFYFP expressed in *E. coli* BL21(DE3) WT, *E. coli* BL21(DE3) Δ livJ, *E. coli* BL21(DE3) Δ livK, and *E. coli* BL21(DE3) Δ livJ Δ livK in response to exogenous gradient concentrations of L-valine. **(d)** Fluorescence ratios of cpSFYFP expressed in *E. coli* BL21(DE3) WT, *E. coli* BL21(DE3) Δ livJ, *E. coli* BL21(DE3) Δ livK, and *E. coli* BL21(DE3) Δ livJ Δ livK in response to exogenous gradient concentrations of L-isoleucine. **(e, f)** Sequential ratiometric images **(e)** and fluorescence ratios **(f)** of permeabilized HEK293FT cells expressing cpSFYFP before and after the addition of 5 mM L-valine, L-leucine, or L-isoleucine. Scale bar, 10 μ m. The micrographs are representative of three experiments that show similar results. Data shown in

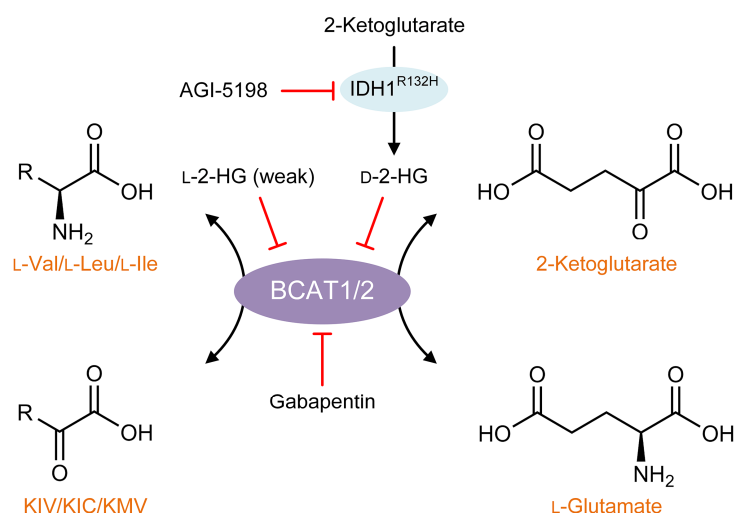
panel **a-d** are means $\pm$ s.d. (n = 3 independent experiments for **a-c**; n = 4 independent experiments for **d**). Source data are provided as a Source Data file.



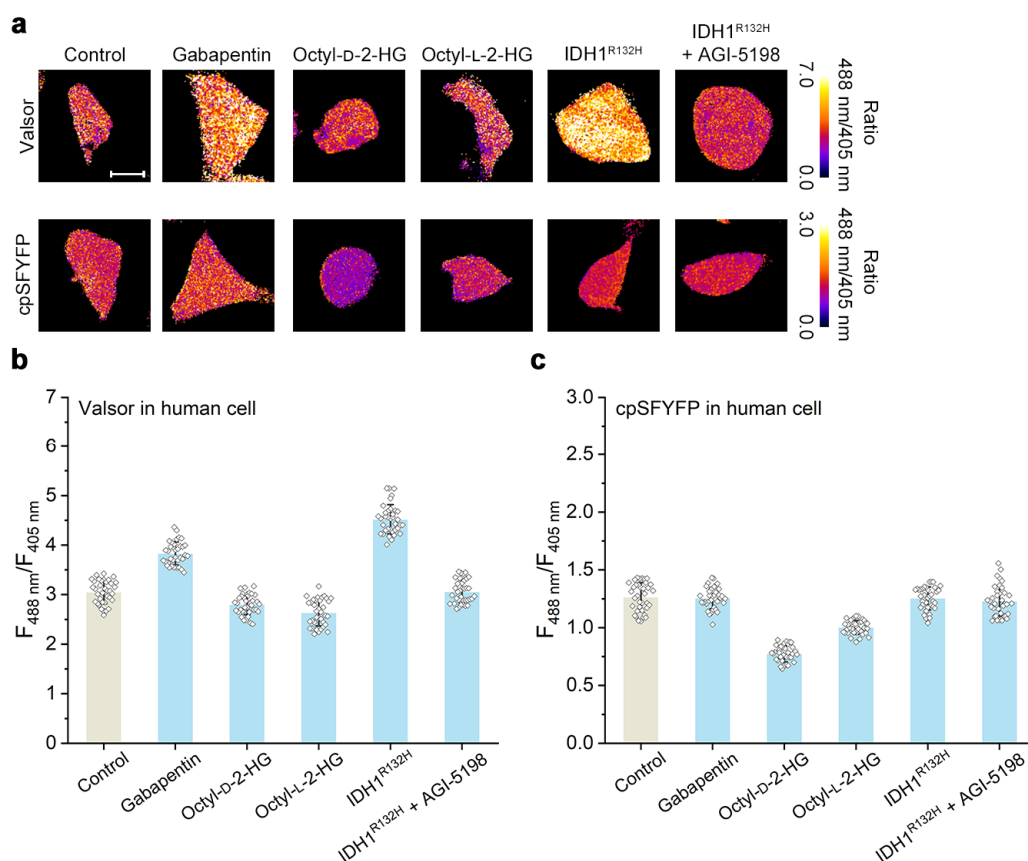
Supplementary Figure 20 Characterization of Valsor in HEK293FT cells. **(a)** Ratiometric images of single HEK293FT cells expressing Valsor in response to exogenous increasing concentrations of L-valine addition. Scale bar, 10 μ m. The micrographs are representative of three experiments that show similar results. **(b)** Dose-response curve of Valsor expressed in HEK293FT cells for increasing concentrations of L-valine. The HEK293FT cells were permeabilized with 80 μ M digitonin. All data shown are means $\pm$ s.d. ($n = 25$ cells). Source data are provided as a Source Data file.



Supplementary Figure 21 Fluorescence responses of Valsor in HEK293FT cells to extreme L-valine metabolic perturbations. **(a, b)** Ratiometric images **(a)** and fluorescence ratios **(b)** of HEK293FT cells expressing Valsor cultured in BCAA-free DMEM, standard DMEM, and DMEM supplemented with 10 mM L-valine. Scale bar, 10 μm . The micrographs are representative of three experiments that show similar results. **(c, d)** Ratiometric images **(c)** and fluorescence ratios **(d)** of HEK293FT cells expressing cpSFYFP cultured in BCAA-free DMEM, standard DMEM, and DMEM supplemented with 10 mM L-valine. Scale bar, 10 μm . The micrographs are representative of three experiments that show similar results. All data shown are means $\pm$ s.d. ($n = 20$ cells). ****, $P < 0.0001$; one-way ANOVA with Tukey's multiple comparisons test. Exact P values are reported in the Source Data file. Source data are provided as a Source Data file.

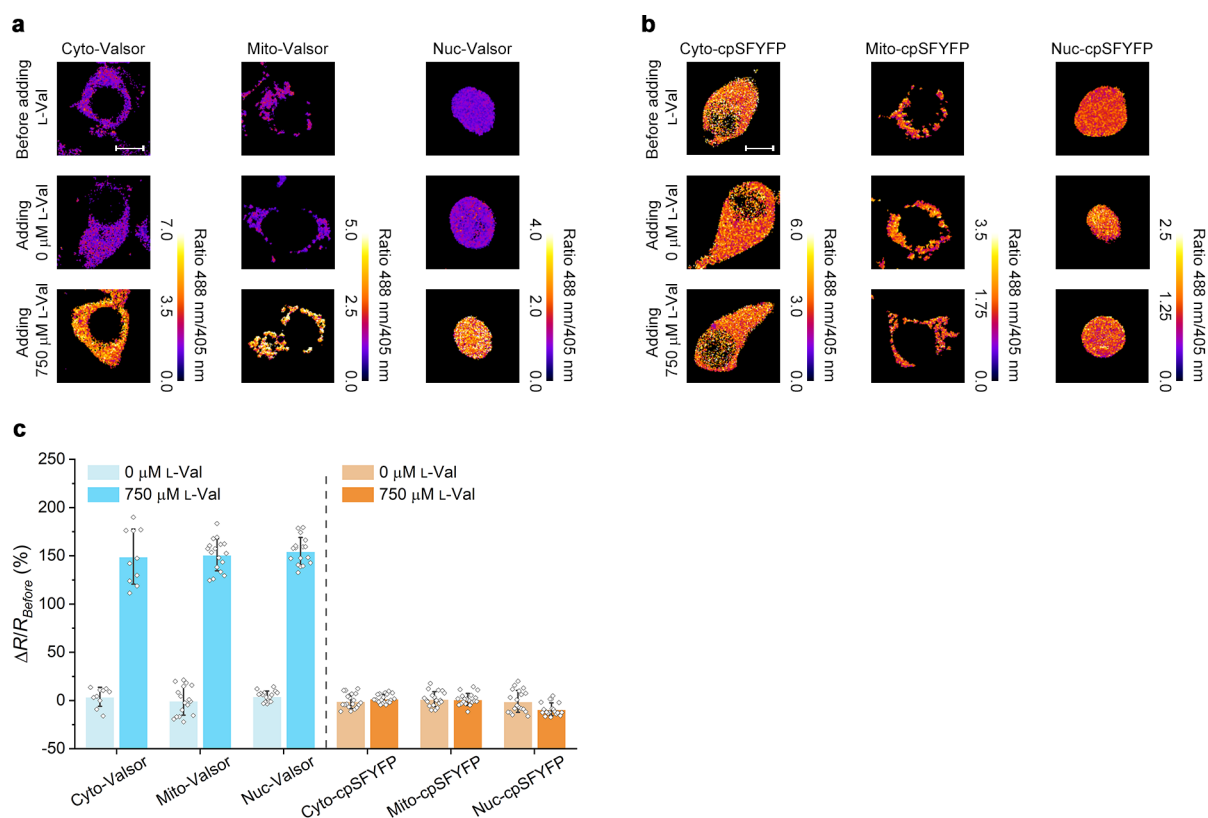


Supplementary Figure 22 Schematic representation of the metabolic pathways of the three branched-chain amino acids (BCAAs) in human cells. The activity of BCAA transaminases (BCAT1 and BCAT2) can be inhibited by gabapentin, L-2-HG (L-2-hydroxyglutarate), or D-2-HG (D-2-hydroxyglutarate). IDH1^{R132H}, a mutant isocitrate dehydrogenase 1 that causes supraphysiologic accumulation of endogenous D-2-HG, can be inhibited by AGI-5198. KIV, 2-ketoisovalerate; KIC, 2-ketoisocaproate; KMV, 2-ketomethylvalerate.



Supplementary Figure 23 Analysis of the effects of different BCAT inhibitors on intracellular L-valine levels in HEK293FT cells using Valsor. **(a)** Ratiometric images of single HEK293FT cells expressing Valsor or cpSFYFP in response to different inhibitors treatment. The concentrations of gabapentin, octyl-D-2-HG (octyl-D-2-hydroxyglutarate), octyl-L-2-HG (octyl-L-2-hydroxyglutarate), and AGI-5198 used were 2 mM, 1 mM, 1 mM, and 3 μ M, respectively. The fluorescence ratios were determined 20 h after treatment with different inhibitors. Scale bar, 10 μ m. The micrographs are representative of three experiments that show similar results. **(b, c)** Fluorescence ratios of HEK293FT cells expressing Valsor **(b)** or cpSFYFP **(c)** in response to different inhibitors treatment. Different inhibitor treatments (especially octyl-D-2-HG and octyl-L-2-HG) may lead to potential intracellular pH fluctuations, which affected the fluorescence ratio of cpSFYFP. cpSFYFP-

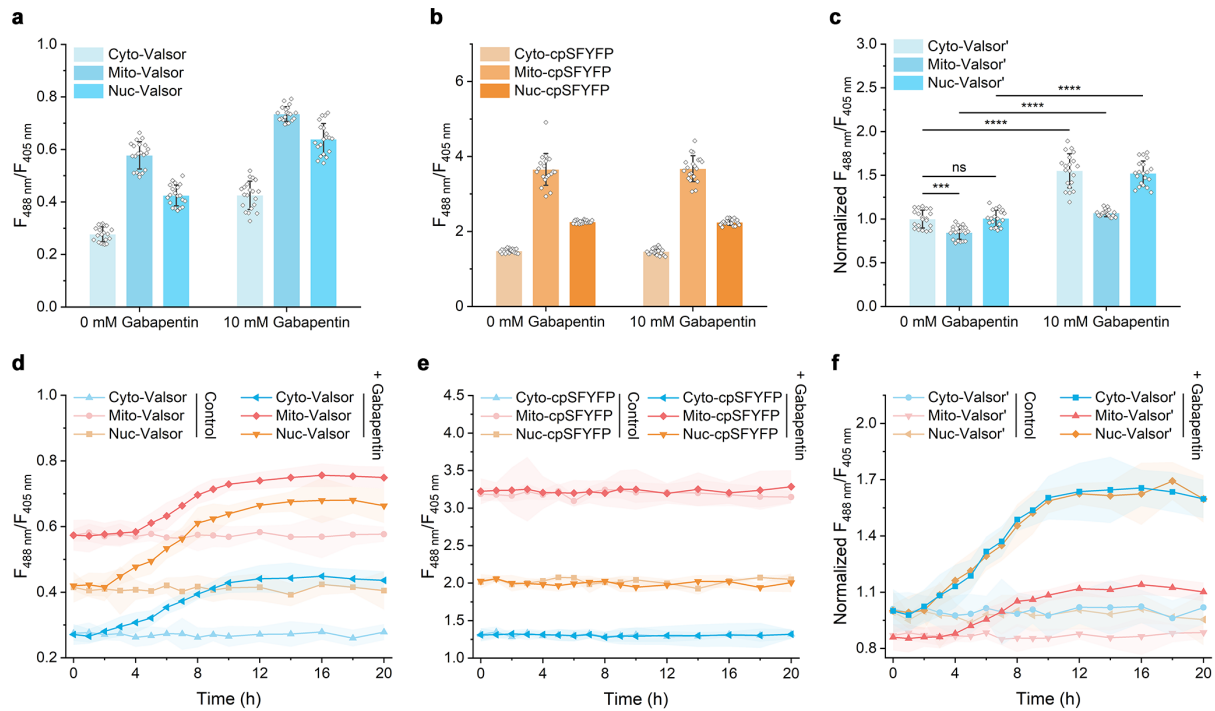
corrected fluorescence ratios of Valsor were shown in Fig. 3i. All data shown are means $\pm$ s.d. (n = 36 cells). Source data are provided as a Source Data file.



Supplementary Figure 24 Characterization of different subcellularly localized Valsor in HEK293FT cells. **(a, b)** Ratiometric images of single HEK293FT cells expressing Cyto-Valsor, Mito-Valsor, and Nuc-Valsor **(a)**, or Cyto-cpSFYFP, Mito-cpSFYFP, and Nuc-cpSFYFP **(b)** before and after the addition of 0 or 750 μ M L-valine. Scale bar, 10 μ m. The micrographs are representative of three experiments that show similar results. **(c)**

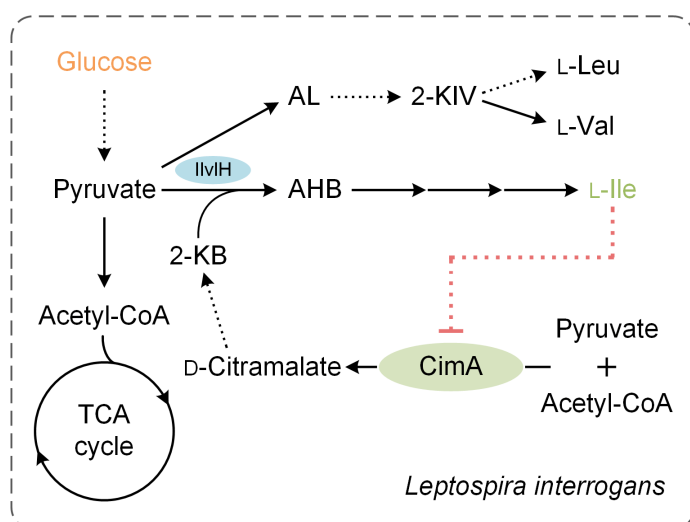
Fluorescence responses of different subcellularly localized Valsor or cpSFYFP to exogenous 750 μ M L-valine addition. The HEK293FT cells were permeabilized with 80 μ M digitonin.

Data shown in panel **c** are means $\pm$ s.d. (n = 10, 10, 17, 17, 17, 17, 19, 19, 19, 19, 19, 19 cells from left to right). Source data are provided as a Source Data file.



Supplementary Figure 25 Analysis of subcellular distribution of L-valine using different subcellularly localized Valsor. **(a, b)** Fluorescence ratios of HEK293FT cells expressing different subcellularly localized Valsor **(a)** or cpSFYFP **(b)** in response to 10 mM gabapentin addition. Cells were treated with 0 or 10 mM gabapentin, and the fluorescence ratios were determined after 20 h. The pH values varied between different subcellular compartments, which affected the fluorescence ratio of cpSFYFP. The fluorescence ratios of different subcellularly localized Valsor required correction using cpSFYFP. **(c)** cpSFYFP-corrected fluorescence ratios of HEK293FT cells expressing different subcellularly localized Valsor. **(d, e)** Time courses of the fluorescence ratio changes of HEK293FT cells expressing different subcellularly localized Valsor **(d)** or cpSFYFP **(e)** in response to 10 mM gabapentin addition. Cells were treated with 0 or 10 mM gabapentin, and the fluorescence ratios were determined at the indicated time points. **(f)** Time courses of the cpSFYFP-corrected fluorescence ratios of HEK293FT cells expressing different subcellularly localized Valsor. All data shown are

means $\pm$ s.d. (n = 20 cells for **a-c**; n = 14 cells for the gabapentin addition group in **d-f**; n = 10 cells for the control group in **d-f**). ***, $P < 0.001$; ****, $P < 0.0001$; ns, no significant difference ($P \geq 0.05$); one-way ANOVA with Tukey's multiple comparisons test. Exact P values are reported in the Source Data file. Source data are provided as a Source Data file.



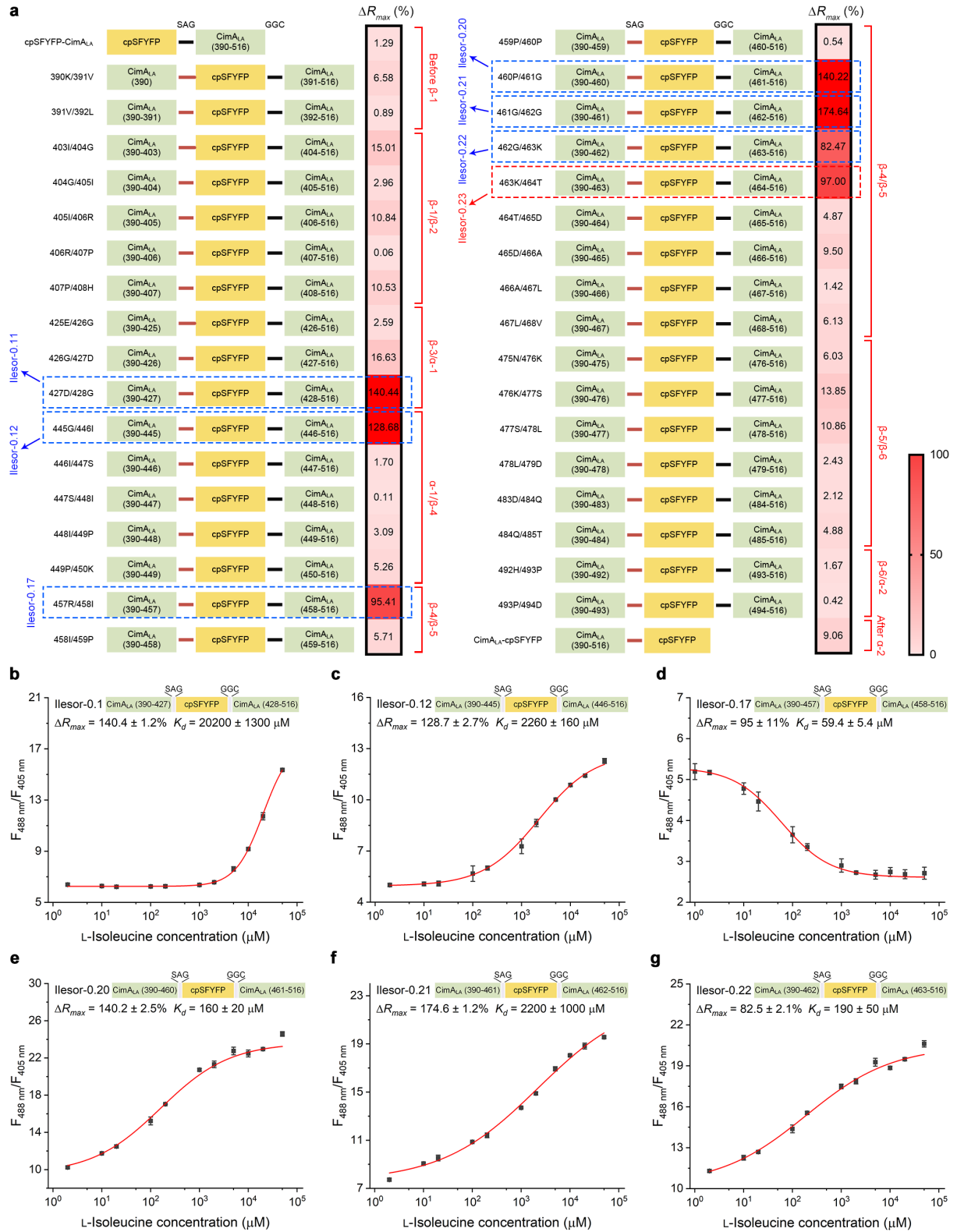
Supplementary Figure 26 Schematic representation of the biosynthetic pathway of L-isoleucine in *Leptospira interrogans*. CimA, D-citramalate synthase.



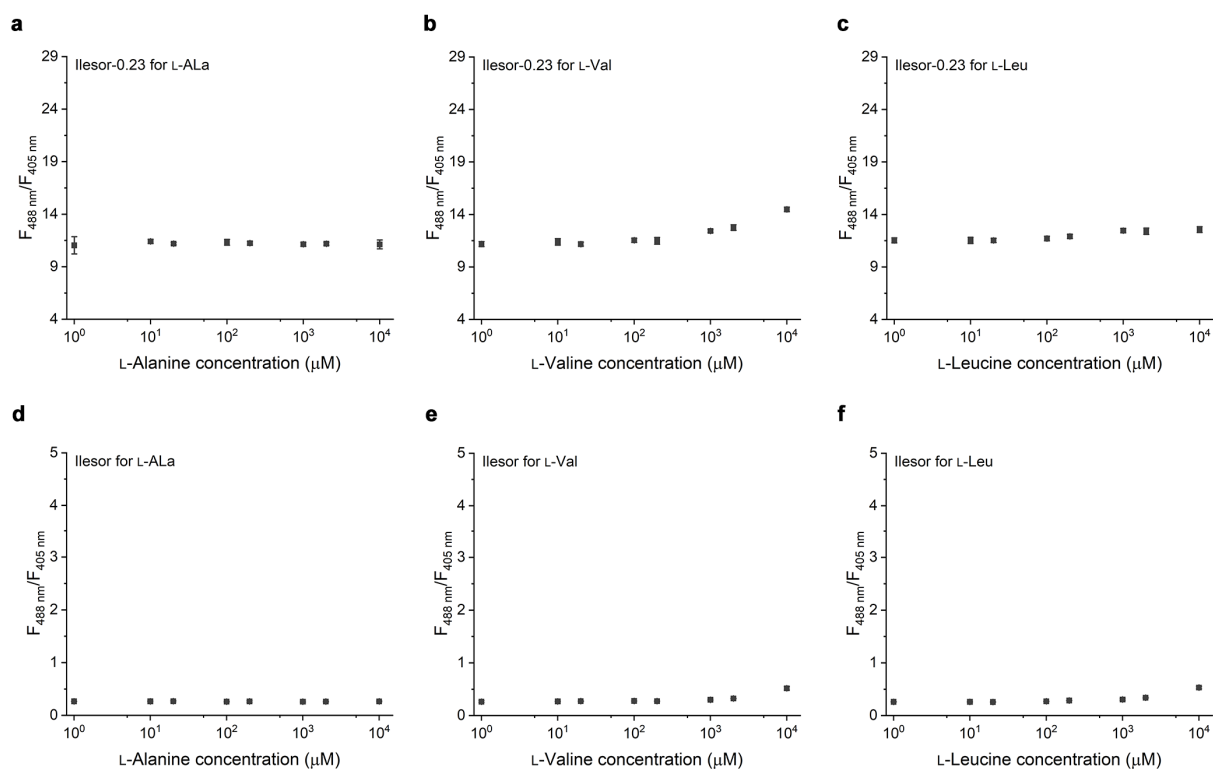
Supplementary Figure 27 Workflow for the development of L-isoleucine biosensor Ilesor.

(a) Schematic representations of the amino acid sequence and the predicted secondary structure of CimA. The catalytic (CimA_{CD}) domain and LeuA regulatory (CimA_{LA}) domain of CimA were shown in purple and green, respectively. The predicted α -helices and β -sheets of the CimA_{LA} domain were indicated with underlines and the corresponding designations were listed above the sequence. (b) Schematic representation of the construction and optimization of Ilesor. The final L-isoleucine biosensor, Ilesor, was constructed by inserting cpSFYFP between the 390K-463K and 464T-516I fragments of CimA, with an N-terminal linker of Phe-Arg-Thr and a C-terminal linker of Pro-His-Leu. (c) Sequence alignment of

CimA, Ilesor-0.23, and Ilesor. The Cim_{ALA} region, the cpSFYFP region, and the N- and C-terminal linkers were indicated.

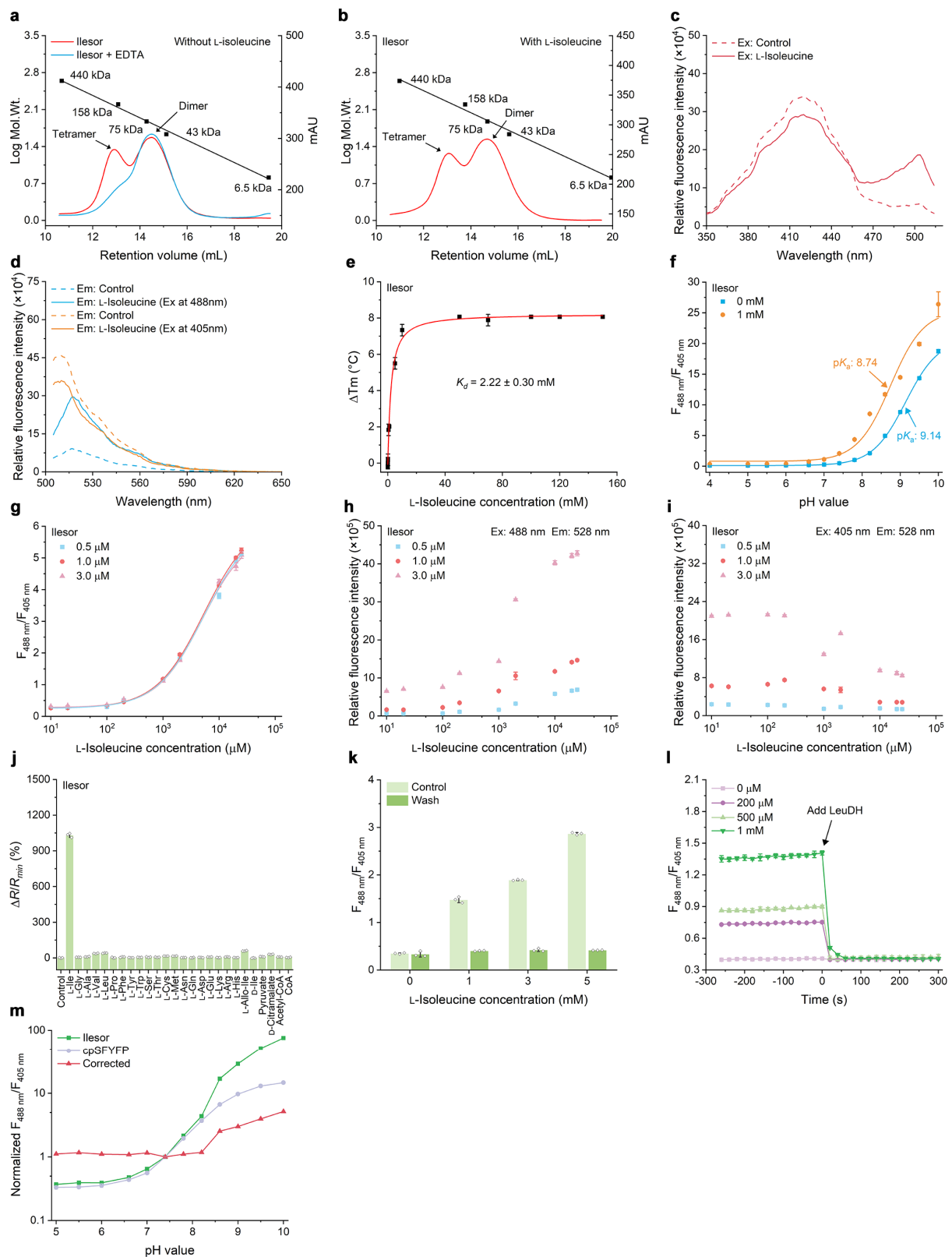


isoleucine were presented in a heatmap, where the color depth represented the magnitude of ΔR_{max} (%). The structure of Ilesor-0.23 was shown in the red dotted box, and the structures of other L-isoleucine biosensor insertion variants (including Ilesor-0.11, Ilesor-0.12, Ilesor-0.17, Ilesor-0.20, Ilesor-0.21, and Ilesor-0.22) were shown in the blue dotted boxes. **(b-g)** Dose-response curves of Ilesor-0.11 **(b)**, Ilesor-0.12 **(c)**, Ilesor-0.17 **(d)**, Ilesor-0.20 **(e)**, Ilesor-0.21 **(f)**, and Ilesor-0.22 **(g)** for increasing concentrations of L-isoleucine. The dose-response curve of Ilesor-0.23 for increasing concentrations of L-isoleucine was indicated in Fig. 4b. All data shown are means $\pm$ s.d. (n = 3 independent experiments). Source data are provided as a Source Data file.



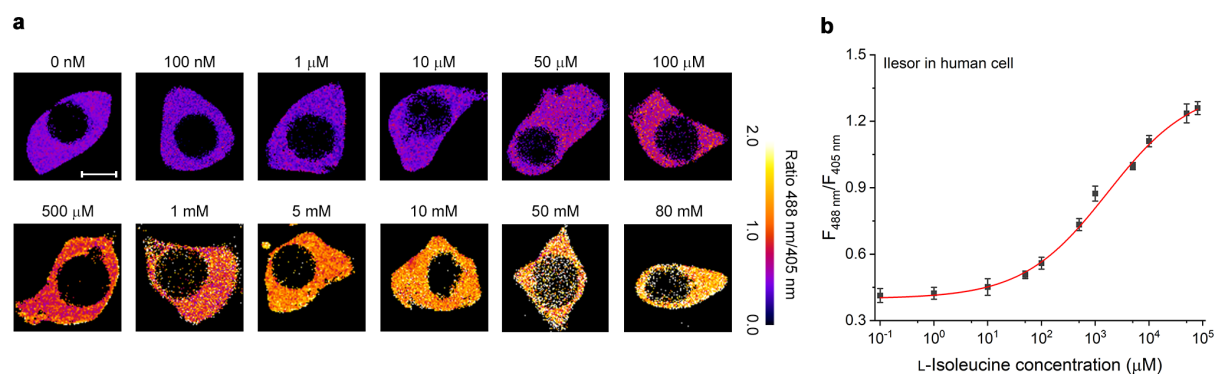
Supplementary Figure 29 Specificity analysis of different L-isoleucine biosensors. **(a-c)**

Dose-response curves of Ilesor-0.23 for increasing concentrations of L-alanine **(a)**, L-valine **(b)**, and L-leucine **(c)**. **(d-f)** Dose-response curves of Ilesor for increasing concentrations of L-alanine **(d)**, L-valine **(e)**, and L-leucine **(f)**. All data shown are means $\pm$ s.d. ($n = 4$ independent experiments). Source data are provided as a Source Data file.

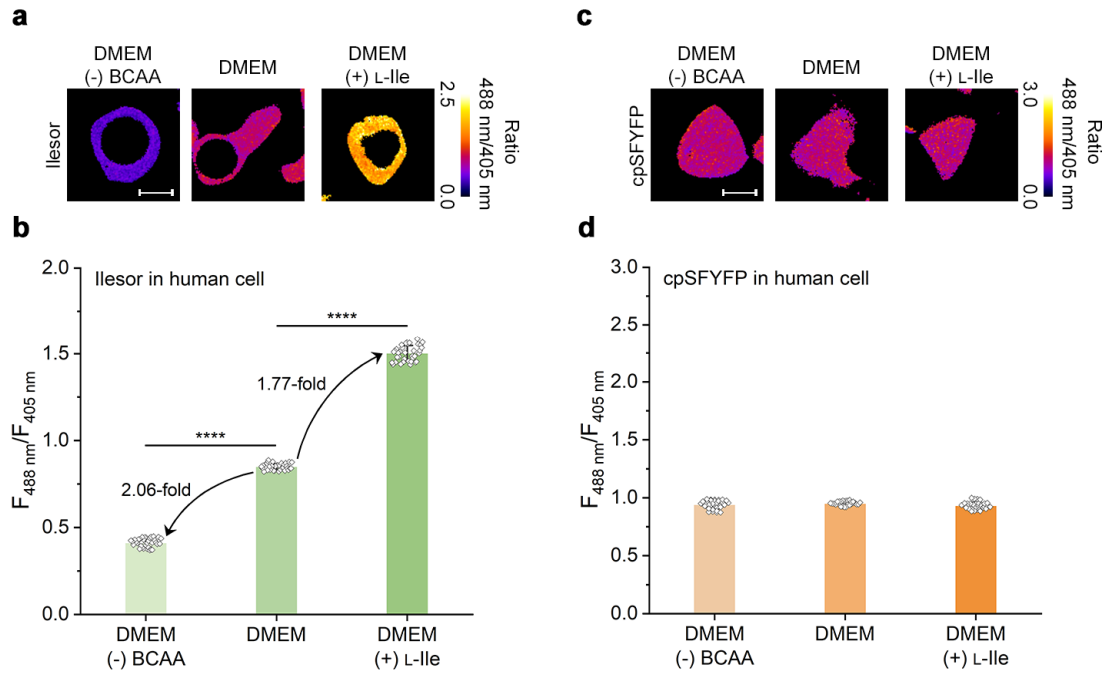


Supplementary Figure 30 Characterization of Ilesor *in vitro*. **(a, b)** Analysis of the oligomeric state of Ilesor in the absence **(a)** or presence **(b)** of 1 mM L-isoleucine by gel

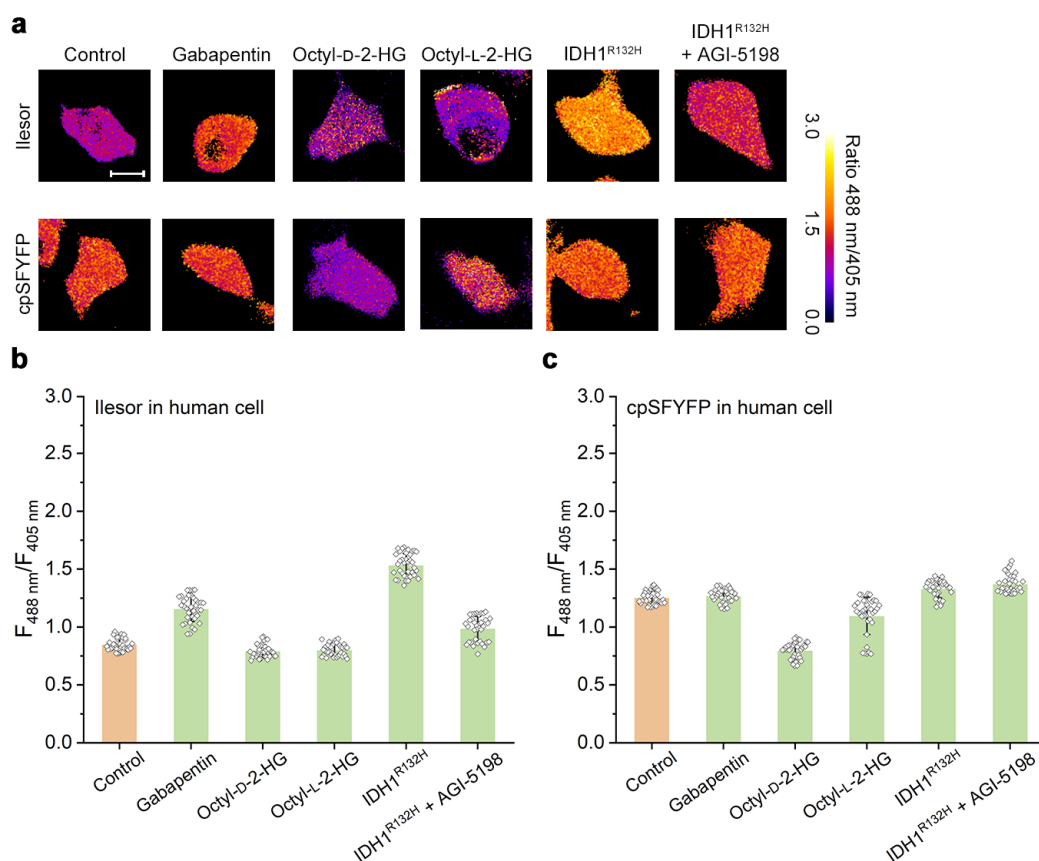
filtration chromatography. 10 mM EDTA was added to chelate Zn^{2+} ions at the side-by-side dimer interface of the Ilesor tetramer. Red curve, chromatogram of purified Ilesor; black line, standard curve for protein molecular mass standards. **(c, d)** Fluorescence excitation **(c)** and emission **(d)** spectra of Ilesor in the absence or presence of 1 mM L-isoleucine. **(e)** Analysis of the binding properties of Ilesor to L-isoleucine by TSA. The ΔT_m values indicated the changes in T_m values compared to the control (with 0 mM L-isoleucine). **(f)** Analysis of the pK_a value of Ilesor in the absence or presence of 1 mM L-isoleucine. **(g-i)** Fluorescence responses of Ilesor to increasing concentrations of L-isoleucine at varying biosensor concentrations (0.5, 1, and 3 μM). The data show the ratiometric readout ($F_{488\text{ nm}}/F_{405\text{ nm}}$) **(g)**, alongside the corresponding single-channel fluorescence intensities, $F_{488\text{ nm}}$ **(h)** and $F_{405\text{ nm}}$ **(i)**. **(j)** Specificity analysis of Ilesor. The fluorescence ratios of Ilesor were determined in the presence of 10 mM indicated metabolites (except L-tyrosine, which was determined at a concentration of 1 mM due to solubility limitations). **(k)** Reversibility analysis of Ilesor. The fluorescence ratios of Ilesor after L-isoleucine addition and subsequent removal were recorded. **(l)** Kinetic analysis of the fluorescence response of Ilesor to L-isoleucine depletion. Indicated concentrations of L-isoleucine were added prior to testing, and 30 mg mL^{-1} LeuDH together with 3 mM NAD^+ were added at time point zero. **(m)** pH-correction of the fluorescence ratio of Ilesor by cpSFYFP. Data were normalized to the ratio at pH 7.4. All data shown are means $\pm$ s.d. ($n = 3$ independent experiments for **c-e, g-m**; $n = 4$ independent experiments for **f**). Source data are provided as a Source Data file.



Supplementary Figure 31 Characterization of Ilesor in HEK293FT cells. **(a)** Ratiometric images of single HEK293FT cells expressing Ilesor in response to exogenous increasing concentrations of L-isoleucine addition. Scale bar, 10 μ m. The micrographs are representative of three experiments that show similar results. **(b)** Dose-response curve of Ilesor expressed in HEK293FT cells for increasing concentrations of L-isoleucine. The HEK293FT cells were permeabilized with 80 μ M digitonin. All data shown are means $\pm$ s.d. (n = 25 cells). Source data are provided as a Source Data file.

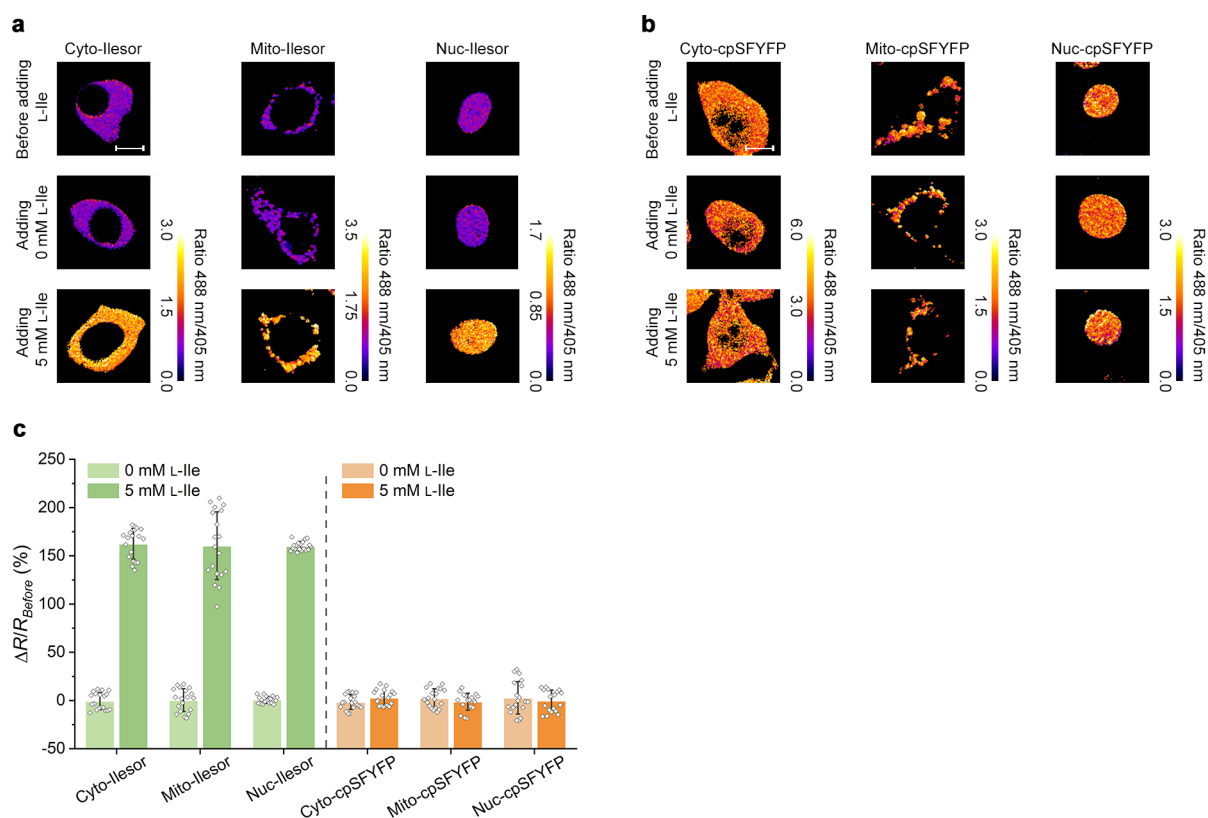


Supplementary Figure 32 Fluorescence responses of Ilesor in HEK293FT cells to extreme L-isoleucine metabolic perturbations. **(a, b)** Ratiometric images **(a)** and fluorescence ratios **(b)** of HEK293FT cells expressing Ilesor cultured in BCAA-free DMEM, standard DMEM, and DMEM supplemented with 10 mM L-isoleucine. Scale bar, 10 μm . The micrographs are representative of three experiments that show similar results. **(c, d)** Ratiometric images **(c)** and fluorescence ratios **(d)** of HEK293FT cells expressing cpSFYFP cultured in BCAA-free DMEM, standard DMEM, and DMEM supplemented with 10 mM L-isoleucine. Scale bar, 10 μm . The micrographs are representative of three experiments that show similar results. All data shown are means $\pm$ s.d. ($n = 36$ cells). ****, $P < 0.0001$; one-way ANOVA with Tukey's multiple comparisons test. Exact P values are reported in the Source Data file. Source data are provided as a Source Data file.



Supplementary Figure 33 Analysis of the effects of different BCAT inhibitors on intracellular L-isoleucine levels in HEK293FT cells using Ilesor. **(a)** Ratiometric images of single HEK293FT cells expressing Ilesor or cpSFYFP in response to different inhibitors treatment. The concentrations of gabapentin, octyl-D-2-HG, octyl-L-2-HG, and AGI-5198 used were 2 mM, 1 mM, 1 mM, and 3 μ M, respectively. The fluorescence ratios were determined 20 h after treatment with different inhibitors. Scale bar, 10 μ m. The micrographs are representative of three experiments that show similar results. **(b, c)** Fluorescence ratios of HEK293FT cells expressing Ilesor **(b)** or cpSFYFP **(c)** in response to different inhibitors treatment. Different inhibitor treatments (especially octyl-D-2-HG and octyl-L-2-HG) may lead to potential intracellular pH fluctuations, which affected the fluorescence ratio of cpSFYFP. cpSFYFP-corrected fluorescence ratios of Ilesor were shown in Fig. 4i. All data

shown are means $\pm$ s.d. (n = 36 cells). Source data are provided as a Source Data file.



Supplementary Figure 34 Characterization of different subcellularly localized Ilesor in

HEK293FT cells. **(a, b)** Ratiometric images of single HEK293FT cells expressing Cyto-

Ilesor, Mito-Ilesor, and Nuc-Ilesor **(a)**, or Cyto-cpSFYFP, Mito-cpSFYFP, and Nuc-

cpSFYFP **(b)** before and after the addition of 0 or 5 mM L-isoleucine. Scale bar, 10 μ m. The

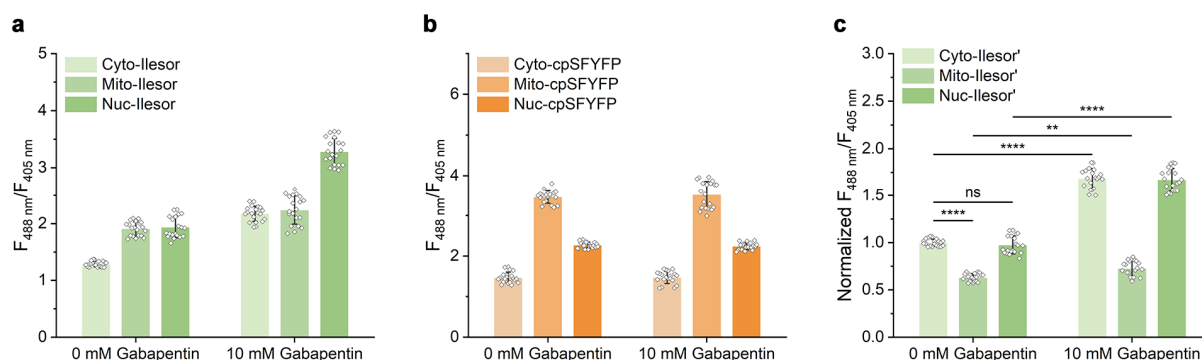
micrographs are representative of three experiments that show similar results. **(c)**

Fluorescence responses of different subcellularly localized Ilesor or cpSFYFP to exogenous 5

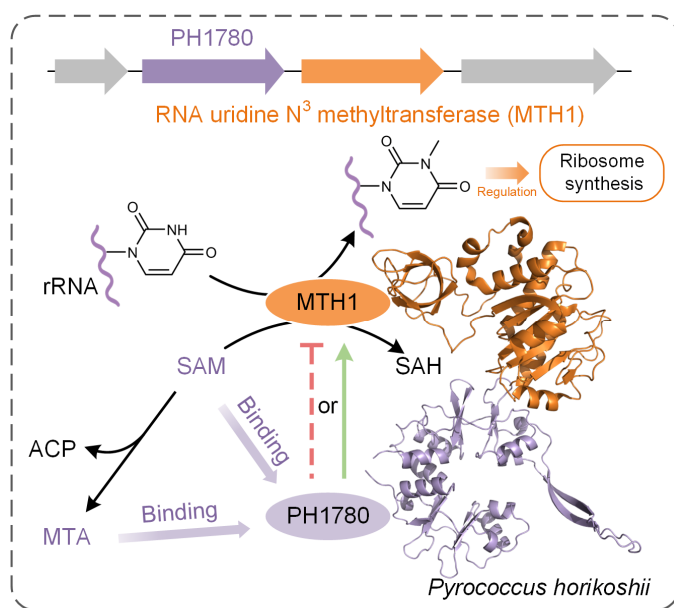
mM L-isoleucine addition. The HEK293FT cells were permeabilized with 80 μ M digitonin.

Data shown in panel **c** are means $\pm$ s.d. (n = 19 cells). Source data are provided as a Source

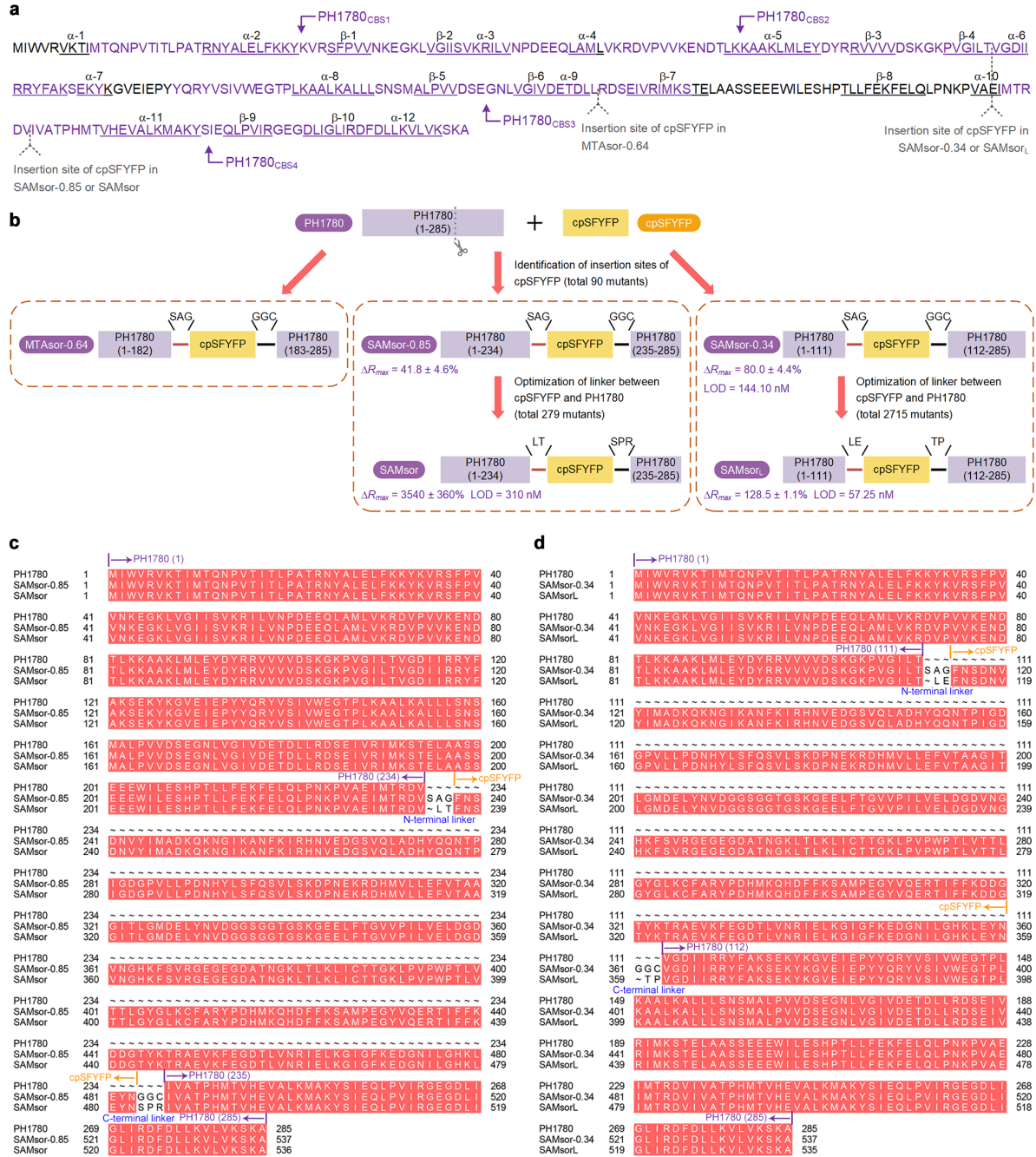
Data file.



Supplementary Figure 35 Analysis of subcellular distribution of L-isoleucine using different subcellularly localized Ilesor. **(a, b)** Fluorescence ratios of HEK293FT cells expressing different subcellularly localized Ilesor **(a)** or cpSFYFP **(b)** in response to 10 mM gabapentin addition. Cells were treated with 0 or 10 mM gabapentin, and the fluorescence ratios were determined after 20 h. The pH values varied between different subcellular compartments, which affected the fluorescence ratio of cpSFYFP. The fluorescence ratios of different subcellularly localized Ilesor required correction using cpSFYFP. **(c)** cpSFYFP-corrected fluorescence ratios of HEK293FT cells expressing different subcellularly localized Ilesor. All data shown are means $\pm$ s.d. ($n = 20$ cells). **, $P < 0.01$; ****, $P < 0.0001$; ns, no significant difference ($P \geq 0.05$); one-way ANOVA with Tukey's multiple comparisons test. Exact P values are reported in the Source Data file. Source data are provided as a Source Data file.



Supplementary Figure 36 Genomic organization and proposed function of PH1780 in *Pyrococcus horikoshii*. PH1780 may serve as a regulatory subunit of MTH1 that regulate MTH1 activity by binding to SAM and MTA. ACP, aminocarboxypropyl.



Supplementary Figure 37 Workflow for the development of SAM biosensors SAMsor and SAMsor_L.

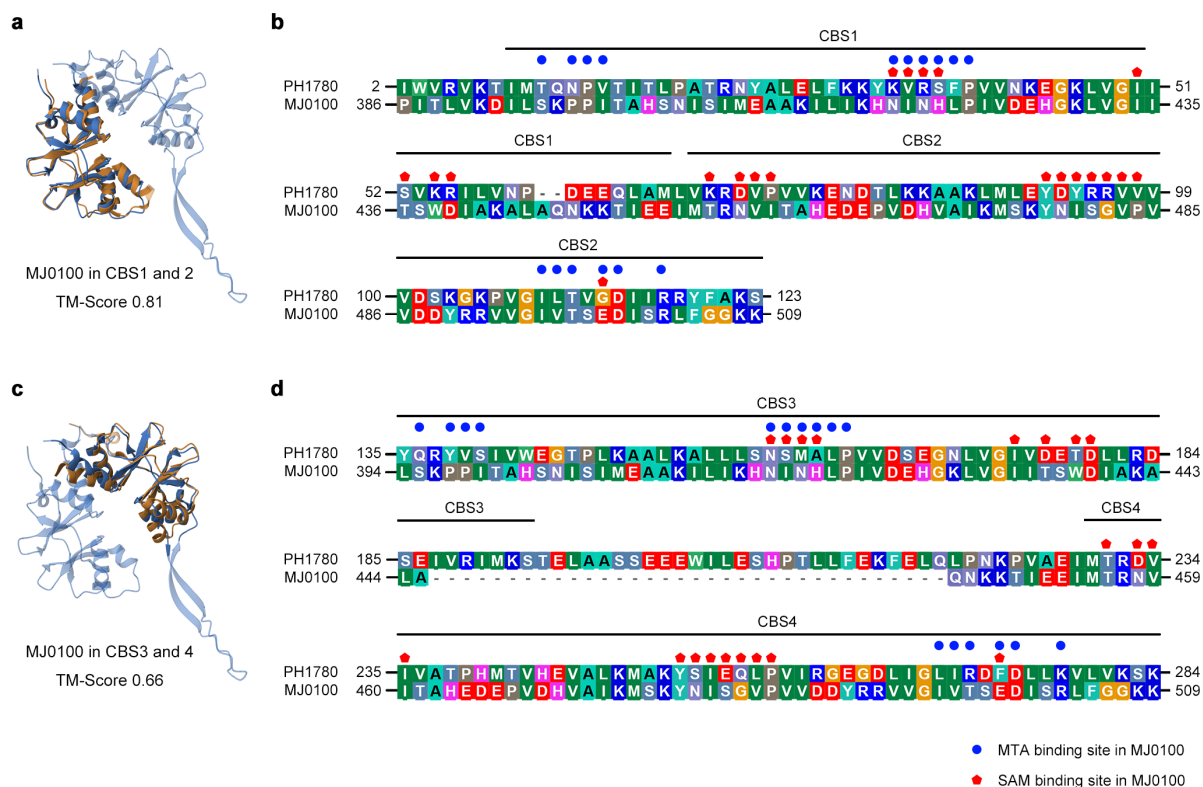
(a) Schematic representations of the amino acid sequence and the predicted

secondary structure of PH1780. The four CBS domains of PH1780 were shown in purple.

The predicted α -helices and β -sheets of PH1780 were indicated with underlines and the

corresponding designations were listed above the sequence. **(b)** Schematic representation of

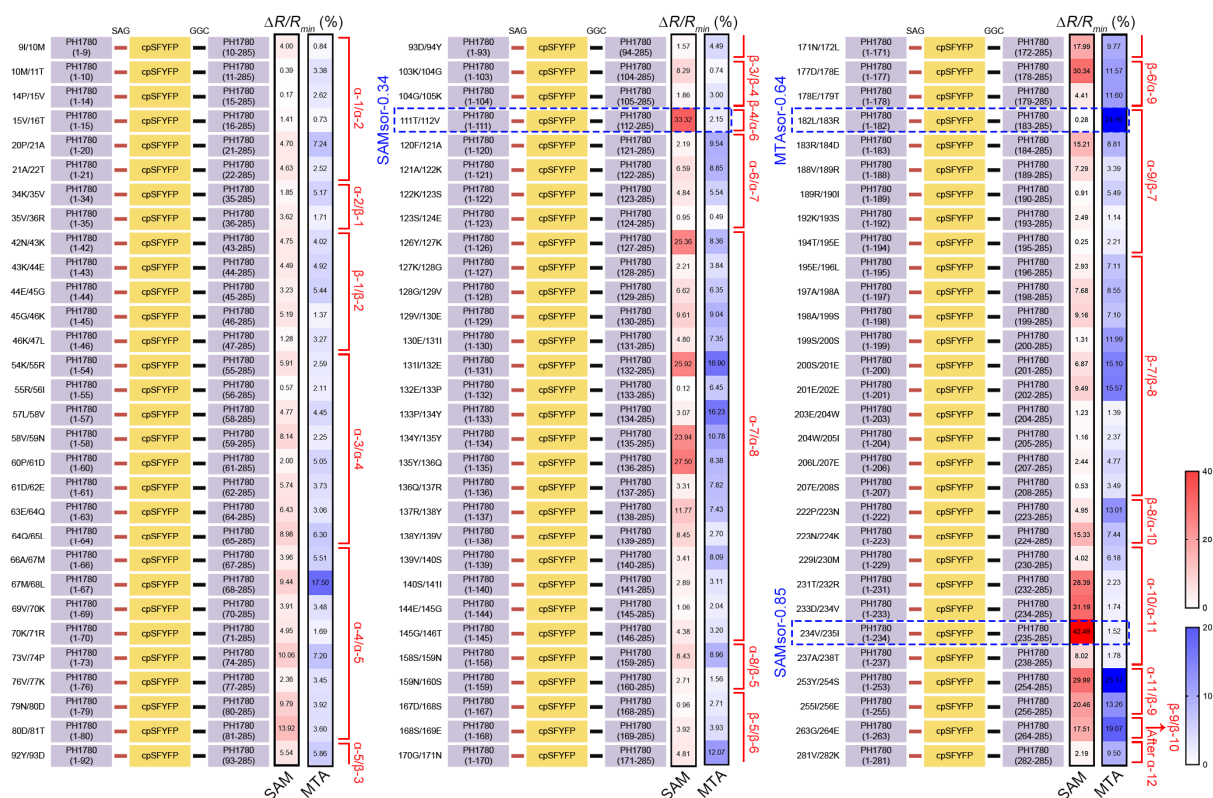
the construction and optimization of MTAsor-0.64, SAMsor, and SAMsor_L. The final MTA biosensor, MTAsor-0.64, was constructed by inserting cpSFYFP between the 1M-182L and 183R-285A fragments of PH1780, with an N-terminal linker of Ser-Ala-Gly and a C-terminal linker of Gly-Gly-Cys. The final SAM biosensor with high detection range, SAMsor, was constructed by inserting cpSFYFP between the 1M-234V and 235I-285A fragments of PH1780, with an N-terminal linker of Leu-Thr and a C-terminal linker of Ser-Pro-Arg. The final SAM biosensor with low detection range, SAMsor_L, was constructed by inserting cpSFYFP between the 1M-111T and 112V-285A fragments of PH1780, with an N-terminal linker of Leu-Glu and a C-terminal linker of Thr-Pro. **(c)** Sequence alignment of PH1780, SAMsor-0.85, and SAMsor. The PH1780 region, the cpSFYFP region, and the N- and C-terminal linkers were indicated. **(d)** Sequence alignment of PH1780, SAMsor-0.34, and SAMsor_L. The PH1780 region, the cpSFYFP region, and the N- and C-terminal linkers were indicated.



Supplementary Figure 38 Analysis of SAM and MTA binding sites in PH1780. (a)

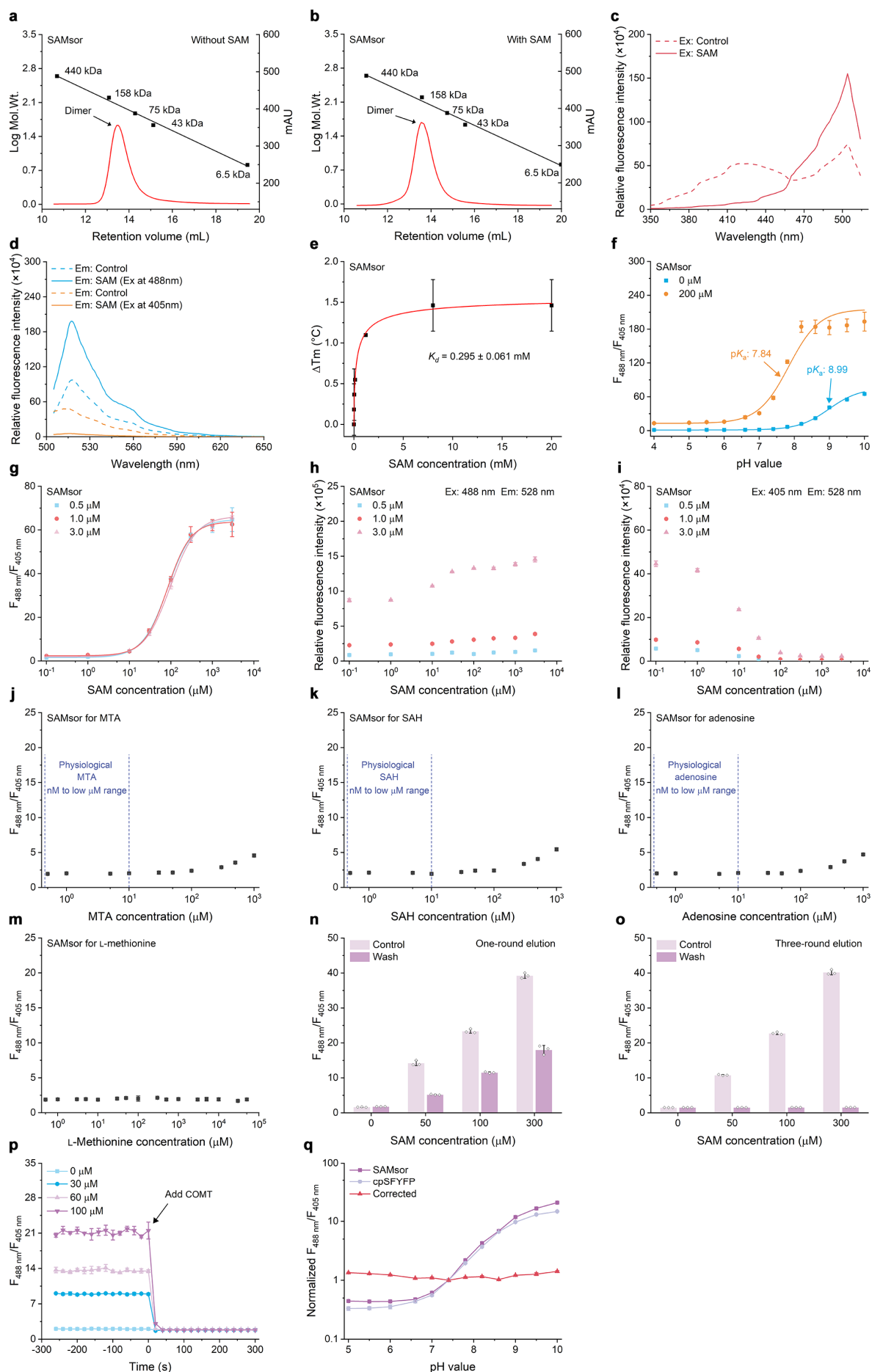
Structural alignment of the regulatory domain of MJ0100 (consisting of two CBS domains) and the CBS1-CBS2 domains of PH1780. The structures of MJ0100 and PH1780 were shown in orange and blue, respectively. **(b)** Sequence alignment of the regulatory domain of MJ0100 and the CBS1-CBS2 domains of PH1780. The designations of the CBS1 and CBS2 domains of PH1780 were indicated above the sequence. The MTA binding sites in MJ0100 and the corresponding sites in PH1780 were indicated by blue circles. The SAM binding sites in MJ0100 and the corresponding sites in PH1780 were indicated by red pentagons. **(c)** Structural alignment of the regulatory domain of MJ0100 and the CBS3-CBS4 domains of PH1780. The structures of MJ0100 and PH1780 were shown in orange and blue, respectively. **(d)** Sequence alignment of the regulatory domain of MJ0100 and the CBS3-CBS4 domains of PH1780. The designations of the CBS3 and CBS4 domains of PH1780 were indicated above

the sequence. The MTA binding sites in MJ0100 and the corresponding sites in PH1780 were indicated by blue circles. The SAM binding sites in MJ0100 and the corresponding sites in PH1780 were indicated by red pentagons. The results of the structure and sequence alignments were obtained using the Structure Alignment Tool on the PDB website (<https://www.rcsb.org/alignment>).



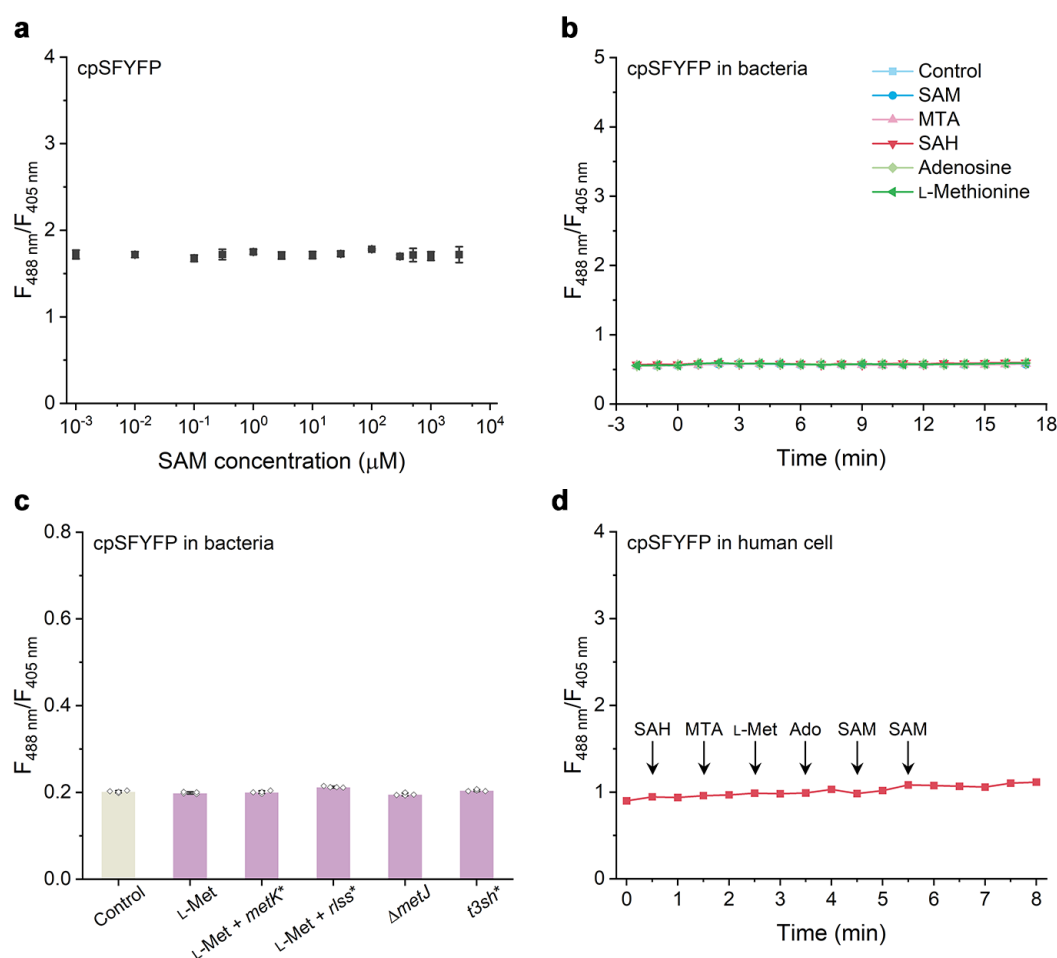
Supplementary Figure 39 Construction of MTAsor-0.64, SAMsor-0.85, and SAMsor-0.34

based on insertion site screening. The response magnitudes ($\Delta R/R_{min}$) of different biosensor variants in response to 100 μ M SAM or MTA were presented in a heatmap, where the color depth represented the $\Delta R/R_{min}$ value (%). The structures of SAMsor-0.85 (234V/235I), MTAsor-0.64 (182L/183R), and SAMsor-0.34 (111T/112V) were shown in the blue dotted boxes. Source data are provided as a Source Data file.



Supplementary Figure 40 Characterization of SAMsor *in vitro*. **(a, b)** Analysis of the oligomeric state of SAMsor in the absence **(a)** or presence **(b)** of 100 μ M SAM by gel filtration chromatography. Red curve, chromatogram of purified SAMsor; black line, standard curve for protein molecular mass standards. **(c, d)** Fluorescence excitation **(c)** and emission **(d)** spectra of SAMsor in the absence or presence of 100 μ M SAM. **(e)** Analysis of the binding properties of SAMsor to SAM by TSA. The ΔT_m values indicated the changes in T_m values compared to the control (with 0 mM SAM). **(f)** Analysis of the pK_a value of SAMsor in the absence or presence of 200 μ M SAM. **(g-i)** Fluorescence responses of SAMsor to increasing concentrations of SAM at varying biosensor concentrations (0.5, 1, and 3 μ M). The data show the ratiometric readout ($F_{488\text{ nm}}/F_{405\text{ nm}}$) **(g)**, alongside the corresponding single-channel fluorescence intensities, $F_{488\text{ nm}}$ **(h)** and $F_{405\text{ nm}}$ **(i)**. **(j-m)** Specificity analysis of SAMsor. The dose-response curves of SAMsor for increasing concentrations of MTA **(j)**, SAH **(k)**, adenosine **(l)**, and L-methionine **(m)** were measured. The physiological concentrations of MTA, SAH, and adenosine in human cells were indicated. **(n, o)** Reversibility analysis of SAMsor. The fluorescence ratios of SAMsor were recorded after SAM addition and subsequent removal through one **(n)** or three **(o)** elution procedures. **(p)** Kinetic analysis of the fluorescence response of SAMsor to SAM depletion. Indicated concentrations of SAM were added prior to testing, and 25 mg mL^{-1} catechol-*O*-methyltransferase from *Solanum lycopersicum* (COMT) together with 100 mM Mg^{2+} , 2 mM dithiothreitol, and 500 μ M catechol were added at time point zero. **(q)** pH-correction of the fluorescence ratio of SAMsor by cpSFYFP. Data were normalized to the ratio at pH 7.4. All data shown are means $\pm$ s.d. ($n = 3$ independent experiments for **c-e, g-i, n-q**; $n = 4$

independent experiments for **f, j-m**). Source data are provided as a Source Data file.



Supplementary Figure 41 Characterization of the control biosensor cpSFYFP for SAM

detection. **(a)** Dose-response curve of cpSFYFP for increasing concentrations of SAM. **(b)**

Time courses of the fluorescence ratio changes of cpSFYFP expressed in permeabilized *E.*

coli BL21(DE3) in response to exogenous SAM (100 μM), MTA (100 μM), SAH (100 μM),

adenosine (100 μM), or L-methionine (500 μM). **(c)** Fluorescence ratios of *E. coli*

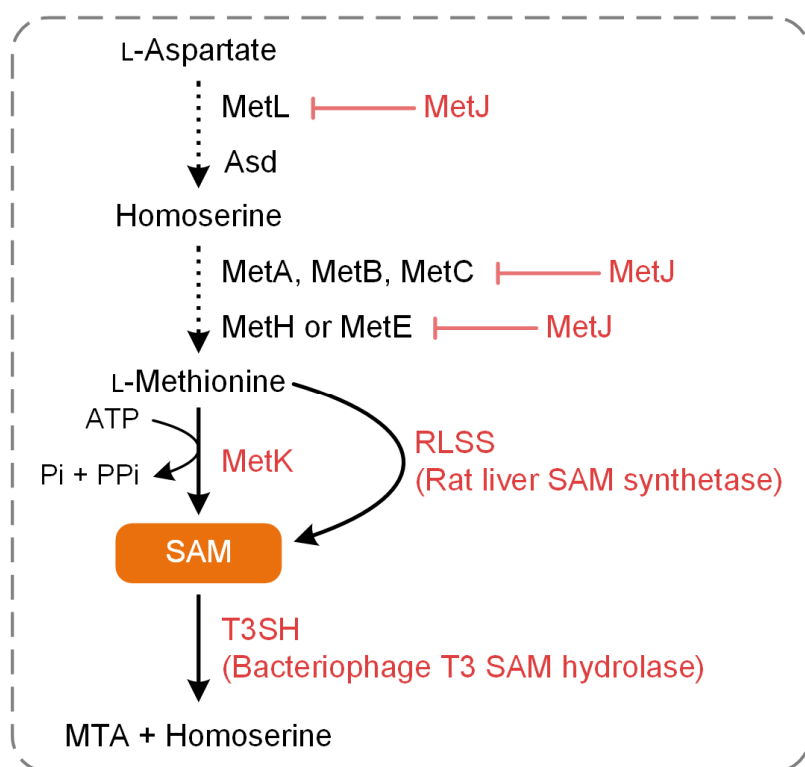
BL21(DE3) expressing cpSFYFP cultured under the indicated conditions. **(d)** Fluorescence

ratios of permeabilized HEK293FT expressing cpSFYFP before and after sequential addition

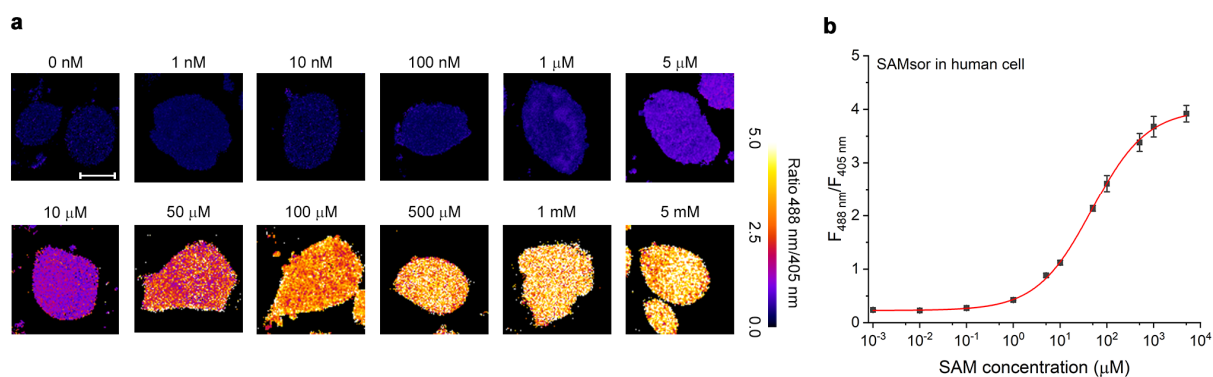
of SAH (50 μM), MTA (100 μM), L-methionine (1 mM), adenosine (100 μM), and SAM (20

μM and 50 μM). Data shown in panel **a-c** are means $\pm$ s.d. ($n = 3$ independent experiments

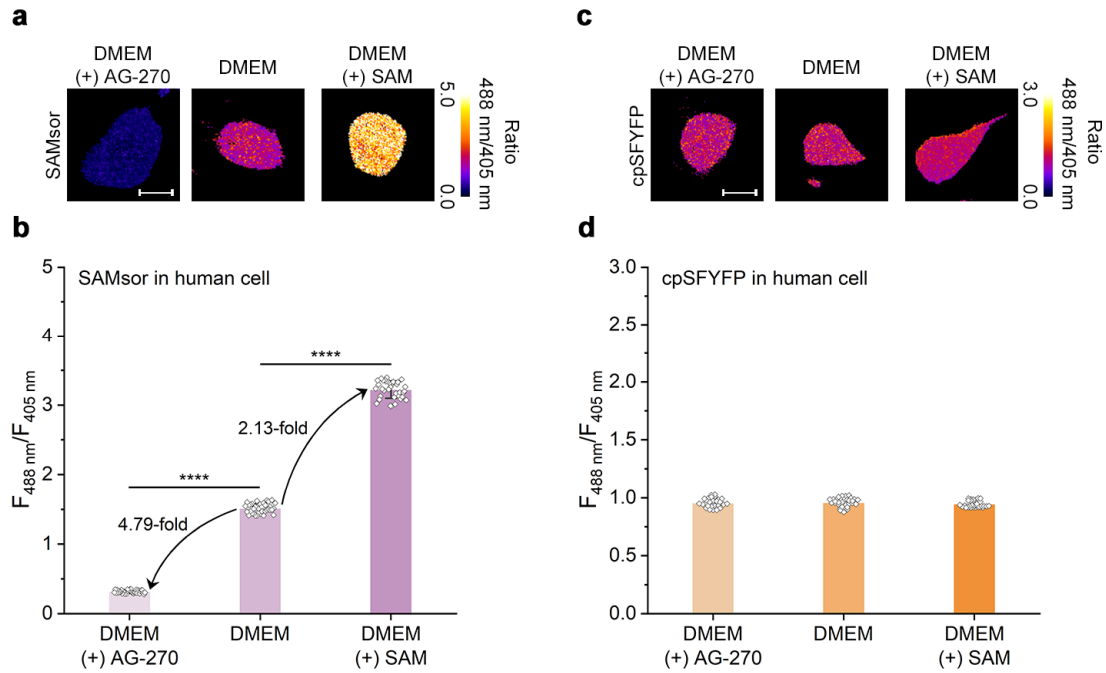
for **a, b**; $n = 4$ independent experiments for **c**). Source data are provided as a Source Data file.



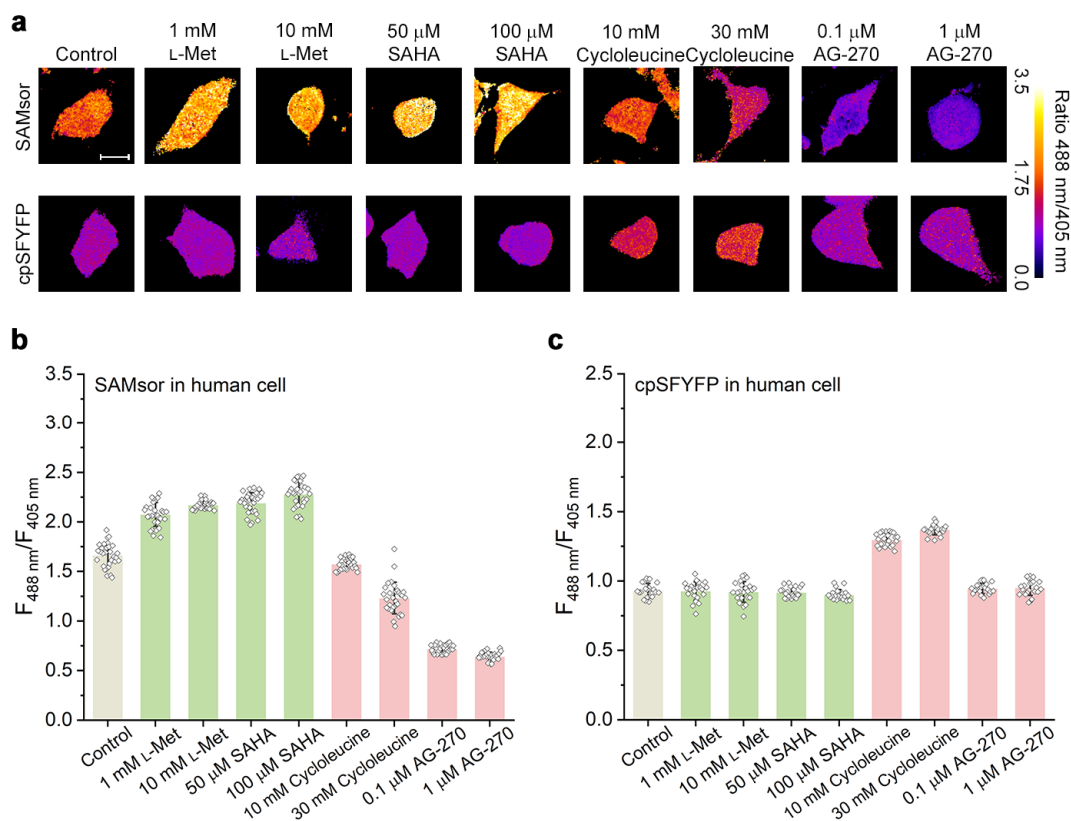
Supplementary Figure 42 Schematic representation of the biosynthetic pathway of SAM in *E. coli* BL21(DE3). RLSS and T3SH were used to modulate endogenous SAM levels by overexpression in *E. coli* BL21(DE3). MetL, aspartokinase; Asd, aspartate semialdehyde dehydrogenase; MetA, homoserine *O*-succinyltransferase; MetB, cystathionine- γ -synthase; MetC, cystathionine- β -lyase; MetH or MetE, L-methionine synthase; MetK, SAM synthetase; MetJ, repressor.



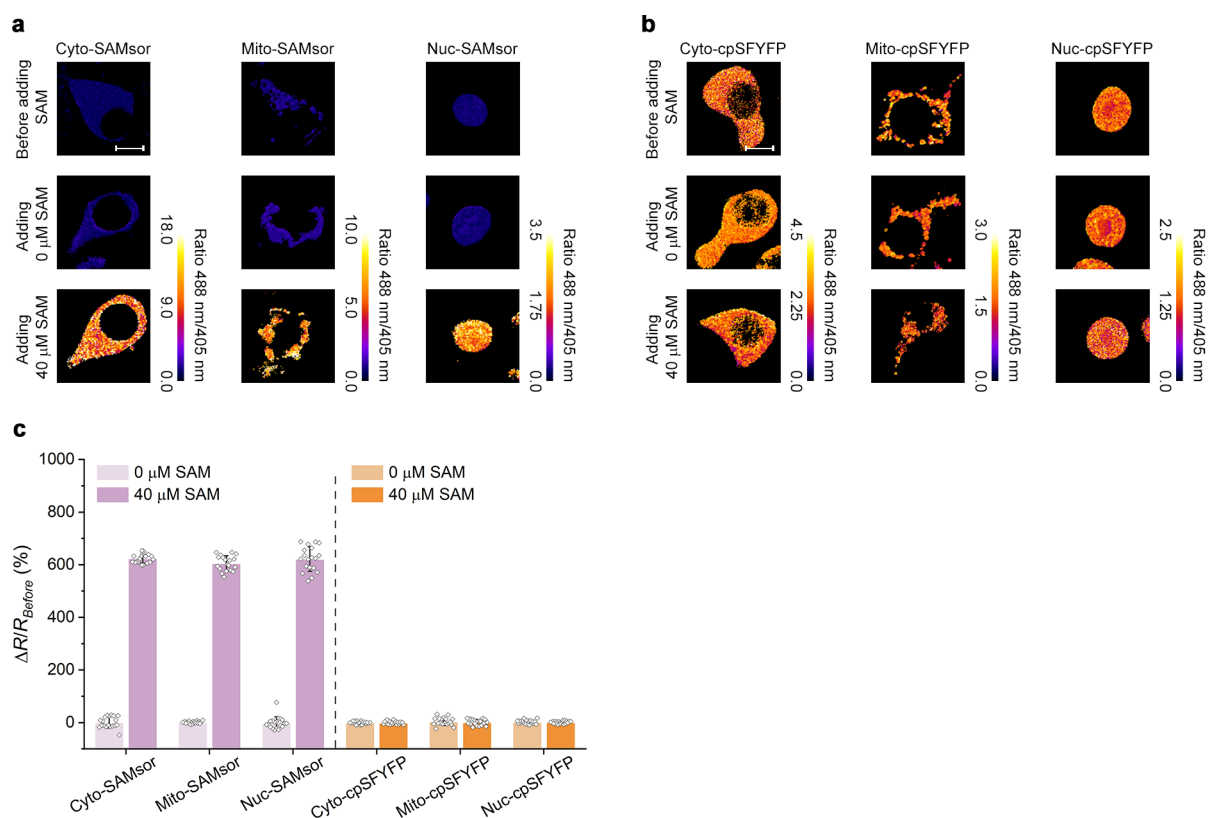
Supplementary Figure 43 Characterization of SAMsor in HEK293FT cells. **(a)** Ratiometric images of single HEK293FT cells expressing SAMsor in response to exogenous increasing concentrations of SAM addition. Scale bar, 10 μ m. The micrographs are representative of three experiments that show similar results. **(b)** Dose-response curve of SAMsor expressed in HEK293FT cells for increasing concentrations of SAM. The HEK293FT cells were permeabilized with 80 μ M digitonin. All data shown are means $\pm$ s.d. (n = 25 cells). Source data are provided as a Source Data file.



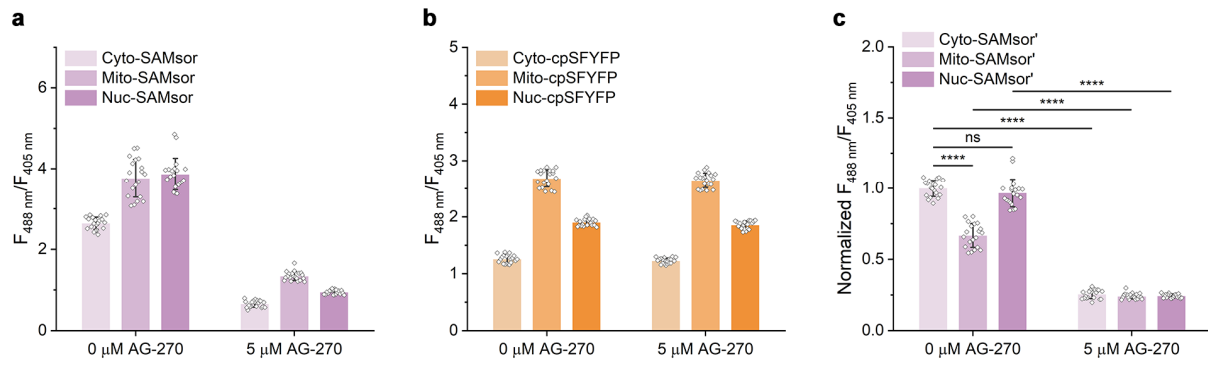
Supplementary Figure 44 Fluorescence responses of SAMsor in HEK293FT cells to extreme SAM metabolic perturbations. **(a, b)** Ratiometric images **(a)** and fluorescence ratios **(b)** of HEK293FT cells expressing SAMsor cultured in DMEM supplemented with 5 μ M AG-270, standard DMEM, and DMEM supplemented with 500 μ M SAM. Scale bar, 10 μ m. The micrographs are representative of three experiments that show similar results. **(c, d)** Ratiometric images **(c)** and fluorescence ratios **(d)** of HEK293FT cells expressing cpSFYFP cultured in DMEM supplemented with 5 μ M AG-270, standard DMEM, and DMEM supplemented with 500 μ M SAM. Scale bar, 10 μ m. The micrographs are representative of three experiments that show similar results. All data shown are means $\pm$ s.d. ($n = 36$ cells). ****, $P < 0.0001$; one-way ANOVA with Tukey's multiple comparisons test. Exact P values are reported in the Source Data file. Source data are provided as a Source Data file.



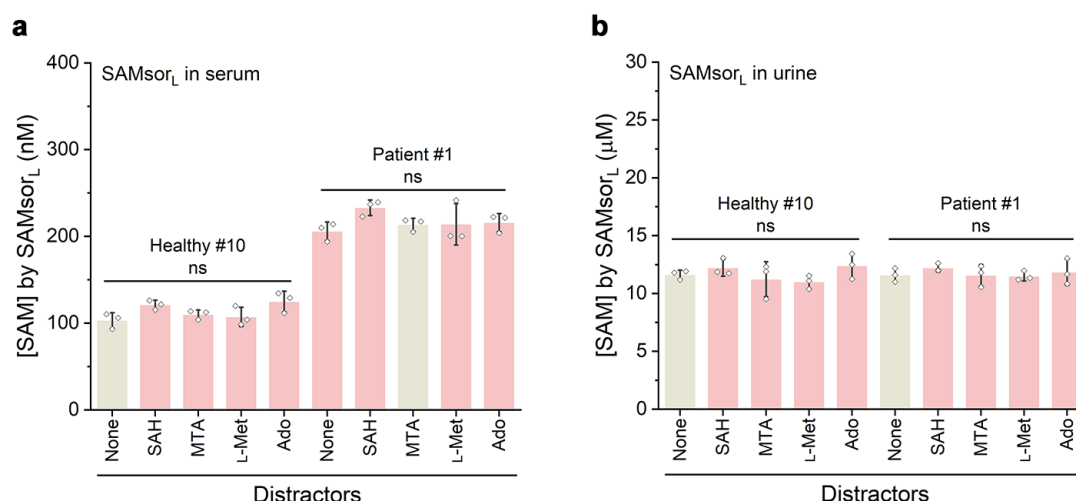
Supplementary Figure 45 Analysis of the effects of different MATase activator or inhibitors on intracellular SAM levels in HEK293FT using SAMsor. **(a)** Ratiometric images of single HEK293FT cells expressing SAMsor or cpSFYFP in response to different MATase activator or inhibitors treatment. The fluorescence ratios were determined 20 h after treatment with different activator or inhibitors. Scale bar, 10 μ m. The micrographs are representative of three experiments that show similar results. **(b, c)** Fluorescence ratios of HEK293FT cells expressing SAMsor **(b)** or cpSFYFP **(c)** in response to different MATase activator or inhibitors treatment. Different inhibitor treatments (especially cycloleucine) may lead to potential intracellular pH fluctuations, which affected the fluorescence ratio of cpSFYFP. cpSFYFP-corrected fluorescence ratios of SAMsor were shown in Fig. 6j. All data shown are means $\pm$ s.d. (n = 30 cells for **b**; n = 23 cells for **c**). Source data are provided as a Source Data file.



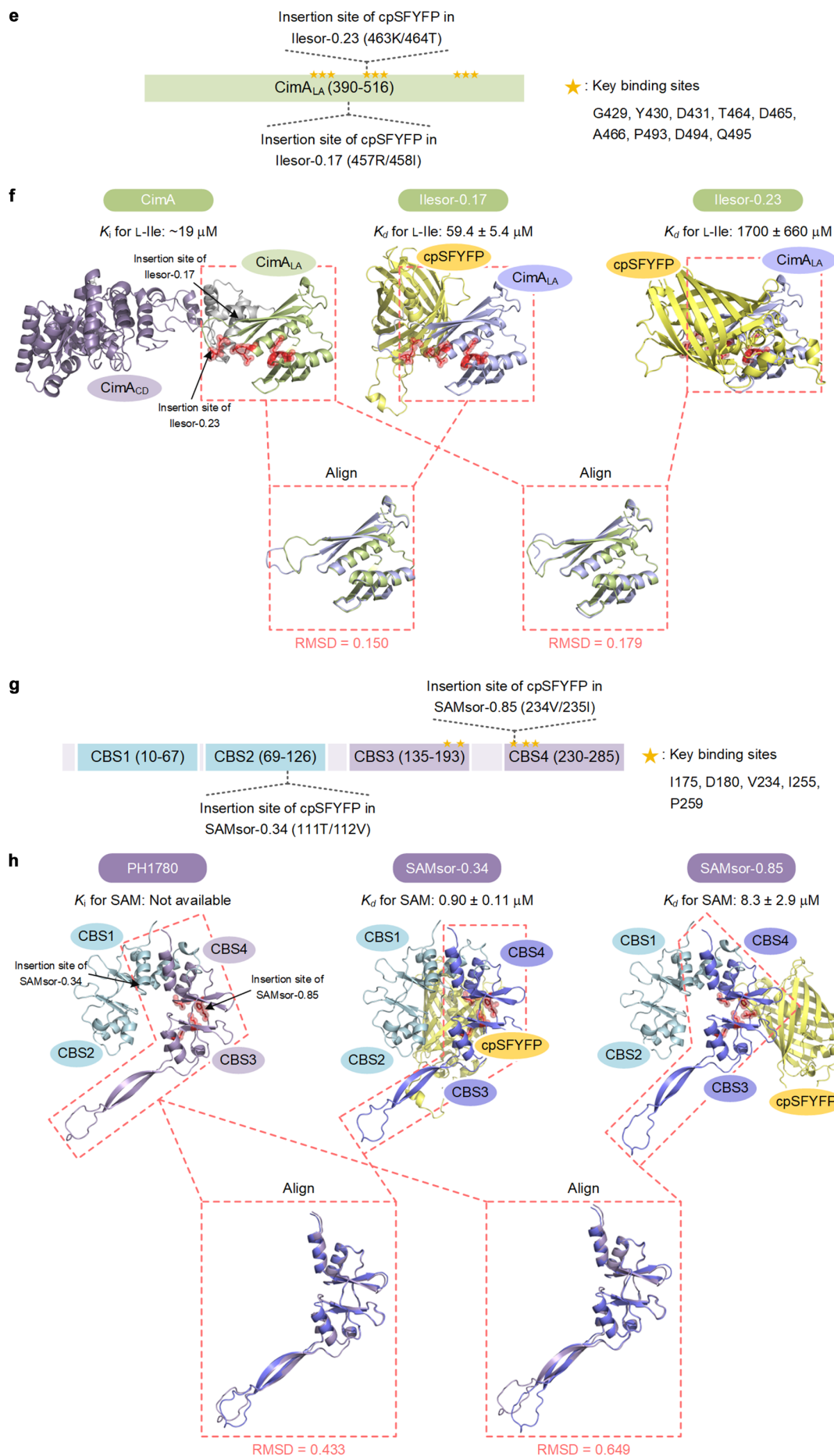
Supplementary Figure 46 Characterization of different subcellularly localized SAMsor in HEK293FT cells. **(a, b)** Ratiometric images of single HEK293FT cells expressing Cyto-SAMsor, Mito-SAMsor, and Nuc-SAMsor **(a)**, or Cyto-cpSFYFP, Mito-cpSFYFP, and Nuc-cpSFYFP **(b)** before and after the addition of 0 or 40 μ M SAM. Scale bar, 10 μ m. The micrographs are representative of three experiments that show similar results. **(c)** Fluorescence responses of different subcellularly localized SAMsor or cpSFYFP to exogenous 40 μ M SAM addition. The HEK293FT cells were permeabilized with 80 μ M digitonin. Data shown in panel **c** are means $\pm$ s.d. (n = 19 cells). Source data are provided as a Source Data file.



Supplementary Figure 47 Analysis of subcellular distribution of SAM using different subcellularly localized SAMsor. **(a, b)** Fluorescence ratios of HEK293FT cells expressing different subcellularly localized SAMsor **(a)** or cpSFYFP **(b)** in response to 5 μM AG-270 addition. Cells were treated with 0 or 5 μM AG-270, and the fluorescence ratios were determined after 20 h. The pH values varied between different subcellular compartments, which affected the fluorescence ratio of cpSFYFP. The fluorescence ratios of different subcellularly localized SAMsor required correction using cpSFYFP. **(c)** cpSFYFP-corrected fluorescence ratios of HEK293FT cells expressing different subcellularly localized SAMsor. All data shown are means $\pm$ s.d. ($n = 20$ cells). ****, $P < 0.0001$; ns, no significant difference ($P \geq 0.05$); one-way ANOVA with Tukey's multiple comparisons test. Exact P values are reported in the Source Data file. Source data are provided as a Source Data file.



Supplementary Figure 48 Analysis of the interference of different SAM analogs on the quantitative detection of SAM by SAMsor_L. **(a, b)** Comparison of the quantitative results of SAMsor_L for SAM in serum **(a)** and urine **(b)** from healthy individuals and patients with lung cancer in the absence or presence of the indicated SAM analogs. The concentration of distractors used in serum was set at 1 μM (for SAH, MTA, adenosine) or 100 μM (for L-methionine) depending on their physiological and pathological concentrations in serum. The concentration of distractors used in urine was set at 10 μM (for SAH, MTA, adenosine) or 100 μM (for L-methionine) depending on their physiological and pathological concentrations in urine. All data shown are means ± s.d. (n = 3 independent experiments). ns, no significant difference ($P \geq 0.05$); one-way ANOVA with Tukey's multiple comparisons test. Exact P values are reported in the Source Data file. Source data are provided as a Source Data file.



Supplementary Figure 49 Analysis of the effect of cpSFYFP insertion sites on the affinity of the resulting biosensor variants. **(a)** Schematic representation of the cpSFYFP insertion sites in the PheA_{ACT} domain of Phesor-0.04 (a low- K_d L-phenylalanine biosensor variant) and Phesor-0.38 (a high- K_d variant). **(b)** Structural alignment of the PheA_{ACT} domains from PheA, Phesor-0.04, and Phesor-0.38. A lower root mean square deviation (RMSD) was observed between the PheA_{ACT} domains of Phesor-0.04 and PheA compared to that between Phesor-0.38 and PheA. **(c)** Schematic representation of the cpSFYFP insertion sites in the IlvH domain of Valsor-0.01 (a low- K_d L-valine biosensor variant) and Valsor-0.03 (a high- K_d variant). **(d)** Structural alignment of the IlvH domains from IlvH, Valsor-0.01, and Valsor-0.03. A lower RMSD was observed between the IlvH domains of Valsor-0.01 and IlvH compared to that between Valsor-0.03 and IlvH. **(e)** Schematic representation of the cpSFYFP insertion sites in the CimA_{LA} domain of Ilesor-0.17 (a low- K_d L-isoleucine biosensor variant) and Ilesor-0.23 (a high- K_d variant). **(f)** Structural alignment of the CimA_{LA} domains from CimA, Ilesor-0.17, and Ilesor-0.23. A lower RMSD was observed between the CimA_{LA} domains of Ilesor-0.17 and CimA compared to that between Ilesor-0.23 and CimA. **(g)** Schematic representation of the cpSFYFP insertion sites in the PH1780 domain of SAMsor-0.34 (a low- K_d SAM biosensor variant) and SAMsor-0.85 (a high- K_d variant). **(h)** Structural alignment of the CBS3-CBS4 domains from PH1780, SAMsor-0.34, and SAMsor-0.85. A lower RMSD was observed between the CBS3-CBS4 domains of SAMsor-0.34 and PH1780 compared to that between SAMsor-0.85 and PH1780. Key ligand-binding sites are denoted with asterisks (panels **a**, **c**, **e**, and **g**) or highlighted in red (panels **b**, **d**, **f**, and **h**). The structures of PheA, Phesor-0.04, Phesor-0.38, Valsor-0.01, Valsor-0.03, CimA, Ilesor-0.17,

Ilesor-0.23, PH1780, SAMsor-0.34, and SAMsor-0.85 were predicted using AlphaFold3. The structure of IlvH was retrieved from PDB entry 2F1F and reference [1]. The ligand-binding sites were identified as follows: the L-phenylalanine binding sites in PheA were predicted using Schrödinger software and the PLIP web server; the L-valine binding sites in IlvH were derived from PDB entry 2F1F and reference [1]; the L-isoleucine binding sites in CimA were derived from PDB entry 3F6G and reference [2]; and the SAM binding sites in PH1780 were identified through structural alignment with the regulatory domain of MJ0100 (Supplementary Fig. 38).

Supplementary Table 1 Information on the names, ligands, and allosteric domains of partial allosteric enzymes.

Protein	Abbreviation	Ligand	Allosteric domain	Species	NCBI number	PDB number	Reference
D-3-Phosphoglycerate dehydrogenase	SerA	L-Serine	ACT	<i>Escherichia coli</i>	WP_001151604.1	1PSD	[3]
Chorismate mutase	PheA	L-Phenylalanine	ACT	<i>Escherichia coli</i>	WP_000200120.1	/	[4]
3-Deoxy-D-arabino-heptulosonate 7-phosphate synthase	AroF	L-Tyrosine	ACT	<i>Thermotoga maritima</i>	WP_004083125.1	3PG9	[5]
Acetolactate synthase regulatory subunit	IlvH	L-Valine	ACT	<i>Escherichia coli</i>	WP_001300383.1	2F1F	[1]
Aspartate kinase III	LysC	L-Lysine	ACT	<i>Escherichia coli</i>	NP_418448.1	2J0X	[6]
Homoserine dehydrogenase	ThrA	L-Threonine	ACT	<i>Escherichia coli</i>	WP_001264707.1	6MX1	[7]
Uridyl transferases-like protein	ACR1	ND ^a	ACT	<i>Arabidopsis</i>	BAB11136.1	/	[8]
2-Isopropylmalate synthase	LeuA	L-Leucine	LeuA regulatory domain	<i>Mycobacterium tuberculosis</i>	WP_230870391.1	3FIG	[9]
D-Citramalate synthase	CimA	L-Isoleucine	LeuA regulatory domain	<i>Leptospira interrogans</i>	WP_000169689.1	3F6G	[2]
Cystathionine β -synthase	CBS	SAM	CBS	<i>Homo sapiens</i>	AAP35818.1	4UUU	[10]
L-Aspartate semialdehyde sulfurtransferase	MJ0100	SAM, MTA	CBS	<i>Methanocaldococcus jannaschii</i>	Q57564.1	3KPB, 3KPC, 3KPD	[11]
Inosine-5'-monophosphate dehydrogenase	IMPDH	ATP, GTP, GDP	CBS	<i>Ashbya gossypii</i>	NP_984977.2	4Z87	[12]
AMP-activated protein kinase γ subunit	AMPK γ subunit	AMP, ATP	CBS	<i>Rattus norvegicus</i>	NP_037142.1	4EAI	[13]

PH1780	PH1780	SAM, MTA	CBS	<i>Pyrococcus horikoshii</i>	WP_048053476.1	/	/
L-Lactate dehydrogenase	LDH	Fructose 1,6-bisphosphate	Lactate dehydrogenase, C-terminal domain	<i>Bifidobacterium longum</i>	WP_007052533.1	1LTH	[14]
Pyruvate kinase	PK	Fructose 1,6-bisphosphate	Pyruvate kinase, C-terminal domain	<i>Saccharomyces cerevisiae</i>	NP_009362.1	1A3W	[15]
Phosphofructokinase A	Pfk A	Phosphoenolpyruvate	ND ^a	<i>Mycobacterium tuberculosis</i>	NP_217526.1	/	[16]
Protein tyrosine phosphatase 1E	PTP1E	RA-GEF2 peptide	PDZ ^b	<i>Homo sapiens</i>	XP_054206630.1	3LNY	[17]
L-Threonine synthase	TS	SAM	ND ^a	<i>Arabidopsis thaliana</i>	NP_194713.1	2C2B, 2C2G	[18]
Glucosamine-6-phosphate deaminase	NagB	N-acetylglucosamine 6-phosphate	ND ^a	<i>Escherichia coli</i>	WP_001237072.1	1HOT	[19]
Bacterial type IV pilus assembly ATPase	PilB	Cyclic-di-GMP, ADP	cdG-binding domain, C-terminal domain	<i>Chloracidobacterium thermophilum</i>	WP_014100180.1	/	[20]
Glucose-1-phosphate adenylyltransferase	GlgC	Fructose 1,6-bisphosphate, AMP	ND ^a	<i>Escherichia coli</i>	NP_417888.1	5L6S, 5L9V	[21]
Fructose-1,6-bisphosphatase	FBPase	Glucose 6-phosphate, AMP	ND ^a	<i>Escherichia coli</i>	WP_000853753.1	2Q8M	[22]

^aND, not determined.

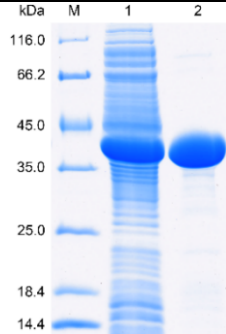
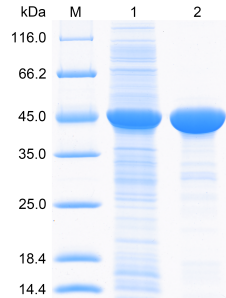
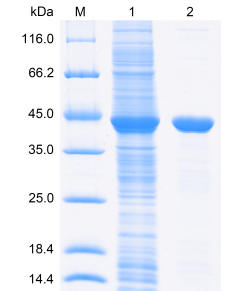
^bPDZ, post-synaptic density-95/discs large/zonula occludens-1 domain.

Supplementary Table 2 Reported concentrations of L-phenylalanine, L-valine, L-isoleucine, and SAM in bacteria, mammalian cells, and human body fluids.

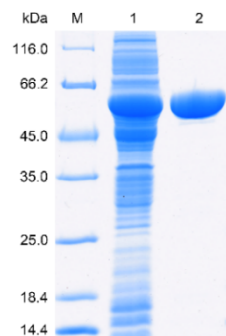
Metabolites	Bacteria	Mammalian cells	Human body fluids
L-Phenylalanine	0.02–3.63 mM (<i>Escherichia coli</i>) ^{[23][24]}	0.05–0.28 mM (HeLa cell) ^[25] ~0.50–4.62 mM (A549 cell and U87-MG cell) ^[26] ~0.84 mM (Mouse kidney epithelial cells) ^[27] ~1.50 mM (A549 cell) ^[28] 0.34–0.81 mM (Hybridoma and CHO cell) ^[29] 0.07–0.56 mM (T cells) ^[30]	35–120 µM (Healthy serum) ^[31] 120–360 µM (Hyperphenylalaninaemia serum) ^[31] > 360 µM (Phenylketonuria serum) ^[31] ~62.89 µM (Healthy urine) ^[32]
L-Valine	~0.82–4.58 mM (<i>Escherichia coli</i>) ^[23] ~50–250 mM (<i>Corynebacterium glutamicum</i>) ^[33]	0.09–0.62 mM (HeLa cell) ^[25] ~0.62–2.44 mM (A549 cell and U87-MG cell) ^[26] ~1.51 mM (Mouse kidney epithelial cells) ^[27] ~2.50 mM (A549 cell) ^[28] 1.55–3.36 mM (Hybridoma and CHO cell) ^[29] 3.38–15.41 mM (T cells) ^[30] 1.36–10.29 mM (Fibroblasts) ^[34]	/
L-Isoleucine	~0.15–0.27 mM (<i>Escherichia coli</i>) ^[23] ~17–100 mM (<i>Corynebacterium glutamicum</i>) ^[35]	0.06–0.55 mM (HeLa cell) ^[25] ~0.58–2.09 mM (A549 cell and U87-MG cell) ^[26] ~1.76 mM (Mouse kidney epithelial cells) ^[27] ~2.10 mM (A549 cell) ^[28] 0.70–1.75 mM (Hybridoma and CHO cell) ^[29] 0.75–6.71 mM (T cells) ^[30]	/
SAM	28–400 µM (<i>Escherichia coli</i>) ^{[36][37]}	~62.99 µM (T cells) ^[30] 354 µM (Fibroblasts) ^[38]	~73.5–120 nM (Serum) ^[41] ~22.1 µM (Urine) ^[41]

1–37 μM (HEK293T cell) ^[39]	~10.2 μM (Urine) ^[42]
10–2,000 μM (Lymphoid cell, HTC116 cell, HEK293 cell) ^[40]	~12.1 μM (Urine) ^[43]

Supplementary Table 3 Expression analysis and amino acid sequences of Phesor, Valsor, Ilesor, SAMsor, and SAMsor_L.

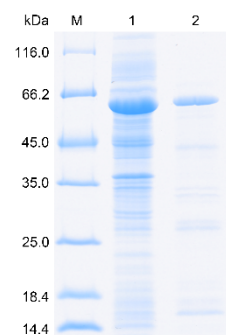
GEFB	Biorecognition element	SDS-PAGE ^a	Sequence
Phesor	ACT domain of PheA		MKAINVSDQVPAKTLLMATGQQAGALVEALLVLRNHNLMTRLESRPIHGNPWEEMF YLDIQANLESAEMQKALKELGEIARLFNSDNVYIMADKQKNGIKANFKIRHNVEDGSVQ LADHYQQNTPIGDGPVLLPDNHYLSFQSVLSKDPNEKRDHMLLEFVTAAGITLGMDEL YNVDGGSGGTGSKGEELFTGVVPILVELDGDVNGHKFSVRGEGEGDATNGKLTCLKICT TGKLPVPWPTLVTTTLGYGLKCFARYPDHMKQHDFFKSAMPEGYVQERTIFFKDDGTYK TRAEVKFEGDTLVNRIELKGIGFKEDGNILGHKLEYNQHLTRSMKVLGCYPSENVVPVDP T
Valsor	IlvH		MRRILSVLLENESGALSRVIGLFSQRGYNIESLTVAPTDDPTLSRMTIQTVGDEKVLQIEK QLHKLVDVLRVSELGQYTFNSDNVYIMADKQKNGIKANFKIRHNVEDGSVQLADHYQQ NTPIGDGPVLLPDNHYLSFQSVLSKDPNEKRDHMLLEFVTAAGITLGMDELYNVDGGS GGTGSKGEEFTGVVPILVELDGDVNGHKFSVRGEGEGDATNGKLTCLKICTTGKLPVP WPTLVTTTLGYGLKCFARYPDHMKQHDFFKSAMPEGYVQERTIFFKDDGTYKTRAEVKF EGDTLVNRIELKGIGFKEDGNILGHKLEYNAGLGAHVEREIMLVKIQASGYGRDEVKRN TEIFRGQIIDVTPSLYTVQLAGTSGKLDAFLASIRDVAKIVEVARSGVVGLSRGDKIMR
Ilesor	LA domain of CimA		MKVLTIKSCNIHSGIGIRPHAQIELEYQGKIHKEISEGDGGYDAFMNALTKITNRLGISIPKL IDYEVRIPPGGKFRFTFNSDNVYIMADKQKNGIKANFKIRHNVEDGSVQLADHYQQNTPIG DGPVLLPDNHYLSFQSVLSKDPNEKRDHMLLEFVTAAGITLGMDELYNVDGGSGGTGS KGEELFTGVVPILVELDGDVNGHKFSVRGEGEGDATNGKLTCLKICTTGKLPVPWPTLV TLGYGLKCFARYPDHMKQHDFFKSAMPEGYVQERTIFFKDDGTYKTRAEVKFEGDTLV NRIELKGIGFKEDGNILGHKLEYNPHLTDALVETRITWNKSLDLEEDQTFKTMGVHPDQT VAHVHATEKMLNQILQPWQI

SAMsor PH1780



MIWVRVKTIMTQNPVTITLPA TRNYALELFKKYKVRSPVNVNKEGKLVGIISVKRILVNP
DEEQLAMLVKRDVPVVKENDTLKKA AKLMLEYDYRRVVVVDSK GKPVGILTVGDIIRR
YFAKSEKYKGVEIEPY YQRYVSIVWEGT PLKAALKALLSNSMALPVVDSEGNLVGIVD
ETDLLRDSEIVRIMKSTELAASSEEEWILESHPTLLFEKFELQLPNKPVAEIMTRDVLTFNS
DNVYIMADKQKNGIKANFKIRHNVEDG SVQLADHYQQNTPIGDGPVLLPDNHYLSFQSV
LSKDPNEKRDHMLLEFVTAAGITLGMDELYNVDGGSGGTGSKGEELFTGVVPILVELD
GDVNGHKFSVRGEGEGDATNGKLT LKLICTTGKLPVPWP TLVTTLGYGLKCFARYPDH
MKQHDFFKSAMPEGYVQERTIFFKDDGTYKTRAEVKFEGDTLVNRIELKGIGFKEDGNIL
GHKLEYNSPRIVATPHMTVHEVALKMAKYSIEQLPVIRGEGDLIGLIRDFDLLKVLVKSK
A

SAMsor_L PH1780



MIWVRVKTIMTQNPVTITLPA TRNYALELFKKYKVRSPVNVNKEGKLVGIISVKRILVNP
DEEQLAMLVKRDVPVVKENDTLKKA AKLMLEYDYRRVVVVDSK GKPVGILTLEFNSDN
VYIMADKQKNGIKANFKIRHNVEDG SVQLADHYQQNTPIGDGPVLLPDNHYLSFQSVLS
KDPNEKRDHMLLEFVTAAGITLGMDELYNVDGGSGGTGSKGEELFTGVVPILVELDGD
VNGHKFSVRGEGEGDATNGKLT LKLICTTGKLPVPWP TLVTTLGYGLKCFARYPDHMK
QHDFFKSAMPEGYVQERTIFFKDDGTYKTRAEVKFEGDTLVNRIELKGIGFKEDGNILGH
KLEYNTPVGDIIRRYFAKSEKYKGVEIEPY YQRYVSIVWEGT PLKAALKALLSNSMALP
VVDSEGNLVGIVDETDLRLDSEIVRIMKSTELAASSEEEWILESHPTLLFEKFELQLPNKP
VAEIMTRDVIVATPHMTVHEVALKMAKYSIEQLPVIRGEGDLIGLIRDFDLLKVLVKSKA

^aSDS-PAGE, sodium dodecyl sulfatepolyacrylamide gel electrophoresis; lane M, molecular weight markers; lane 1, crude extract of *E. coli* BL21(DE3) harboring the expression plasmid for the indicated GEFB; lane 2, purified His₆-tagged indicated GEFB using a HisTrap column. The gel images are representative of three experiments that show similar results.

Supplementary Table 4 Detection properties of Phesor, Valsor, Ilesor, SAMsor, SAMsor_L, and cpSFYFP.

Properties	Phesor	Valsor	Ilesor	SAMsor	SAMsor _L	cpSFYFP
ΔR_{max}	4,030 ± 40%	875 ± 52%	1,325.3 ± 7.1%	3,540 ± 360%	128.5 ± 1.1%	/
K_d	1,760 ± 40 μM	2,170 ± 110 μM	3,620 ± 400 μM	204 ± 21 μM	0.379 ± 0.041 μM	/
Hill slope	1.13 ± 0.03	1.03 ± 0.04	0.98 ± 0.05	1.15 ± 0.04	0.91 ± 0.07	/
LOD (<i>in vitro</i>) ^a	48.52 μM	48.39 μM	26.41 μM	0.31 μM	0.057 μM	/
LOD (in human cells) ^a	67.39 μM	75.17 μM	47.93 μM	0.45 μM	/	/
Oligomeric state (without ligand)	Dimer	Dimer	Dimer and tetramer	Dimer	/	/
Oligomeric state (with ligand)	Dimer	Dimer	Dimer and tetramer	Dimer	/	/
Maximum excitation wavelength	~416 nm and 490 nm	~426 nm and 504 nm	~420 nm and 504 nm	~420 nm and 504 nm	/	/
Maximum emission wavelength	~508 nm (Ex: 405 nm) ~516 nm (Ex: 488 nm)	~509 nm (Ex: 405 nm) ~518 nm (Ex: 488 nm)	~507 nm (Ex: 405 nm) ~517 nm (Ex: 488 nm)	~514 nm (Ex: 405 nm) ~518 nm (Ex: 488 nm)	/	/
pK _a (without ligand)	8.92	8.56	9.14	8.99	/	8.54
pK _a (with ligand) ^b	8.10	8.20	8.74	7.84	/	8.49 (L-phenylalanine) 8.49 (L-valine) 8.69 (L-isoleucine) 8.60 (SAM)
λ _{abs} (ε, M ⁻¹ ·cm ⁻¹) (without ligand) ^c	43365 (416 nm) 3601 (490 nm)	60806 (426 nm) 10981 (504 nm)	44413 (420 nm) 5000 (504 nm)	168092 (420 nm) 46806 (504 nm)	/	/

λ_{abs} (ϵ , $\text{M}^{-1}\cdot\text{cm}^{-1}$)	20320 (416 nm)	43472 (426 nm)	37577 (420 nm)	38347 (420 nm)	/	/
(with ligand) ^c	55679 (490 nm)	34114 (504 nm)	15533 (504 nm)	98780 (504 nm)		
Φ (without ligand) ^c	0.17 (416 nm)	0.09 (426 nm)	0.22 (420 nm)	0.20 (420 nm)	/	/
	0.38 (490 nm)	0.40 (504 nm)	0.11 (504 nm)	0.11 (504 nm)		
Φ (with ligand) ^c	0.12 (416 nm)	0.05 (426 nm)	0.17 (420 nm)	0.06 (420 nm)	/	/
	0.31 (490 nm)	0.40 (504 nm)	0.37 (504 nm)	0.12 (504 nm)		
Brightness	7419 (416 nm)	5171 (426 nm)	9634 (420 nm)	32795 (420 nm)	/	/
(without ligand) ^c	1357 (490 nm)	4436 (504 nm)	549 (504 nm)	5074 (504 nm)		
Brightness	2395 (416 nm)	2134 (426 nm)	6235 (420 nm)	2441 (420 nm)	/	/
(with ligand) ^c	17347 (490 nm)	13810 (504 nm)	5761 (504 nm)	11790 (504 nm)		

^aThe LOD is calculated by interpolating the average background counts + (or –) $3 \times$ standard deviation value^[44].

^bThe pK_a values are determined in the presence of 1 mM L-phenylalanine, 1 mM L-valine, 1 mM L-isoleucine, or 200 μM SAM.

^cThe photophysical properties of Phesor, Valsor, Ilesor, and SAMsor were measured at room temperature in the absence or presence of their respective ligands (1 mM L-phenylalanine, 1 mM L-valine, 1 mM L-isoleucine, and 200 μM SAM). Extinction coefficient (ϵ) was determined at the corresponding absorption wavelength (λ_{abs}). Quantum yield (Φ) was measured using EGFP ($\Phi_{\text{EGFP}} = 0.6$) as a reference. Brightness was calculated as the product of ϵ and Φ .

Supplementary Table 5 Comparison of detection performance between HPLC and the biosensors developed in this study.

Properties	HPLC	Phesor	Valsor	Ilesor	SAMsor	SAMsor _L
Real-time analysis ^a	Incompatibility	Compatibility	Compatibility	Compatibility	Compatibility	/
<i>In situ</i> analysis ^a	Incompatibility	Compatibility	Compatibility	Compatibility	Compatibility	/
Sample Pretreatment ^b	Deproteinization, derivatization	None	None	None	/	None
Analysis cost ^b	~\$4/sample ^c	< \$0.1/sample ^d	< \$0.1/sample ^d	< \$0.1/sample ^d	/	< \$0.1/sample ^d
Sample requirement ^b	5–20 µL (injection volume) > 100 µL (total sample volume)	10 µL	10 µL	10 µL	/	10 µL
LOD ^b	6 µM for L-Phe ^e 1 µM for L-Val and L-Ile ^f 0.050 µM for SAM ^g	48.52 µM	48.39 µM	26.41 µM	/	0.057 µM
Analysis time ^b	> 30 min	< 2 min	< 2 min	< 2 min	/	< 2 min
Analysis throughput ^b	1/detection	384/detection	384/detection	384/detection	/	384/detection

^aDetection performance for applications in living cells.

^bDetection performance for applications *in vitro*.

^cCalibrated based on the quoted price for sample testing from Core Facilities for Life and Environmental Sciences (State Key Laboratory of Microbial Technology, Shandong University). Costs for equipment maintenance, chromatographic column, and analytical reagents are not included.

^dCalibrated based on the quoted price for sample testing from Core Facilities for Life and Environmental Sciences (State Key Laboratory of Microbial Technology, Shandong University). Cost for protein purification are also included.

^eThe indicated LOD corresponds to an injection volume of 20 µL, based on data from Reference [45].

^fThe indicated LOD was determined in this study using an injection volume of 5 µL.

^gThe indicated LOD was determined in this study using an injection volume of 20 µL.

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