

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

Corresponding Author: Professor Chao Gao

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

A version of this paper was originally rejected for publication by Nature Communications, however that decision was reconsidered after appeal by the authors.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

This manuscript presents a strategy for constructing genetically encoded fluorescent biosensors (GEFBs) using the regulatory domains of allosteric enzymes as ligand-binding elements. Specifically, the authors inserted cpSFYFP into the regulatory domains of chorismate mutase, 2-acetolactate synthase, and D-citramalate synthase to generate sensors for L-phenylalanine, L-valine, and L-isoleucine. The authors also developed SAM and MTA sensors by inserting cpSFYFP into the CBS domain, a multi-ligand-binding regulatory domain. While the use of regulatory domains from allosteric enzymes as novel ligand-binding modules is a conceptual advance that adds diversity to the biosensor toolkit, I have several substantial concerns about the overall novelty and practical applicability of the work in its current form.

Major Concerns:

1. Suitability of the sensors for intracellular imaging

The authors claim that the four biosensors can be used to monitor intracellular metabolite levels with high spatiotemporal resolution. However, the reported K_d values are relatively high: ~1660 μM (Phesor), ~2300 μM (Valsor), ~3600 μM (Ilesor), and ~200 μM (SAMsor). In contrast, the physiological concentrations of these metabolites in mammalian cells are much lower: for instance, L-phenylalanine, valine, and isoleucine typically range from tens to several hundreds of μM (ACS Sensors, 2019, 4(12): 3333–3342), and SAM is typically around 300 μM (Communications Biology, 2022, 5(1): 313). Among the four sensors, only SAMsor exhibits a K_d value that approaches the physiological concentration range of its target metabolite. However, even in this case, Figures 6i and 6m show that upon stimulation, the fluorescence changes in *E. coli* and HEK293FT cells compared to controls were still less than one-fold. For the other three sensors—Phesor, Valsor, and Ilesor—whose K_d values are several-fold higher than the physiological concentrations of their respective target metabolites, Figures 2m, 3m, and 4m show that under the relevant stimuli, the observed fluorescence changes in cells were small, with fluorescence ratios increasing by less than 1x compared to control. Moreover, the authors did not provide the corresponding live-cell imaging data for Figures 2m, 3m, 4m, and 6m.

The authors should address this limitation by improving sensors accordingly, rather than broadly claiming suitability for high-resolution intracellular imaging. The claim of "high spatiotemporal resolution" is currently overstated.

2. Validation of biosensors for precise in vitro detection

In lines 152–158, the authors simulate phenylketonuria (PKU) conditions by adding 1 or 3 mM L-phenylalanine to healthy serum and urine samples. However, physiological upper limits for serum L-phenylalanine in PKU diagnosis are around 120 μM . Using 1 mM greatly exceeds this range and undermines the relevance of the test. Therefore, validation experiments should be conducted within a physiologically meaningful range (e.g., ~50–200 μM) to better reflect clinical conditions and to demonstrate the practical utility of the biosensor in realistic diagnostic scenarios. Additionally, while validation with HPLC was performed for Valsor and Ilesor, details on the sample matrices used are lacking. Were interfering substances added to the same sample or to different ones? If only a single sample was tested, the conclusion about robustness of detection may be premature. Furthermore, no standard curve information was provided for the biosensors, making it difficult to assess their quantitative agreement with HPLC measurements.

3. Structure and logic of the manuscript

Currently, each sensor is presented as a separate, largely independent module. While this is understandable, it dilutes the central message. Since the authors emphasize that they have developed a "versatile method for constructing metabolite GEFBs using regulatory domains of allosteric enzymes," the structure should reflect a unified workflow: (1) selection of regulatory domain, (2) identification of insertion sites, (3) linker optimization, (4) functional testing. A schematic summarizing this generalizable design pipeline would be highly valuable. In addition, rather than offering superficial validation of all four sensors, the authors might consider presenting in-depth application of one sensor (e.g., optimization for better intracellular resolution or more rigorous in vitro testing) to highlight the method's potential.

4. cpSFYFP insertion strategy

It would be helpful to know whether any general rules emerged regarding cpSFYFP insertion sites in different regulatory domains. Can these be clustered in conserved motifs or loops? A summary of insertion-site mapping would be valuable for guiding future efforts.

Minor Points:

1. Figure 1i and Figure 2d show redundant ligand specificity data for Phesor. One could be moved to the supplementary materials.
2. Line 267: Please spell out CBS domain in full on first mention.
3. Lines 74-75 state "two specific biosensors for the previously unavailable distinction of L-isoleucine and L-valine." However, Wang et al. (Nat Commun, 2023, 14:6680) had already reported a biosensor for L-isoleucine using a similar approach. This prior work should be acknowledged.

In summary, while the conceptual framework of using allosteric regulatory domains as sensing modules for GEFBs is valuable, the practical implementation in this manuscript requires more rigorous validation and clearer focus to meet the novelty and impact expectations of Nature Communications.

Reviewer #2

(Remarks to the Author)

Kang et al. report the development of genetically encoded fluorescent biosensors for L-phenylalanine, L-valine, L-isoleucine, and S-adenosyl-L-methionine (SAM), named Phesor, Valsor, Ilesor, and SAMsor, respectively. The authors use the regulatory domains of enzymes as sensing domains to engineer these biosensors. They develop and characterize the biosensors, demonstrating their capability to detect changes in target concentrations both in vitro and in living mammalian cells. The authors suggest that these biosensors may significantly contribute to the field by providing a novel platform for biosensor development.

The manuscript is well-written and contributes valuable insights into biosensor technology, making it a strong candidate for publication in Nature Communications. However, I recommend addressing the following major and minor concerns prior to publication:

Major Issues:

1. Lines 111–112: the authors note that the PheAACT, used as a sensing domain in Phesor, forms a dimer. This raises questions about whether L-phenylalanine binding to one Phesor monomer might affect the other. Clarifying whether this interaction influences the binding dynamics and reporting the Hill coefficient for L-phenylalanine would be informative. An analysis of the oligomeric state of each biosensor would enhance the manuscript.
2. Lines 137–138: Discrepancies in the reported excitation (405/488 nm) and emission (528 nm) peaks of Phesor compared to Supplementary Fig. 3a need correction. Furthermore, similar spectral data for Valsor, Ilesor, and SAMsor should also be included as it is fundamental for characterizing these biosensors.
3. The manuscript presents fluorescence response and kinetics data upon target concentration increase but lacks corresponding data for decreases. Wash-out method used in this study lacks temporal information for turn-off response. Demonstrating reversible behavior through additional kinetic studies for the turn-off response would provide a more comprehensive understanding of the biosensors' functionality.
4. Figure 6e indicates that SAMsor does not fully recover its initial fluorescence intensity in the absence of SAM, suggesting it is not fully reversible. The implications of this for practical applications should be discussed.
5. The claimed stability of biosensor fluorescence across pH changes is based on a limited range (6.6–8.2). Expanding this range (e.g., 5–10) and reporting the pKa values of each biosensor (and cpSFYFP control) would substantiate the authors' claims more robustly.
6. The authors apply each biosensor in live HEK293FT cells: Phesor (Fig. 2m), Valsor (Fig. 3m), Ilesor (Fig. 4m), and SAMsor (Fig. 6m). Pharmacological stimulations can perturb intracellular pH. To more rigorously demonstrate that these biosensors specifically enable monitoring the change in target concentrations in living mammalian cells, the authors should show the same data set using cpSFYFP control as well.

7. The statistical methods currently applied need reconsideration. For the multiple comparisons made in Figs. 1e,f,g,h,i, 2m, 3i,m, 4i,m, 5c, 6i,m, analysis of variance (ANOVA) followed by appropriate post hoc tests should be used to ensure robust statistical validity.

8. While the dissociation constants (K_ds) of each biosensor are reported, a discussion relating these values to the physiological and pathological concentrations of the metabolites in bacteria and mammalian cells would contextualize the biosensors' applicability.

9. Regarding the specificity, Figs. 2d, 3f, and 4f are based on 1 mM metabolites. However, Phesor, Valsor, and Ilesor are not saturated in 1 mM L-phenylalanine, L-valine, L-isoleucine, respectively. Thus, the authors should show the data of 10 mM metabolites for the characterization of specificity of these biosensors. Especially, Fig. 4f indicates that L-valine and L-leucine induce substantial fluorescence response of Ilesor. This fact raises concern on the specificity of Ilesor for the BCAAs. The authors should show the titration data of Ilesor for L-valine and L-leucine. Unexpectedly, Fig. 6d is based on different concentrations of each metabolite. SAM at 200 μ M is not enough to saturate the SAMsor's fluorescence response. The authors should show the data of 1 mM SAM and other metabolites. If there are substantial fluorescence response of SAMsor upon 1 mM of MTA, SAH, and Ado, the authors should show the titration data of SAMsor for these metabolites as well.

Minor Issues:

1. Line 430–431: The authors mention “The regulatory domains of allosteric enzymes can also be integrated with signal transduction systems like Förster resonance energy transfer (FRET) or bioluminescence resonance energy transfer (BRET) to develop other types of biosensors”. This claim is not experimentally validated in this study and should be moved from the summary to a more speculative section in the Discussion.

2. Line 583: The title of the method section is “Characterization of Phesor, Valsor, Ilesor, and SAMsor in living bacteria”. However, in this method, the bacteria are not live due to permeabilization process. The sample prepared in this protocol should be just called crude extract protein. The authors should revise the main text and Methods accordingly.

3. Line 663: Related to the comment 2, the authors describe as “Live-cell imaging of HEK293FT cells expressing either...”. However, in this protocol, HEK293FT cells are dead cells due to permeabilization with digitonin. The authors should revise the main text and Methods accordingly.

4. Line 652: Details regarding the species for which codon optimization was performed should be clarified.

5. Line 701: “(Schrödinger, USA)” should be “(Schrödinger, USA)”.

Reviewer #3

(Remarks to the Author)

This study presents a novel method for developing genetically encoded fluorescent biosensors (GEFBs) using the regulatory domains of allosteric enzymes as biorecognition elements. The authors demonstrate the versatility of this approach by constructing biosensors for four specific metabolites, L-phenylalanine, L-valine, L-isoleucine, and SAM. The study highlights the potential of allosteric regulatory domains as versatile tools for biosensor design, providing a way of biosensor development and addressing gaps in metabolite detection.

From the authors' description and the experimental results of this work, I do not particularly see the urgency of developing these metabolite biosensors and have not understood what specific situations would require the use of the biosensors developed in this work. About half of the results in this work are from bacteria, what are the advantages of using these biosensors compared to traditional methods such as HPLC in these common systems? The authors claim that these biosensors can monitor their targeted metabolites with high spatiotemporal resolution, but there are not results regarding the spatial resolution. The imaging results obtained in cultured HEK293FT cells do not distinguish subcellular localization, which did not showcase the advantage of fluorescence biosensors in providing spatial information under microscopic imaging.

In general, only experiments conducted in living organisms are referred to as “in vivo.” It may not be quite appropriate to refer to the results obtained in cultured cells as “in vivo” and the entire field might be more interested in seeing the application of these powerful sensors for metabolite imaging and detection in higher systems. If these biosensors are only applied in bacteria and cultured cells, the significance of this work will be greatly limited. If there are unique advantages of the biosensors in in vitro diagnostics, this work lacks related results in this regard to demonstrate that these biosensors can indeed play an important role in the actual testing of patient samples.

Other comments:

Fig. 1c, 2h, 3b, 4b, 5a, and 6h demonstrates the detailed metabolic pathways associated with these metabolites to be detected. However, these figures contain a large number of components that are irrelevant to the sensors, and their content is not the main findings of this work. I doubt whether it is necessary to retain these in the main figures.

The development and characterization are similar between different sensors, while some details are not clearly described such as the residues mutated in the evolution.

The on-kinetics of Phesor are better. Slow kinetics of Valsor and Ilesor

In Fig. 2g, 3h, 4h, and 6g, it seems that the on-kinetics of Phesor and SAMsor are good while those of Valsor and Ilesor are relatively slow. Moreover, the apparent K_d of Valsor and Ilesor are also higher (over 2 mM). Is it possible to discuss the

reasons for such differences based on the inherent properties of the allosteric domain used for sensor development, thus provide theoretical bases for improving the kinetics and affinity of these sensors?

Reviewer #4

(Remarks to the Author)

This is a thoroughly interesting manuscript. The regulatory domains of allosteric enzymes are used as the binding domain in fluorescent probes by fusing to cpSFYFP. They have shown that they can elicit fluorescence response to the allosteric domain binding ligands. What is attractive about this work is the versatility they have shown. They have used ACT domains from both homo and heteromeric enzyme assemblies, used a LeuA domain and used a multiligand binding CBS domain. Not only have they shown that these engineered proteins show sensitivity in their fluorescent response to both in vitro and in vivo.

I am very supportive of the publication of these results and believe they are of wide interest to those interested in protein engineering and protein based sensors. Such approaches have a wide range of useful applications - many of which are referenced in this manuscript.

There are a number of aspects of this manuscript that warrant attention before publication. Many of these comments relate to the relatively scant experimental detail that is provided.

1. While the response to the ligands is clear, the dissociation constants measured through fluorescence are high and the levels of ligands used in these assays are generally high - in the mM range. I'm aware that many of these allosteric domains should have micro molar dissociation constants. There is not comment on the disruption to binding. While the mutagenesis results support the binding sites are indeed intact I would be more comfortable with some alternative assessment of binding and binding stoichiometry - also see comments below about quaternary structure.

2. I found it frustrating that I could not be certain of the sequence of many of the constructs used in the study - in some instances it wasn't precisely clear what the engineered proteins were or what had been optimised through random mutagenesis.

3. These AA binding domains do rely on the appropriate quaternary structure to form binding sites. There is no comment on this or assessment of quaternary structure of any of the sensors. As quaternary structure for some of these relies on the catalytic domain interfaces is it possible that appropriate quaternary structures have not assembled - or does this relate to the poor dissociation constants? I'm concerned about the impact of protein conc on relative fluorescence here and would like to see results that demonstrate predictable response.

4. I had some concerns over the use of normalised fluorescence data - while this was useful for some presentations - it felt like it was hiding the absolute changes that should be available for inspection in the supplementary material.

5. Repeat experiments - I was unsure of the nature of the replicates given in some places - in particular for the cellular work were these biological replicates?

6. All the measurements must be reported to the appropriate significant figures that correspond to the accuracy of the measurement. Sometimes 5 or 6 sf are used in measurements or errors! Reporting figures such as 1659.85 ± 110.02 or 444.08 ± 97.38 just suggests that the accuracy of the data is not well understood. Sometimes also % values in the text are reported without errors which makes the excess significant figures even more misleading.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

Reviewer #1 (Remarks to the Author):

I would like to thank the authors for their comprehensive response and the immense effort put into revising the manuscript. The addition of extensive new data, particularly the development of the high-affinity SAMsorL, the detailed subcellular imaging, and the validation with authentic clinical samples, has significantly strengthened the work.

Regarding my previous Major Concern 1 (Suitability for intracellular imaging), the new subcellular localization experiments convincingly demonstrate that these sensors can resolve metabolite fluctuations in specific organelles. This effectively highlights the unique value of these genetically encoded sensors over traditional methods, addressing my concerns regarding spatiotemporal resolution. Similarly, my concerns regarding the manuscript's structure and the generalizability of the design strategy (previous Major Concerns 3 and 4) have been well addressed. The inclusion of a unified workflow schematic successfully articulates the design pipeline, enhancing the logical flow. I also appreciate the detailed analysis of insertion sites relative to secondary structures and ligand-binding pockets. This analysis provides valuable insights into the

structure-function relationship—specifically how the distance between the insertion site and the binding pocket modulates affinity—thereby offering a clearer guide for future engineering efforts.

However, regarding the validation of biosensors for in vitro detection, I have a remaining specific concern regarding the characterization of the results. Specifically, the claim that the current L-phenylalanine sensor (Phesor) is suitable for "rapid" and "point-of-care diagnostics" is not supported by the methods described.

In the Discussion (Lines 515–519), the authors state: "Compared to traditional HPLC- or mass spectrometry-based methods, Phesor is easy to handle, cost-effective, rapid, and compatible with high-throughput assays (Supplementary Table 5), making it an ideal tool for point-of-care diagnostics of l-phenylalanine-related diseases." However, a close examination of the "Preparation of human body fluid samples" section (Lines 792–800) reveals that the sample preparation for dried blood spots involves "sonicating for 20 min," "stirring overnight," and "completely drying in a vacuum centrifuge" before resuspension. A workflow requiring overnight stirring and bulky equipment fundamentally contradicts the definition of "rapid" and "point-of-care diagnostics". It appears that this laborious processing is necessary to concentrate the samples because the selected Phesor variant ($K_d \approx 1.7$ mM) has a relatively low affinity compared to the physiological phenylalanine levels in healthy or mild-PKU blood samples (~ 50 – 200 μ M). I note that the authors identified a high-affinity variant during screening (Phesor-0.04, $K_d \approx 57$ μ M) but chose to optimize the lower-affinity Phesor-0.38 variant. While the resulting Phesor ($K_d \approx 1.7$ mM) has a high dynamic range suitable for intracellular imaging of elevated metabolite levels, its use for serum detection necessitates the extensive sample pre-concentration mentioned above.

While the experimental validation is now sufficient and demonstrates the sensor's robustness in complex matrices, the textual claims regarding "easy to handle" and "point-of-care diagnostics" in the Discussion (specifically Lines 515–519) are not supported by the described workflow and should be rephrased. The current workflow does not offer a timeline advantage over traditional HPLC methods, and acknowledging this limitation would provide a more balanced view for the readers.

Minor Issue:

On page 55 of the Supplementary Information file, Supplementary Figure 37 appears to be displayed incompletely or cut off. Please check and correct this in the final version.

In summary, the manuscript is now of high quality and fits the scope of Nature Communications. With the textual modifications suggested above to clarify the practical positioning of the in vitro assay, I support the publication of this work.

Reviewer #2

(Remarks to the Author)

The authors have thoroughly revised the manuscript in response to the reviewers' comments. The revisions have substantially improved the quality, clarity, and rigor of the study. The reviewer is supportive of publication in Nature Communications. However, several points should be further addressed to strengthen the manuscript.

Major issue #4.

The authors now convincingly demonstrate the complete reversibility of SAMsor using purified protein with additional elution steps. This represents an important technical improvement. However, the process leading to this refinement, as well as the authors' speculation that the originally observed incomplete reversibility may have resulted from residual SAM remaining in the assay system, would be highly valuable information for end users.

To provide transparency and practical guidance, the original dataset (Fig. 6e in the initial submission) should be retained in the revised manuscript, accompanied by a clear explanation of the experimental optimization and the rationale for the updated results. Including both versions would enhance the manuscript's instructional value and reproducibility.

Major issue #5.

The reported pKa values of each biosensor in the presence and absence of ligand are very similar. It is important to clarify whether this observation is consistent with the large ratiometric response (F_{488}/F_{405}) observed around pH ~ 7 (Fig. 3e).

If the pKa values remain largely unchanged upon ligand binding, this would suggest that the primary mechanism of fluorescence modulation is unlikely to arise from a shift in the equilibrium between the neutral and anionic chromophore states. Instead, the response may predominantly reflect changes in extinction coefficient, quantum yield, chromophore environment, or other structural factors.

To clarify this point, the authors should present full pH titration curves for each biosensor in both the ligand-bound and ligand-free states (currently referenced in Fig. 3h), rather than reporting only the ligand-bound state. These curves would allow readers to directly evaluate whether ligand binding alters the protonation equilibrium or instead modulates fluorescence through alternative mechanisms.

An expanded discussion addressing the likely molecular basis of the fluorescence response in these four biosensors would further strengthen the mechanistic insight of the study.

Reviewer #3

(Remarks to the Author)

The authors have thoroughly addressed the concerns raised in the first round of review. I particularly appreciate the addition of applying GEFBs in both clinical and bioprocess contexts, as well as the development of sensor variants with

compartment-specific localization. These revisions have significantly strengthened the manuscript. That said, I still have several comments that could further improve the clarity of the manuscript and enhance the presentation of the tools' capabilities.

Major comments:

1. The authors used degenerate primers for random linker mutations. The authors should provide more details on the nature of these mutations (such as linker length and amino acid compositions) and the theoretical diversity of a mutation library. For most GEFBs, only several hundred variants were screened, whereas six randomized linker residues could in principle generate up to 20^6 possible combinations.
2. The authors demonstrate that the ratiometric GEFB responses are independent of sensor concentration. To further support this point, I suggest including the corresponding single-channel fluorescence responses for comparison.
3. What is the difference between $\Delta R_{\max}$ and $\Delta R/R_{\min}$, as both metrics are frequently used throughout the manuscript? I also notice that some sensor variants exhibit negatively-going responses, such as Phesor-0.38, Valsor-0.03, MTAsor-0.64 and SAMsorL. For such sensors, reporting $\Delta R_{\max}$ and $\Delta R/R_{\min}$ may be less appropriate, particularly for imaging in living cells or for quantifying exogenous ligands, since achieving $R_{\min}$ requires saturate ligand concentrations, which are usually hard to reach. I therefore suggest present $\Delta R/R_0$ (R without ligands) instead of $\Delta R_{\max}$ in the dose-response curves like Fig. 7d and in high-throughput screening results like Fig. 7c.
4. The authors have increased the utility of these tools by generating GEFB variants targeted to different subcellular compartments, which revealed lower amino acid / SAM levels in mitochondria compared with the cytosol and nucleus. To further highlight the advantage of high spatiotemporal resolution, I suggest using these compartment-localized sensors to monitor spatial and temporal differences in drug-induced amino acid fluctuations through time-lapse imaging. For example, the subcellularly targeted Ilesor and Valsor could be used to examine valine and isoleucine dynamics in response to gabapentin or octyl-D-2-HG, or different SAMsor variants could be applied to track SAHA- and AG-270-regulated SAM synthesis. Such experiments could potentially provide additional insight into the compartment-specific and kinetic features of valine/isoleucine/SAM metabolism.
5. In the table comparing detection performance between HPLC and biosensors, the authors say that HPLC needs $> 100 \mu\text{L}$ sample, which is unusual given the very high sensitivity of HPLC. I suggest revisiting this point for accuracy. Additionally, it may be more informative for readers to compare the concentration detection ranges (or limits of detection) of these approaches, rather than focusing on sample volume requirements.

Minor comments:

1. In Fig. 1b, do the colors represent different secondary structures? If so, this should be explained in the figure legend.
2. In line 97, "feedback inhibits the activities of PheAcM and PheAPDT" should be "act as a feedback inhibitor of PheACM and PheAPDT activities".
3. For the high-throughput screening results (e.g., Fig. 2b, 3c, 4c, 6b and 7c), it would improve clarity to label the starting and final versions. For example, in Fig. 2b, the variants corresponding to Phesor-0.38 and the final Phesor could be explicitly marked.
4. In line 376-379, the descriptions of "diminished" and "abolished" response appear to be reversed, as mutated MTAsor-0.64 still show some response to MTA. Besides, the two panels lack statistic comparison (at least no significance indicators are labeled), and therefore stating "significantly" is not appropriate.
5. In Fig. 6e, the authors use $100 \mu\text{M}$ MTA and $10 \mu\text{M}$ SAH to demonstrate the selectivity of SAMor. However, even $10 \mu\text{M}$ MTA induce a modest response. According to Supplementary Fig. 40, the physiological concentrations of MTA, adenosine and SAH fall within a similar range. Therefore, it will be more appropriate to use SAH at $100 \mu\text{M}$ in Fig. 6e for a more balanced comparison.
6. In heatmaps like supplementary Fig. 4a, the legend of $\Delta R_{\max}$ is missing a "%" symbol. In addition, I can not find such heatmaps for Valsor development.
7. The upper parts of some supplementary figures including Fig. 2, 4 and 37 appear to be cropped or incomplete. For example, the label " $\Delta R_{\max}$ " in supplementary Fig. 4 is partially obscured.
8. Using structural modeling, the authors found that the low-Kd variants tend to have cpSFYFP insertion sites distal to the ligand-binding pockets. It will be clearer to highlight the ligand-binding sites in the structures. In addition, the figure legends should specify whether these structures were obtained from experimental PDB entries or predicted (e.g., by AF3).
9. The authors state that PLIP website was used for binding site prediction for PheA. Was the same approach applied to the other sensors as well?
10. Compared with the 12-hour permeabilization shown in Fig. 4h of the original manuscript, the signal kinetics after 24-hour permeabilization in current Fig. 4e seems even slower. How to explain this unexpected result?
11. AlphaFold3 is not a simple homology modeling tool. Please use the term "structural modeling" or "structural prediction" instead of "homology modeling".

Reviewer #4

(Remarks to the Author)

This manuscript has undergone a thorough revision in response to the reviewers comments. The manuscript has undergone considerable change addressing my primary concerns about the data that had been presented. I believe appropriate data is now included in both the main manuscript and the supplementary information to allow for publication.

Version 2:

Reviewer comments:

Reviewer #2

(Remarks to the Author)

The authors have carefully and comprehensively revised the manuscript in response to the reviewer's comments. The revised version is scientifically sound, clearly presented, and significantly improved in both rigor and clarity. I am fully convinced that this work will have a substantial impact on the field of biosensor engineering, and I strongly support its publication in Nature Communications.

Reviewer #3

(Remarks to the Author)

The authors have provided a thorough, point-by-point response to all my concerns and have incorporated the requested additional data. The revised manuscript is now robust and presents a complete narrative. The work is in an excellent state for publication, and I would like to thank the authors for their diligence and hard work.

Open Access This Peer Review File is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made.

In cases where reviewers are anonymous, credit should be given to 'Anonymous Referee' and the source.

The images or other third party material in this Peer Review File are included in the article's Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder.

To view a copy of this license, visit <https://creativecommons.org/licenses/by/4.0/>

Reviewers' comments:

Reviewer #1 (Remarks to the Author):

This manuscript presents a strategy for constructing genetically encoded fluorescent biosensors (GEFBs) using the regulatory domains of allosteric enzymes as ligand-binding elements. Specifically, the authors inserted cpSFYFP into the regulatory domains of chorismate mutase, 2-acetolactate synthase, and D-citramalate synthase to generate sensors for L-phenylalanine, L-valine, and L-isoleucine. The authors also developed SAM and MTA sensors by inserting cpSFYFP into the CBS domain, a multi-ligand-binding regulatory domain. While the use of regulatory domains from allosteric enzymes as novel ligand-binding modules is a conceptual advance that adds diversity to the biosensor toolkit, I have several substantial concerns about the overall novelty and practical applicability of the work in its current form.

Major Concerns:

1. Suitability of the sensors for intracellular imaging

The authors claim that the four biosensors can be used to monitor intracellular metabolite levels with high spatiotemporal resolution. However, the reported K_d values are relatively high: ~1660 μM (Phesor), ~2300 μM (Valsor), ~3600 μM (Ilesor), and ~200 μM (SAMsor). In contrast, the physiological concentrations of these metabolites in mammalian cells are much lower: for instance, L-phenylalanine, valine, and isoleucine typically range from tens to several hundreds of μM (ACS Sensors, 2019, 4(12): 3333–3342), and SAM is typically around 300 μM (Communications Biology, 2022, 5(1): 313). Among the four sensors, only SAMsor exhibits a K_d value that approaches the physiological concentration range of its target metabolite. However, even in this case, Figures 6i and 6m show that upon stimulation, the fluorescence changes in *E. coli* and HEK293FT cells compared to controls were still less than one-fold. For the other three sensors—Phesor, Valsor, and Ilesor—whose K_d values are several-fold higher than the physiological concentrations of their respective target metabolites, Figures 2m, 3m, and 4m show that under the relevant stimuli, the observed fluorescence changes in cells were small, with fluorescence ratios increasing by less than 1x compared to control. Moreover, the authors did not provide the corresponding live-cell

imaging data for Figures 2m, 3m, 4m, and 6m.

The authors should address this limitation by improving sensors accordingly, rather than broadly claiming suitability for high-resolution intracellular imaging. The claim of “high spatiotemporal resolution” is currently overstated.

2. Validation of biosensors for precise *in vitro* detection

In lines 152–158, the authors simulate phenylketonuria (PKU) conditions by adding 1 or 3 mM L-phenylalanine to healthy serum and urine samples. However, physiological upper limits for serum L-phenylalanine in PKU diagnosis are around 120 μ M. Using 1 mM greatly exceeds this range and undermines the relevance of the test. Therefore, validation experiments should be conducted within a physiologically meaningful range (e.g., ~50–200 μ M) to better reflect clinical conditions and to demonstrate the practical utility of the biosensor in realistic diagnostic scenarios. Additionally, while validation with HPLC was performed for Valsor and Ilesor, details on the sample matrices used are lacking. Were interfering substances added to the same sample or to different ones? If only a single sample was tested, the conclusion about robustness of detection may be premature. Furthermore, no standard curve information was provided for the biosensors, making it difficult to assess their quantitative agreement with HPLC measurements.

3. Structure and logic of the manuscript

Currently, each sensor is presented as a separate, largely independent module. While this is understandable, it dilutes the central message. Since the authors emphasize that they have developed a “versatile method for constructing metabolite GEFBs using regulatory domains of allosteric enzymes,” the structure should reflect a unified workflow: (1) selection of regulatory domain, (2) identification of insertion sites, (3) linker optimization, (4) functional testing. A schematic summarizing this generalizable design pipeline would be highly valuable. In addition, rather than offering superficial validation of all four sensors, the authors might consider presenting in-depth application of one sensor (e.g., optimization for better intracellular resolution or more rigorous *in vitro* testing) to highlight the method’s potential.

4. cpSFYFP insertion strategy

It would be helpful to know whether any general rules emerged regarding cpSFYFP insertion sites in different regulatory domains. Can these be clustered in conserved motifs or

loops? A summary of insertion-site mapping would be valuable for guiding future efforts.

Minor Points:

1. Figure 1i and Figure 2d show redundant ligand specificity data for Phesor. One could be moved to the supplementary materials.
2. Line 267: Please spell out CBS domain in full on first mention.
3. Lines 74-75 state “two specific biosensors for the previously unavailable distinction of L-isoleucine and L-valine.” However, Wang *et al.* (Nat Commun, 2023, 14:6680) had already reported a biosensor for L-isoleucine using a similar approach. This prior work should be acknowledged.

In summary, while the conceptual framework of using allosteric regulatory domains as sensing modules for GEFBs is valuable, the practical implementation in this manuscript requires more rigorous validation and clearer focus to meet the novelty and impact expectations of *Nature Communications*.

Reviewer #2 (Remarks to the Author):

Kang *et al.* report the development of genetically encoded fluorescent biosensors for L-phenylalanine, L-valine, L-isoleucine, and S-adenosyl-L-methionine (SAM), named Phesor, Valsor, Ilesor, and SAMsor, respectively. The authors use the regulatory domains of enzymes as sensing domains to engineer these biosensors. They develop and characterize the biosensors, demonstrating their capability to detect changes in target concentrations both *in vitro* and in living mammalian cells. The authors suggest that these biosensors may significantly contribute to the field by providing a novel platform for biosensor development.

The manuscript is well-written and contributes valuable insights into biosensor technology, making it a strong candidate for publication in *Nature Communications*. However, I recommend addressing the following major and minor concerns prior to publication:

Major Issues:

1. Lines 111–112: the authors note that the Phe_{ACT}, used as a sensing domain in Phesor, forms a dimer. This raises questions about whether L-phenylalanine binding to one Phesor monomer might affect the other. Clarifying whether this interaction influences the

binding dynamics and reporting the Hill coefficient for L-phenylalanine would be informative. An analysis of the oligomeric state of each biosensor would enhance the manuscript.

2. Lines 137–138: Discrepancies in the reported excitation (405/488 nm) and emission (528 nm) peaks of Phesor compared to Supplementary Fig. 3a need correction. Furthermore, similar spectral data for Valsor, Ilesor, and SAMsor should also be included as it is fundamental for characterizing these biosensors.

3. The manuscript presents fluorescence response and kinetics data upon target concentration increase but lacks corresponding data for decreases. Wash-out method used in this study lacks temporal information for turn-off response. Demonstrating reversible behavior through additional kinetic studies for the turn-off response would provide a more comprehensive understanding of the biosensors' functionality.

4. Figure 6e indicates that SAMsor does not fully recover its initial fluorescence intensity in the absence of SAM, suggesting it is not fully reversible. The implications of this for practical applications should be discussed.

5. The claimed stability of biosensor fluorescence across pH changes is based on a limited range (6.6–8.2). Expanding this range (e.g., 5–10) and reporting the pK_a values of each biosensor (and cpSFYFP control) would substantiate the authors' claims more robustly.

6. The authors apply each biosensor in live HEK293FT cells: Phesor (Fig. 2m), Valsor (Fig. 3m), Ilesor (Fig. 4m), and SAMsor (Fig. 6m). Pharmacological stimulations can perturb intracellular pH. To more rigorously demonstrate that these biosensors specifically enable monitoring the change in target concentrations in living mammalian cells, the authors should show the same data set using cpSFYFP control as well.

7. The statistical methods currently applied need reconsideration. For the multiple comparisons made in Figs. 1e,f,g,h,i, 2m, 3i,m, 4i,m, 5c, 6i,m, analysis of variance (ANOVA) followed by appropriate post hoc tests should be used to ensure robust statistical validity.

8. While the dissociation constants (K_{ds}) of each biosensor are reported, a discussion relating these values to the physiological and pathological concentrations of the metabolites in bacteria and mammalian cells would contextualize the biosensors' applicability.

9. Regarding the specificity, Figs. 2d, 3f, and 4f are based on 1 mM metabolites.

However, Phesor, Valsor, and Ilesor are not saturated in 1 mM L-phenylalanine, L-valine, L-isoleucine, respectively. Thus, the authors should show the data of 10 mM metabolites for the characterization of specificity of these biosensors. Especially, Fig. 4f indicates that L-valine and L-leucine induce substantial fluorescence response of Ilesor. This fact raises concern on the specificity of Ilesor for the BCAAs. The authors should show the titration data of Ilesor for L-valine and L-leucine. Unexpectedly, Fig. 6d is based on different concentrations of each metabolite. SAM at 200 μ M is not enough to saturate the SAMsor's fluorescence response. The authors should show the data of 1 mM SAM and other metabolites. If there are substantial fluorescence response of SAMsor upon 1 mM of MTA, SAH, and Ado, the authors should show the titration data of SAMsor for these metabolites as well.

Minor Issues:

1. Line 430–431: The authors mentions “The regulatory domains of allosteric enzymes can also be integrated with signal transduction systems like Förster resonance energy transfer (FRET) or bioluminescence resonance energy transfer (BRET) to develop other types of biosensors”. This claim is not experimentally validated in this study and should be moved from the summary to a more speculative section in the Discussion.

2. Line 583: The title of the method section is “Characterization of Phesor, Valsor, Ilesor, and SAMsor in living bacteria”. However, in this method, the bacteria are not live due to permeabilization process. The sample prepared in this protocol should be just called crude extract protein. The authors should revise the main text and Methods accordingly.

3. Line 663: Related to the comment 2, the authors describe as “Live-cell imaging of HEK293FT cells expressing either...”. However, in this protocol, HEK293FT cells are dead cells due to permeabilization with digitonin. The authors should revise the main text and Methods accordingly.

4. Line 652: Details regarding the species for which codon optimization was performed should be clarified.

5. Line 701: “((Schrödinger, USA))” should be “(Schrödinger, USA)”.

Reviewer #3 (Remarks to the Author):

This study presents a novel method for developing genetically encoded fluorescent

biosensors (GEFBs) using the regulatory domains of allosteric enzymes as biorecognition elements. The authors demonstrate the versatility of this approach by constructing biosensors for four specific metabolites, L-phenylalanine, L-valine, L-isoleucine, and SAM. The study highlights the potential of allosteric regulatory domains as versatile tools for biosensor design, providing a way of biosensor development and addressing gaps in metabolite detection.

From the authors' description and the experimental results of this work, I do not particularly see the urgency of developing these metabolite biosensors and have not understood what specific situations would require the use of the biosensors developed in this work. About half of the results in this work are from bacteria, what are the advantages of using these biosensors compared to traditional methods such as HPLC in these common systems? The authors claim that these biosensors can monitor their targeted metabolites with high spatiotemporal resolution, but there are not results regarding the spatial resolution. The imaging results obtained in cultured HEK293FT cells do not distinguish subcellular localization, which did not showcase the advantage of fluorescence biosensors in providing spatial information under microscopic imaging.

In general, only experiments conducted in living organisms are referred to as "*in vivo*." It may not be quite appropriate to refer to the results obtained in cultured cells as "*in vivo*" and the entire field might be more interested in seeing the application of these powerful sensors for metabolite imaging and detection in higher systems. If these biosensors are only applied in bacteria and cultured cells, the significance of this work will be greatly limited. If there are unique advantages of the biosensors in *in vitro* diagnostics, this work lacks related results in this regard to demonstrate that these biosensors can indeed play an important role in the actual testing of patient samples.

Other comments:

Fig. 1c, 2h, 3b, 4b, 5a, and 6h demonstrates the detailed metabolic pathways associated with these metabolites to be detected. However, these figures contain a large number of components that are irrelevant to the sensors, and their content is not the main findings of this work. I doubt whether it is necessary to retain these in the main figures.

The development and characterization are similar between different sensors, while some

details are not clearly described such as the residues mutated in the evolution.

The on-kinetics of Phesor are better. Slow kinetics of Valsor and Ilesor

In Fig. 2g, 3h, 4h, and 6g, it seems that the on-kinetics of Phesor and SAMsor are good while those of Valsor and Ilesor are relatively slow. Moreover, the apparent K_d of Valsor and Ilesor are also higher (over 2 mM). Is it possible to discuss the reasons for such differences based on the inherent properties of the allosteric domain used for sensor development, thus provide theoretical bases for improving the kinetics and affinity of these sensors?

Reviewer #4 (Remarks to the Author):

This is a thoroughly interesting manuscript. The regulatory domains of allosteric enzymes are used as the binding domain in fluorescent probes by fusing to cpSFYFP. They have shown that they can elicit fluorescence response to the allosteric domain binding ligands. What is attractive about this work is the versatility they have shown. They have used ACT domains from both homo and heteromeric enzyme assemblies, used a LeuA domain and used a multiligand binding CBS domain. Not only have they shown that these engineered proteins show sensitivity in their fluorescent response to both *in vitro* and *in vivo*.

I am very supportive of the publication of these results and believe they are of wide interest to those interested in protein engineering and protein based sensors. Such approaches have a wide range of useful applications - many of which are referenced in this manuscript.

There are a number of aspects of this manuscript that warrant attention before publication. Many of these comments relate to the relatively scant experimental detail that is provided.

1. While the response to the ligands is clear, the dissociation constants measured through fluorescence are high and the levels of ligands used in these assays are generally high - in the mM range. I'm aware that many of these allosteric domains should have micro molar dissociation constants. There is no comment on the disruption to binding. While the mutagenesis results support the binding sites are indeed intact I would be more comfortable with some alternative assessment of binding and binding stoichiometry - also see comments below about quaternary structure.

2. I found it frustrating that I could not be certain of the sequence of many of the

constructs used in the study - in some instances it wasn't precisely clear what the engineered proteins were or what had been optimised through random mutagenesis.

3. These AA binding domains do rely on the appropriate quaternary structure to form binding sites. There is no comment on this or assessment of quaternary structure of any of the sensors. As quaternary structure for some of these relies on the catalytic domain interfaces is it possible that appropriate quaternary structures have not assembled - or does this relate to the poor dissociation constants? I'm concerned about the impact of protein conc on relative fluorescence here and would like to see results that demonstrate predictable response.

4. I had some concerns over the use of normalised fluorescence data - while this was useful for some presentations - it felt like it was hiding the absolute changes that should be available for inspection in the supplementary material.

5. Repeat experiments - I was unsure of the nature of the replicates given in some places - in particular for the cellular work were these biological replicates?

6. All the measurements must be reported to the appropriate significant figures that correspond to the accuracy of the measurement. Sometimes 5 or 6 sf are used in measurements or errors! Reporting figures such as 1659.85 ± 110.02 or 444.08 ± 97.38 just suggests that the accuracy of the data is not well understood. Sometimes also % values in the text are reported without errors which makes the excess significant figures even more misleading.

Responses to the reviewers' comments point-by-point:

We sincerely thank the reviewers for their constructive comments and thoughtful suggestions, which have been very helpful in improving our manuscript. We have carefully considered all comments and revised the manuscript accordingly. A detailed point-by-point response to each comment is provided below:

To Reviewer #1:

This manuscript presents a strategy for constructing genetically encoded fluorescent biosensors (GEFBs) using the regulatory domains of allosteric enzymes as ligand-binding elements. Specifically, the authors inserted cpSFYFP into the regulatory domains of chorismate mutase, 2-acetolactate synthase, and D-citramalate synthase to generate sensors for L-phenylalanine, L-valine, and L-isoleucine. The authors also developed SAM and MTA sensors by inserting cpSFYFP into the CBS domain, a multi-ligand-binding regulatory domain. While the use of regulatory domains from allosteric enzymes as novel ligand-binding modules is a conceptual advance that adds diversity to the biosensor toolkit, I have several substantial concerns about the overall novelty and practical applicability of the work in its current form.

Response: We sincerely appreciate your recognition of our work as a “conceptual advance that adds diversity to the biosensor toolkit”. We also thank you for your constructive criticism regarding the practical applicability and novelty, which have been crucial for expanding the scope and depth of our work. In accordance with your suggestions, we have performed extensive new experiments and rigorous data analyses in the revised manuscript. Our key responses are summarized as follows:

(1) Biosensor affinity. In the revised manuscript, we established a generalized method for strategically adjusting the affinity of allosteric regulatory domain-based GEFBs by manipulating the cpSFYFP insertion site. The affinities of the exemplified biosensors were all rationally designed to align with the physiological and pathological concentration ranges of target metabolites. In particular, we developed a new, highly sensitive SAM biosensor with a ~500-fold increase in affinity by screening over 2,800 variants, specifically for

clinical diagnostics. We also established a structural basis for tailoring GEFBs with predictable affinities. This structure-function relationship will expand the toolbox for metabolic imaging and *in vitro* diagnostics across varying concentration scales.

(2) Biosensor practical applicability in living cells. We have provided comprehensive characterization data, such as determining the maximum response magnitude, to demonstrate the robust performance of the developed biosensors in living cells. **Furthermore, we achieved subcellular localization for all four exemplified biosensors.** Through subcellular-level tracking of target metabolites, we have demonstrated their spatial resolution within living cells. Therefore, these biosensors enable the specific detection of L-phenylalanine, L-valine, L-isoleucine, and SAM in living cells with previously unavailable spatiotemporal resolution.

(3) Biosensor practical applicability *in vitro*. **Surpassing the request for rigorous *in vitro* testing of a single biosensor, we have comprehensively validated all developed biosensors.** Specifically, we have demonstrated **the utility of the L-phenylalanine biosensor and the newly developed highly sensitive SAM biosensor for point-of-care testing (POCT) of phenylketonuria and lung cancer using authentic clinical specimens.** These biosensors offer practical tools for the clinical diagnostics and therapeutic monitoring of related diseases. In addition, we have demonstrated **the utility of the L-valine biosensor and the L-isoleucine biosensor for continuous bioprocess monitoring using authentic fermentation systems.** Such biosensor-based high-throughput screening may accelerate the engineering of L-valine and L-isoleucine producing strains.

Overall, we believe the revised manuscript demonstrates substantial improvements in the “novelty and practical applicability”, and we hope that these revisions meet your satisfaction.

Major Concerns:

1. Suitability of the sensors for intracellular imaging

The authors claim that the four biosensors can be used to monitor intracellular metabolite levels with high spatiotemporal resolution. However, the reported K_d values are relatively high: $\sim 1660 \mu M$ (Phesor), $\sim 2300 \mu M$ (Valsor), $\sim 3600 \mu M$ (Ilesor), and $\sim 200 \mu M$

*(SAMsor). In contrast, the physiological concentrations of these metabolites in mammalian cells are much lower: for instance, L-phenylalanine, valine, and isoleucine typically range from tens to several hundreds of μM (ACS Sensors, 2019, 4(12): 3333–3342), and SAM is typically around 300 μM (Communications Biology, 2022, 5(1): 313). Among the four sensors, only SAMsor exhibits a K_d value that approaches the physiological concentration range of its target metabolite. However, even in this case, Figures 6i and 6m show that upon stimulation, the fluorescence changes in *E. coli* and HEK293FT cells compared to controls were still less than one-fold. For the other three sensors—Phesor, Valsor, and Ilesor—whose K_d values are several-fold higher than the physiological concentrations of their respective target metabolites, Figures 2m, 3m, and 4m show that under the relevant stimuli, the observed fluorescence changes in cells were small, with fluorescence ratios increasing by less than 1x compared to control. Moreover, the authors did not provide the corresponding live-cell imaging data for Figures 2m, 3m, 4m, and 6m.*

The authors should address this limitation by improving sensors accordingly, rather than broadly claiming suitability for high-resolution intracellular imaging. The claim of “high spatiotemporal resolution” is currently overstated.

Response: Thanks for your valuable and insightful comments. We sincerely apologize for the confusion caused by our insufficient discussion on aligning biosensor detection ranges with physiological concentrations. Herein, we respectfully note that the intracellular concentrations of these target metabolites may vary significantly depending on cell type and culture condition. Following your comments and those of Reviewer #2, we compiled the reported physiological and pathological concentrations of L-phenylalanine, L-valine, L-isoleucine, and SAM in both bacteria and mammalian cells (**Table 1**). These concentration ranges, along with the calculated limits of detection (LOD) for Phesor, Valsor, Ilesor, and SAMsor, have now been incorporated into their respective dose-response curves. As shown in **Fig. 1**, all four biosensors exhibit detection ranges that appropriately encompass the physiological concentrations of the target ligands in mammalian cells, as well as the majority of concentration ranges in bacteria.

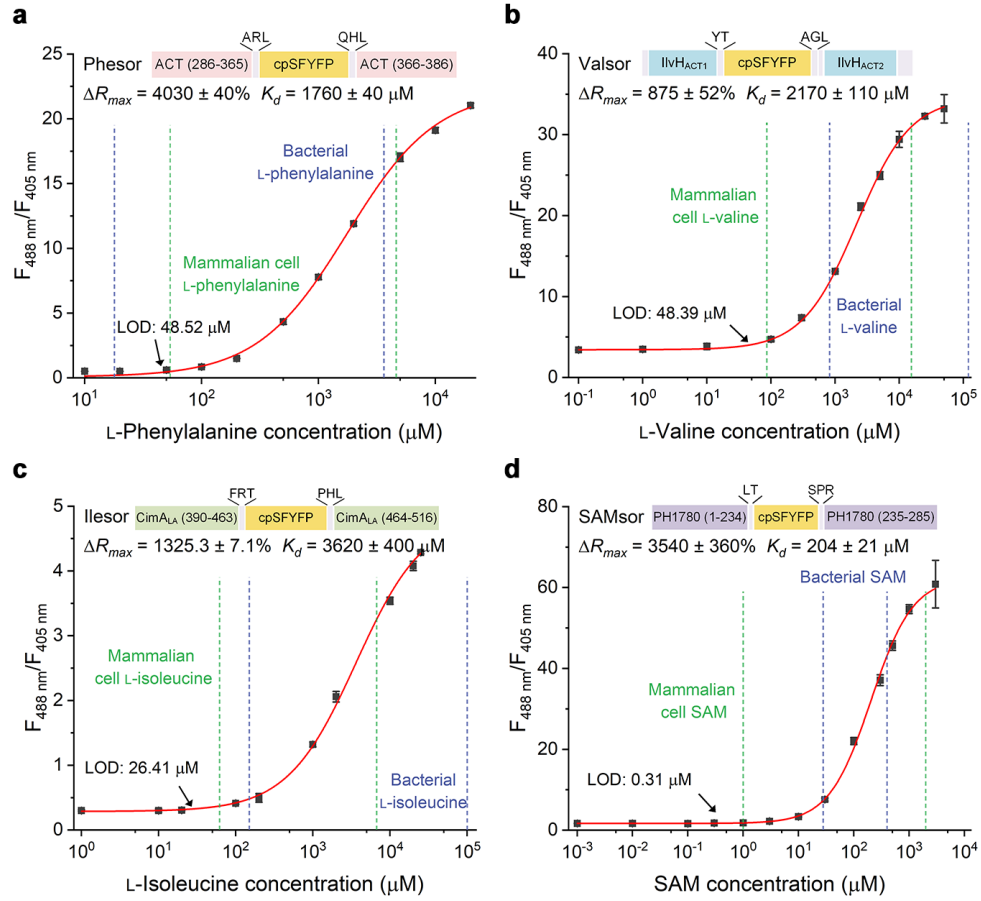


Figure 1 Characterization of Phesor, Valsor, Ilesor, and SAMsor *in vitro*. **(a-d)** Dose-response curve of Phesor **(a)**, Valsor **(b)**, Ilesor **(c)**, and SAMsor **(d)** for increasing concentrations of L-phenylalanine, L-valine, L-isoleucine, and SAM. The structural composition of the four biosensors and physiological concentrations of target ligands in bacteria and mammalian cell were shown. All data shown are means $\pm$ s.d. (n = 3 independent experiments). (Fig. 2c, Fig. 3d, Fig. 4d, and Fig. 6c in the revised manuscript)

Table 1 Reported concentrations of L-phenylalanine, L-valine, L-isoleucine, and SAM in bacteria, mammalian cells, and human body fluids.**(Supplementary Table 2 in the revised manuscript)**

Metabolites	Bacteria	Mammalian cells	Human body fluids
L-Phenylalanine	0.02–3.63 mM (<i>Escherichia coli</i>) ^{[1][2]}	0.05–0.28 mM (HeLa cell) ^[3] ~0.50–4.62 mM (A549 cell and U87-MG cell) ^[4] ~0.84 mM (Mouse kidney epithelial cells) ^[5] ~1.50 mM (A549 cell) ^[6] 0.34–0.81 mM (Hybridoma and CHO cell) ^[7] 0.07–0.56 mM (T cells) ^[8]	35–120 µM (Healthy serum) ^[9] 120–360 µM (Hyperphenylalaninaemia serum) ^[9] > 360 µM (Phenylketonuria serum) ^[9] ~62.89 µM (Healthy urine) ^[10]
L-Valine	~0.82–4.58 mM (<i>Escherichia coli</i>) ^[11] ~50–250 mM (<i>Corynebacterium glutamicum</i>) ^[11]	0.09–0.62 mM (HeLa cell) ^[3] ~0.62–2.44 mM (A549 cell and U87-MG cell) ^[4] ~1.51 mM (Mouse kidney epithelial cells) ^[5] ~2.50 mM (A549 cell) ^[6] 1.55–3.36 mM (Hybridoma and CHO cell) ^[7] 3.38–15.41 mM (T cells) ^[8] 1.36–10.29 mM (Fibroblasts) ^[12]	/
L-Isoleucine	~0.15–0.27 mM (<i>Escherichia coli</i>) ^[11] ~17–100 mM (<i>Corynebacterium glutamicum</i>) ^[13]	0.06–0.55 mM (HeLa cell) ^[3] ~0.58–2.09 mM (A549 cell and U87-MG cell) ^[4] ~1.76 mM (Mouse kidney epithelial cells) ^[5] ~2.10 mM (A549 cell) ^[6] 0.70–1.75 mM (Hybridoma and CHO cell) ^[7] 0.75–6.71 mM (T cells) ^[8]	/
SAM	28–400 µM (<i>Escherichia coli</i>) ^{[14][15]}	~62.99 µM (T cells) ^[8] 354 µM (Fibroblasts) ^[16] 1–37 µM (HEK293T cell) ^[17] 10–2,000 µM (Lymphoid cell, HTC116 cell, HEK293 cell) ^[18]	~73.5–120 nM (Serum) ^[19] ~22.1 µM (Urine) ^[19] ~10.2 µM (Urine) ^[20] ~12.1 µM (Urine) ^[21]

1. Bennett, B.D. *et al.* Absolute metabolite concentrations and implied enzyme active site occupancy in *Escherichia coli*. *Nat. Chem. Biol.* **5**, 593–599 (2009).
2. Liu, Y. *et al.* Genetic engineering of *Escherichia coli* to improve L-phenylalanine production. *BMC Biotechnol.* **18**, 5 (2018).
3. Yoshida, T., Nakajima, H., Takahashi, S., Kakizuka, A. & Imamura, H. OLIVE: a genetically encoded fluorescent biosensor for quantitative imaging of branched-chain amino acid levels inside single living cells. *ACS Sens.* **4**, 3333–3342 (2019).
4. Gauthier-Coles, G. *et al.* Quantitative modelling of amino acid transport and homeostasis in mammalian cells. *Nat. Commun.* **12**, 5282 (2021).
5. Park, J.O. *et al.* Metabolite concentrations, fluxes and free energies imply efficient enzyme usage. *Nat. Chem. Biol.* **12**, 482–489 (2016).
6. Bröer, S. & Gauthier-Coles, G. Amino acid homeostasis in mammalian cells with a focus on amino acid transport. *J. Nutr.* **152**, 16–28 (2022).
7. Heidemann, R., Lütkemeyer, D., Büntemeyer, H. & Lehmann, J. Effects of dissolved oxygen levels and the role of extra- and intracellular amino acid concentrations upon the metabolism of mammalian cell lines during batch and continuous cultures. *Cytotechnology* **26**, 185–197 (1998).
8. O’Keeffe, S., Sim, H., McCue, E. & Park, J.O. Ensemble quantitation of absolute metabolite concentrations in T cells reveals conserved features of immunometabolism. *bioRxiv* (2025).
9. van Spronsen, F.J. *et al.* Phenylketonuria. *Nat. Rev. Dis. Primers* **7**, 36 (2021).
10. Xie, G. *et al.* A metabolite array technology for precision medicine. *Anal. Chem.* **93**, 5709–5717 (2021).
11. Ruklisha, M., Paegle, L. & Denina, I. L-Valine biosynthesis during batch and fed-batch cultivations of *Corynebacterium glutamicum*:: relationship between changes in bacterial growth rate and intracellular metabolism. *Process Biochem.* **42**, 634–640 (2007).
12. Kok, G. *et al.* Isoleucine-to-valine substitutions support cellular physiology during isoleucine deprivation. *Nucleic Acids Res.* **53**, gkae1184 (2025).
13. Morbach, S., Sahm, H. & Eggeling, L. L-Isoleucine production with *Corynebacterium glutamicum*: further flux increase and limitation of export. *Appl. Environ. Microbiol.* **62**, 4345–4351 (1996).
14. Halliday, N.M. *et al.* Quantitative liquid chromatography-tandem mass spectrometry profiling of activated methyl cycle metabolites involved in LuxS-dependent quorum sensing in *Escherichia coli*. *Anal. Biochem.* **403**, 20–29 (2010).
15. Posnick L.M. & Samson, L.D. Influence of *S*-adenosylmethionine pool size on spontaneous mutation, dam methylation, and cell growth of *Escherichia coli*. *J. Bacteriol.* **181**, 6756–6762 (1999).
16. Fukumoto, K. *et al.* Excess *S*-adenosylmethionine inhibits methylation via catabolism to adenine. *Commun. Biol.* **5**, 313 (2022).
17. Gu, X. *et al.* SAMTOR is an *S*-adenosylmethionine sensor for the mTORC1 pathway. *Science* **358**, 813–818 (2017).
18. Ye, C. & Tu, B.P. Sink into the epigenome: histones as repositories that influence cellular metabolism. *Trends Endocrinol. Metab.* **29**, 626–637 (2018).
19. Bravo, A.C. *et al.* Analysis of *S*-adenosylmethionine and *S*-adenosylhomocysteine: method optimisation and profiling in healthy adults upon short-term dietary

intervention. *Metabolites* **12**, 373 (2022).

20. Stabler, S.P. & Allen, R.H. Quantification of serum and urinary S-adenosylmethionine and S-adenosylhomocysteine by stable-isotope-dilution liquid chromatography-mass spectrometry. *Clin. Chem.* 50, 365–372 (2004).
21. Ivanov, A.V. *et al.* Determination of S-adenosylmethionine, S-adenosylhomocysteine, and methylthioadenosine in urine using solvent-modified micellar electrokinetic chromatography. *Electrophoresis* **41**, 209–214 (2020).

In addition, Phesor, Valsor, Ilesor, and SAMsor possessed high response magnitudes for their target ligands (~4030%, ~846%, ~1325%, and ~3540%) (**Fig. 1**), which are comparable to those of recently reported biosensors, such as the L-arginine biosensor (~1540%; *Cell Metab.*, 2025, 37:291–304), the ATP biosensor (~1400%; *Proc. Natl. Acad. Sci. USA*, 2024, 121:e2314604121), and the adenosine biosensor (~929%; *Nat. Commun.*, 2025, 16:4245). However, as rightly noted, the fluorescence responses of these biosensors in living cells were observed to be less than 100% under certain stimulations. We speculated that these weak fluorescence changes may be attributed to limited perturbations in the intracellular concentrations of target ligands under the tested conditions. Therefore, we investigated the performance of the four biosensors under extreme metabolic perturbations. As shown in **Fig. 2**, Phesor-expressing HEK293FT cells cultured in L-phenylalanine-free or L-phenylalanine-supplemented DMEM (high glucose Dulbecco's Modified Eagle Medium) exhibited a 3.39-fold decrease and a 2.39-fold increase in fluorescence ratios, respectively, compared to those cultured in standard DMEM. Similarly, Valsor, Ilesor, and SAMsor expressed in HEK293FT cells all exhibited dramatic fluorescence responses under the tested extreme conditions, with maximum ratio changes of ~4.41-fold, ~3.65-fold, and ~10.21-fold, respectively (**Fig. 3**, **Fig. 4**, and **Fig. 5**).

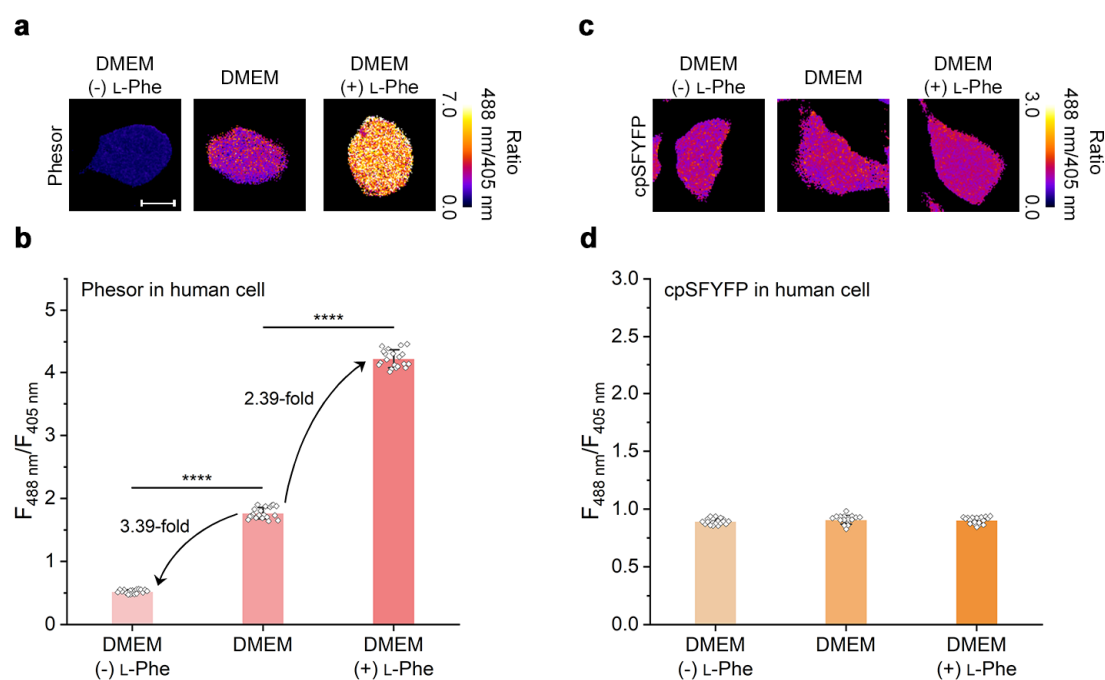


Figure 2 Fluorescence response of Phesor in HEK293FT cells to extreme L-phenylalanine metabolic

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. **(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 μ m. All data shown are means $\pm$ s.d. ($n = 20$ cells). ****, $P < 0.0001$; one-way ANOVA test with Tukey's Multiple Comparison Test. **(Supplementary Fig. 9 in the revised manuscript)**

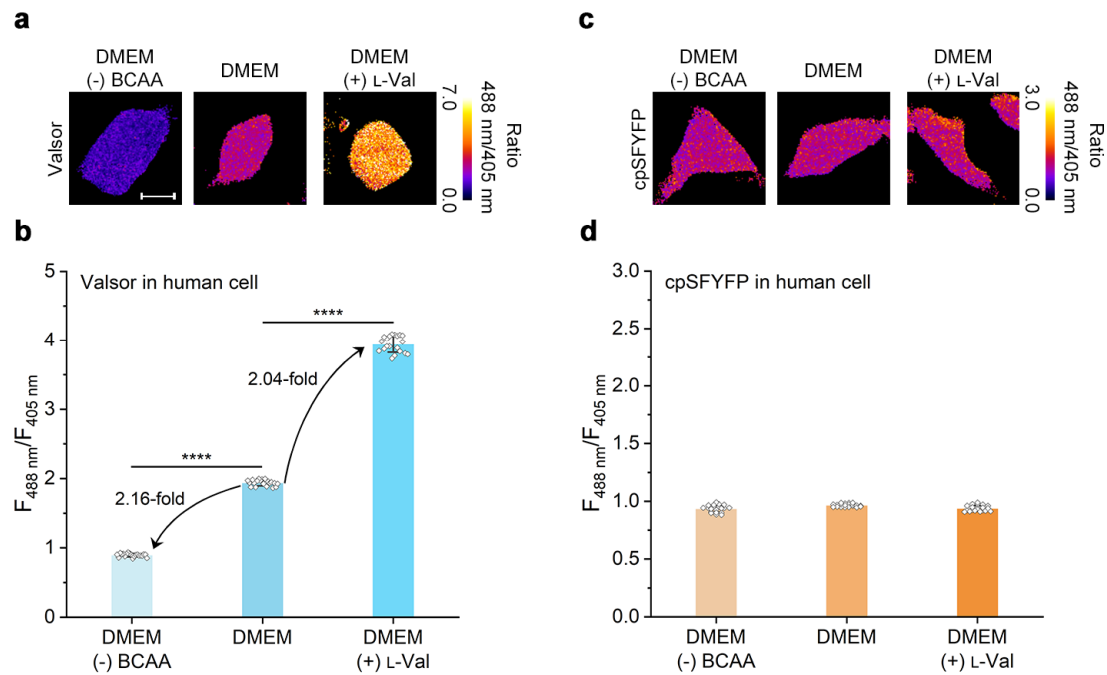


Figure 3 Fluorescence response of Valsor in HEK293FT cells to extreme L-valine metabolic perturbations. **(a, b)** Ratiometric images **(a)** and fluorescence ratios **(b)** of HEK293FT cells expressing Valsor cultured in BCAA-free DMEM, standard DMEM, and DMEM supplemented with 10 mM L-valine. **(c, 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 μ m. All data shown are means $\pm$ s.d. ($n = 20$ cells). ****, $P < 0.0001$; one-way ANOVA test with Tukey's Multiple Comparison Test. **(Supplementary Fig. 21 in the revised manuscript)**

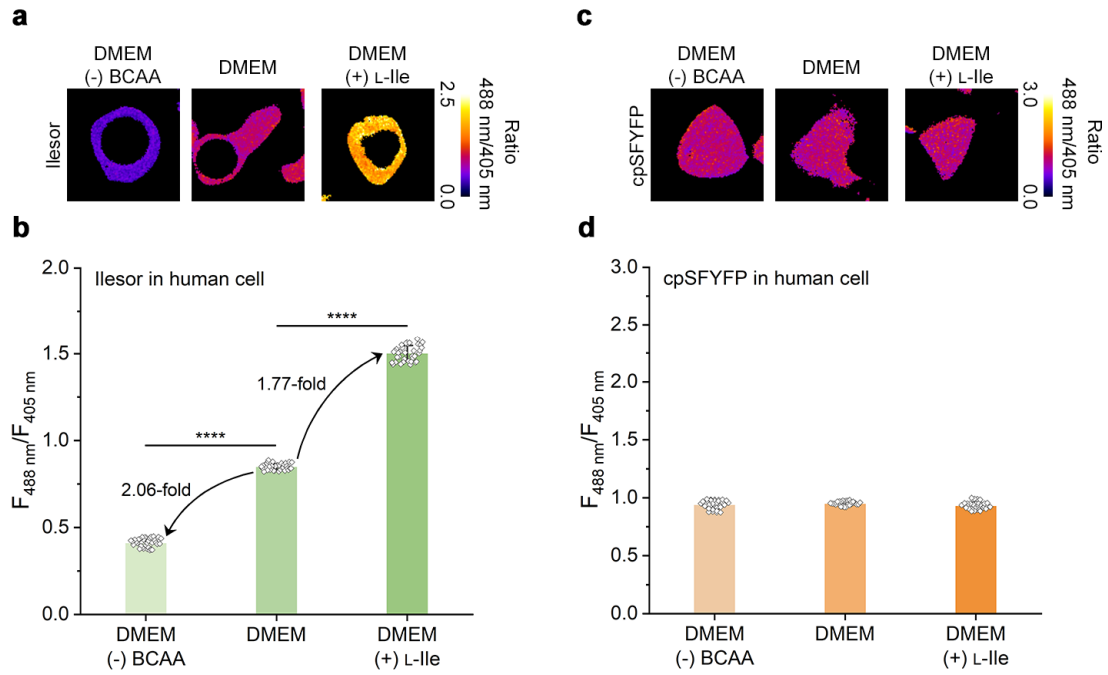


Figure 4 Fluorescence response of Ilesor in HEK293FT cells to extreme L-isoleucine metabolic perturbations. **(a, b)** Ratiometric images **(a)** and fluorescence ratios **(b)** of HEK293FT cells expressing Ilesor cultured in BCAA-free DMEM, standard DMEM, and DMEM supplemented with 10 mM L-isoleucine. **(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 . All data shown are means $\pm$ s.d. ($n = 36$ cells). ****, $P < 0.0001$; one-way ANOVA test with Tukey's Multiple Comparison Test. **(Supplementary Fig. 32 in the revised manuscript)**

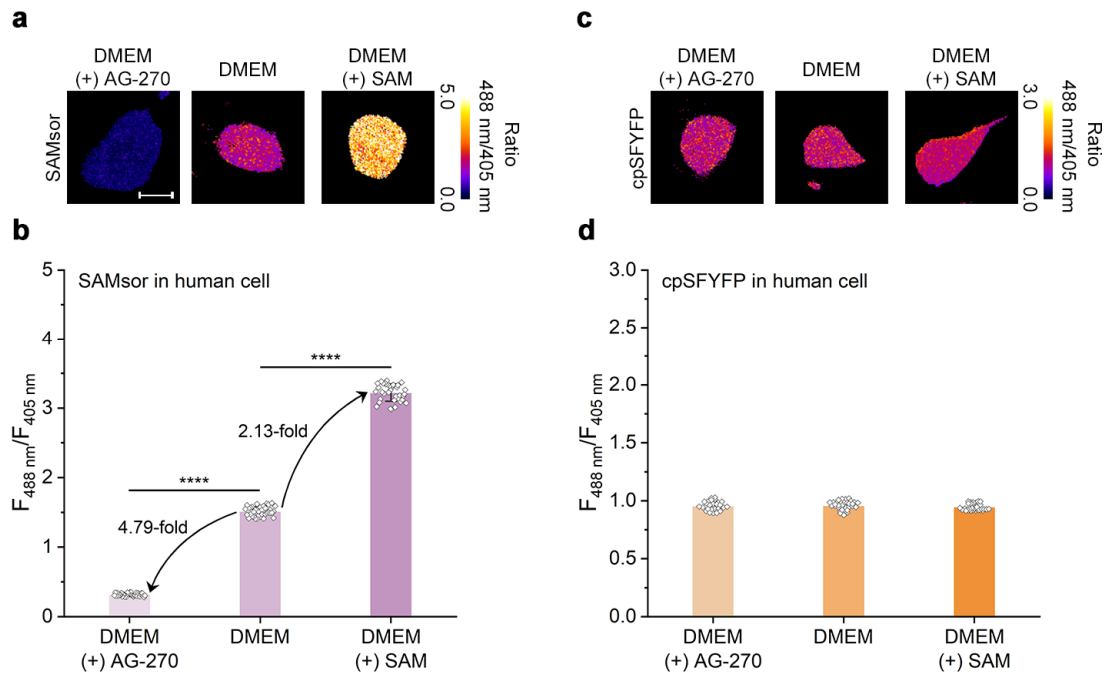


Figure 5 Fluorescence response of SAMsor in HEK293FT cells to extreme SAM metabolic perturbations. **(a, b)** Ratiometric images **(a)** and fluorescence ratios **(b)** of HEK293FT cells expressing SAMsor cultured in DMEM supplemented with 5 μ M AG-270, standard DMEM, and DMEM supplemented with 500 μ M SAM. **(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. All data shown are means $\pm$ s.d. (n = 36 cells). ****, $P < 0.0001$; one-way ANOVA test with Tukey's Multiple Comparison Test. **(Supplementary Fig. 44 in the revised manuscript)**

In addition, we would like to respectfully note that genetically encoded amino acid biosensors are often rationally designed with relatively high K_d values to better match the high physiological ligand concentrations. For example, Asanova *et al.* recently developed an L-isoleucine biosensor (non-specific), Nelle, with a tuned K_d value of 5 mM (*ACS Sens.*, 2024, 9:5135–5147). The authors noted that “Nelle has 12–33,000-fold lower affinity to isoleucine amino acid (K_d of 0.00015–0.4 mM vs K_d of 5 mM, respectively). The affinity of the Nelle indicator to isoleucine and leucine amino acids is appropriate for their detection in the cytosol of mammalian cells; the concentration of isoleucine and leucine in the cytosol of the mammalian cells in standard medium is around 0.4–2 and 0.6–2 mM, respectively, and depends on the composition of the external medium”. Similarly, an L-aspartate-specific biosensor was recently reported with a high K_d (1.4 mM) through extensive screening to align with physiological L-aspartate concentrations (0.1–10 mM) (*Cell Chem. Biol.*, 2024, 31:1529–1541).

Notably, the K_d values of allosteric regulatory domain-based GEFBs can be strategically modulated by adjusting the insertion site of cpSFYFP within the biorecognition element. The K_d values for all four biosensors, Phesor, Valsor, Ilesor, and SAMsor, were rationally tuned to match the physiological concentrations of their target ligands. Taking Phesor as an example, its development involved screening insertion sites of cpSFYFP within the Phe_{ACT} domain of chorismate mutase PheA, which yielded intermediate variant Phesor-0.38, followed by linker optimization. Notably, multiple biosensor variants with a wide range of affinities (K_d from $\sim$ 57 to $\sim$ 3,830 μ M) were identified during the insertion site screening (**Fig. 6**). Among them, Phesor-0.04, with cpSFYFP inserted near the N-terminus of Phe_{ACT}, exhibited the lowest K_d ($\sim$ 57 μ M) (**Fig. 6c**). In

contrast, Phesor-0.38, with cpSFYFP inserted in the interior of PheA_{ACT}, exhibited a relatively higher K_d (~440 μ M) (Fig. 6e). Due to its suitable affinity for detecting L-phenylalanine in both living cells and human body fluids, Phesor-0.38 was selected for subsequent linker optimization.

a
MTSENPLALREKISALDEKLALLAERRELADEVGKAKLLSHRPVRDIDRERDLLERLITLGAHHLDAHYITRLFQLIEDSVLTQQALLQQLNKNINPHSARIAFLGPKGS
YSHLAARQYAARHFEQIESGCAKFADIFNQVETGQADYAVVPIENTSSGAINDVYDLLQHTLSIVGEMTLTIDHCLLVSGTTDLSTINTVYSHPPQFPQCSKFLNRYPH
WKIEYTESTSAAMEKVAQAKSPHVAALGSEAGGTLGLQVLERIANQRQNFTRFVLARKAINVSDQVPAKTTLLMATGQQAGALVEALLVRNHNLMITRLESRIPIHG
NPWEEMFYLDIQANLESAEMQKALKELGEHTRSMKVLGCYPSENVVPDPT

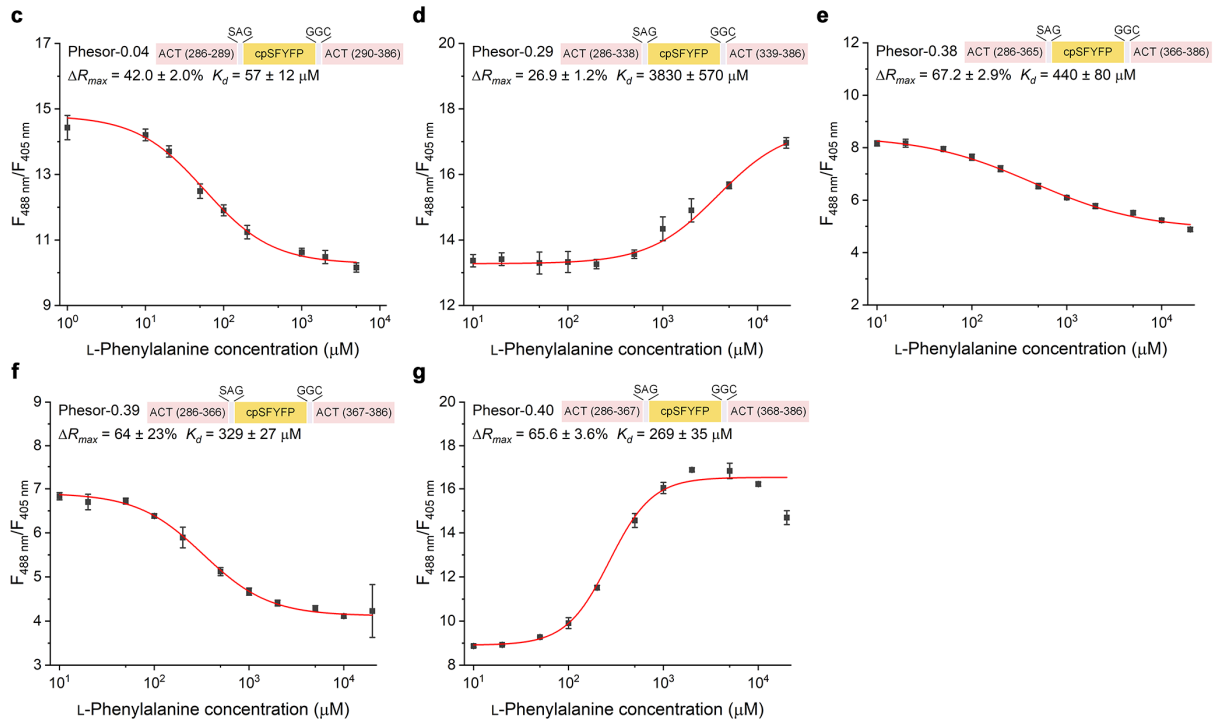
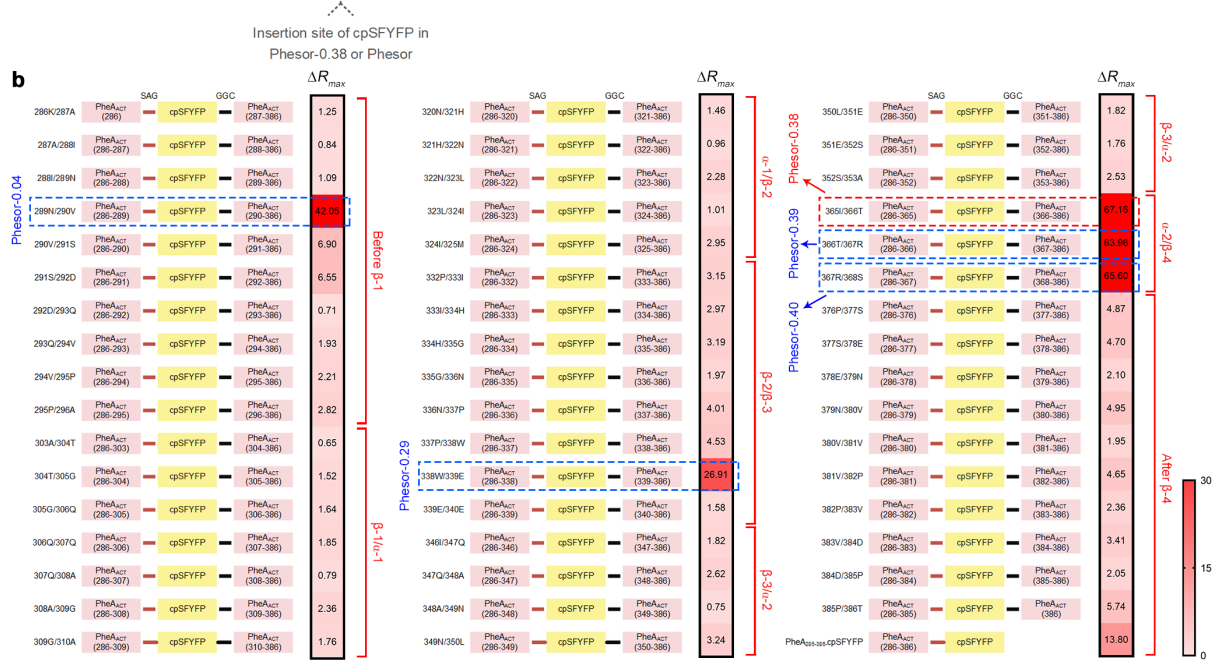


Figure 6 Construction of Phesor-0.38 (precursor of Phesor) by insertion sites screening. **(a)** Schematic representations of the amino acid sequence and the predicted secondary structure of PheA. The chorismate mutase (PheA_{CM}) domain, prephenate dehydratase (PheA_{PDT}) domain, and ACT (PheA_{ACT}) 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 of the L-phenylalanine biosensor Phesor-0.38 based on insertion sites screening. The ΔR_{max} of different biosensor variants 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. **(c-g)** Dose-response curves of Phesor-0.04 **(c)**, Phesor-0.29 **(d)**, Phesor-0.38 **(e)**, Phesor-0.39 **(f)**, and Phesor-0.40 **(g)** for increasing concentrations of L-phenylalanine. The low K_d value variant Phesor-0.04 together with the high K_d value variant Phesor-0.38 (precursor of Phesor) were submitted to further bioinformatics analysis. All data shown are means $\pm$ s.d. ($n \geq 3$ independent experiments). **(Fig. 2a, Supplementary Fig. 2a, and Supplementary Fig. 4 in the revised manuscript)**

Consistent with the observations in Phesor, multiple insertion variants spanning a range of K_d values were also obtained during the development of Valsor (K_d from ~ 35 to ~ 414 μM), Ilesor (K_d from ~ 59 to $\sim 17,000$ μM), and SAMsor (K_d from ~ 0.9 to ~ 8.3 μM) (Fig. 7, Fig. 8, and Fig. 9). From these variants, Valsor-0.03 (K_d of ~ 414 μM), Ilesor-0.23 (K_d of $\sim 1,700$ μM), and SAMsor-0.85 (K_d of ~ 8.3 μM) were selected for further linker optimization due to their moderate affinities (Fig. 7e, Fig. 8i, and Fig. 9d). A detailed analysis of the correlation between the cpSFYFP insertion site and biosensor affinity is provided in our response to Comment 4.

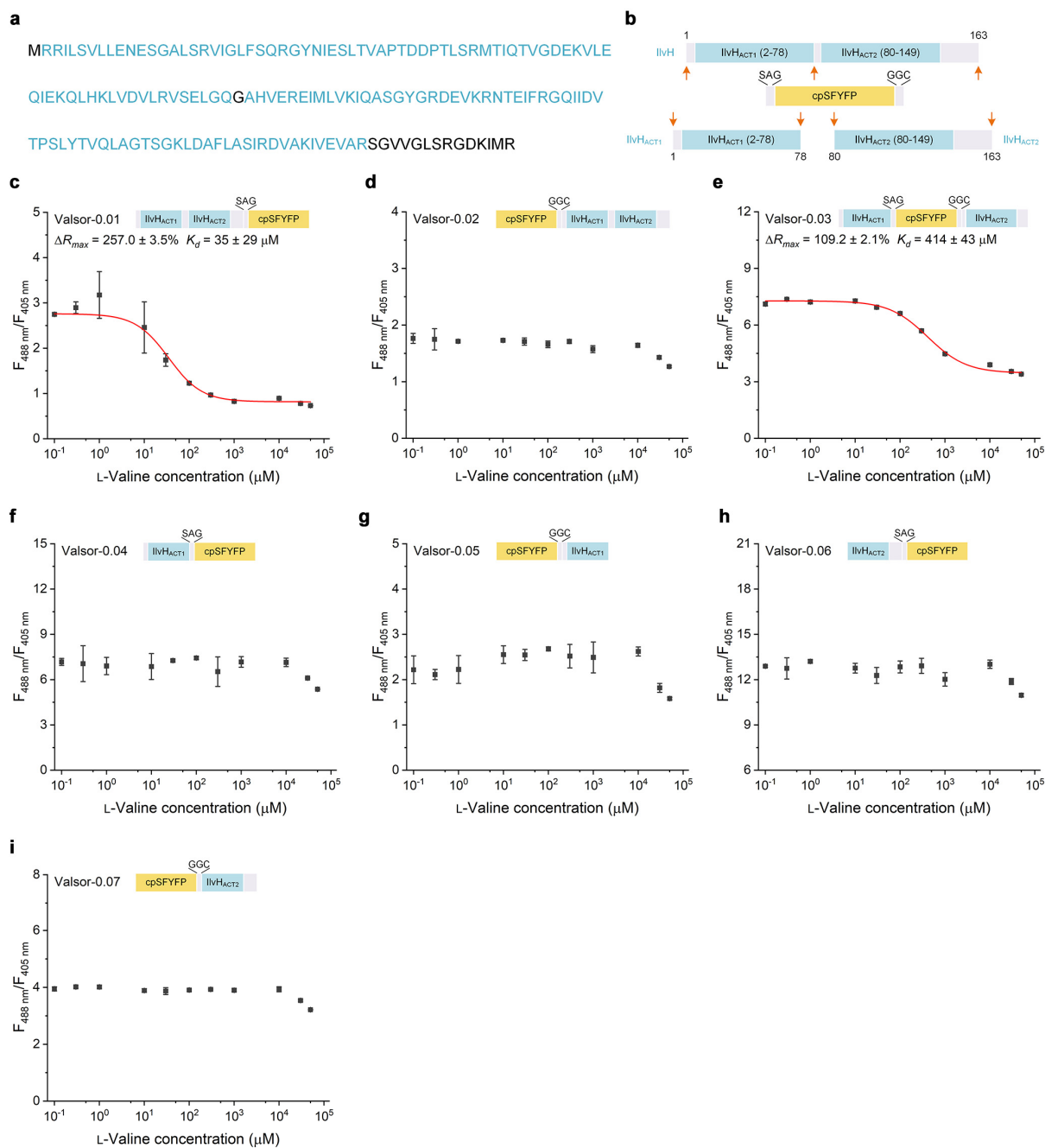


Figure 7 Construction of Valsor-0.03 (precursor of Valsor) by insertion sites screening. **(a)** Schematic representation of the amino acid sequence of IlvH. The two ACT domains of IlvH were shown in cyan. **(b)** Schematic representation of the construction of the initial seven L-valine biosensor insertion variants. **(c-i)** Dose-response curves of Valsor-0.01 **(c)**, Valsor-0.02 **(d)**, Valsor-0.03 **(e)**, Valsor-0.04 **(f)**, Valsor-0.05 **(g)**, Valsor-0.06 **(h)**, and Valsor-0.07 **(i)** for increasing concentrations of L-valine. The low K_d value variant Valsor-0.01 together with the high K_d value variant Valsor-0.03 (precursor of Valsor) were submitted to further bioinformatics analysis. All data shown are means $\pm$ s.d. ($n = 3$ independent experiments). (**Fig. 3b** and **Supplementary Fig. 16** in the revised manuscript)

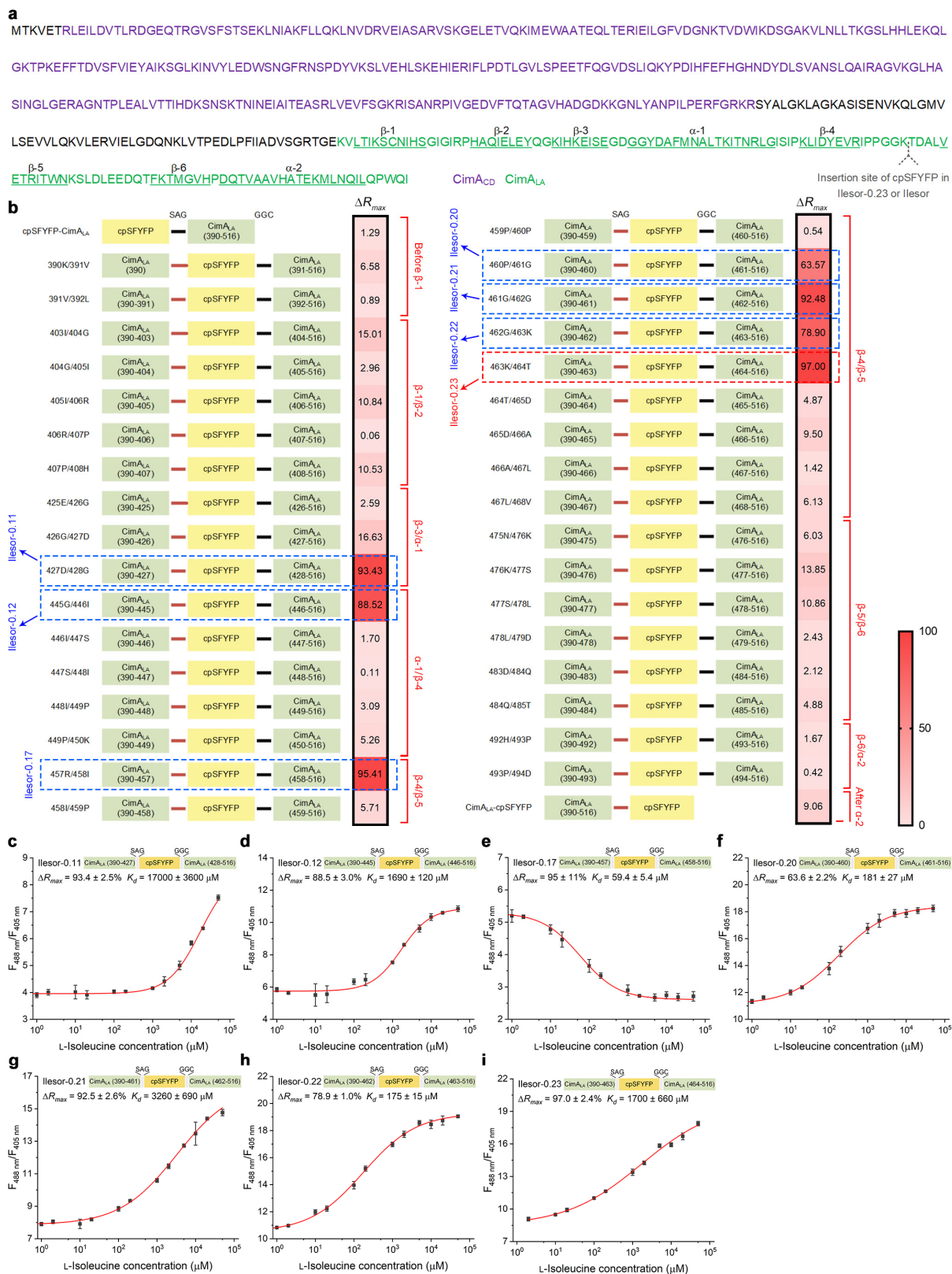


Figure 8 Construction of Ilesor-0.23 (precursor of Ilesor) by insertion sites screening. **(a)** Schematic representations of the amino acid sequence and the predicted secondary structure of CimA. The catalytic (CimA_{CD}) domain and LeuA regulatory (CimA_{LA}) domain of CimA were shown in purple and 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 of the L-isoleucine biosensor Ilesor-0.23 based on insertion sites screening. The ΔR_{max} of different biosensor variants to L-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. **(c-i)** Dose-response curves of Ilesor-0.11 **(c)**, Ilesor-0.12 **(d)**, Ilesor-0.17 **(e)**, Ilesor-0.20 **(f)**, Ilesor-0.21 **(g)**, Ilesor-0.22 **(h)**, and Ilesor-0.23 **(i)** for increasing concentrations of L-isoleucine. The low K_d value variant Ilesor-0.17 together with the high K_d value variant Ilesor-0.23 (precursor of Ilesor) were submitted to further bioinformatics analysis. All data shown are means $\pm$ s.d. (n = 3 independent experiments) **(Fig. 4b, Supplementary Fig. 27a, and Supplementary Fig. 28 in the revised manuscript)**

(234V/235I), MTAsor-0.64 (182L/183R), and SAMsor-0.34 (111T/112V) were shown in the blue dotted boxes. **(c, d)** Dose-response curves of SAMsor-0.34 **(c)** and SAMsor-0.85 **(d)** for increasing concentrations of SAM. The low K_d value variant SAMsor-0.34 (precursor of SAMsor_L) together with the high K_d value variant SAMsor-0.85 (precursor of SAMsor) were submitted to further bioinformatics analysis. All data shown are means $\pm$ s.d. ($n = 3$ independent experiments). (**Fig. 5e, Fig. 7b, Supplementary Fig. 37a, and Supplementary Fig. 39 in the revised manuscript**)

Theoretically, other biosensor variants with different K_d values could also be further optimized via linker engineering. SAM is recognized as an important biomarker for evaluating human disease and health status. Elevated SAM levels in human body fluids are highly associated with the occurrence of diseases including digestive cancer, lung cancer, and pneumonia (**Fig. 10a**). The LOD of SAMsor for SAM was calculated to be 310 nM (**Fig. 1d**), which exceeds the reported serum SAM concentrations in healthy individuals (~ 73.5 –120 nM) (**Table 1**). Fortunately, among the 90 insertion variants screened, we identified SAMsor-0.34, with cpSFYFP inserted between residues 111T/112V of PH1780, exhibited higher sensitivity to SAM (**Fig. 9c**). Dose-response curve analysis indicated that SAMsor-0.34 achieved a ΔR_{max} of $80.0 \pm 4.4\%$, a low K_d of 900 ± 110 nM, and a low LOD of 144.10 nM for SAM (**Fig. 9c and Fig. 10b**). This variant was therefore selected both for validating the feasibility of developing GEFBs with different detection ranges from a single allosteric regulatory domain and for realizing quantitative SAM detection in human body fluids.

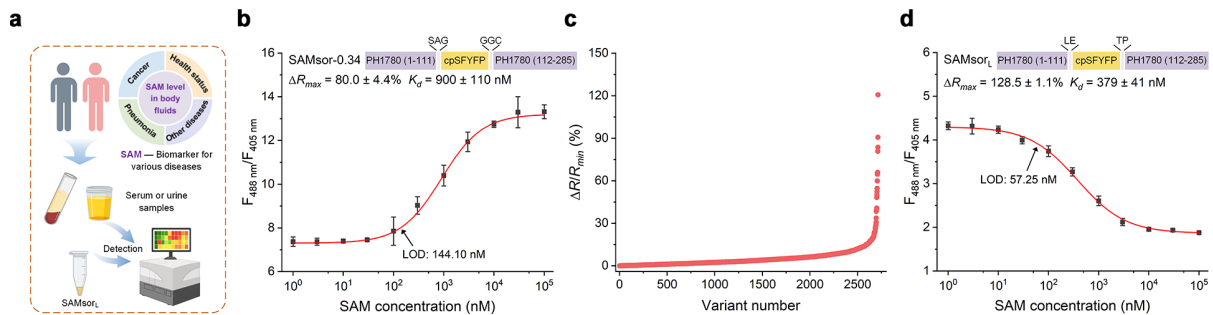


Figure 10 Optimization and application of the SAM biosensor SAMsor_L with low detection range. **(a)** Schematic representation of the workflow for quantitative detection of SAM in human serum and urine samples using SAMsor_L. **(b)** Dose-response curve of SAMsor-0.34 for increasing concentrations of SAM. The structural composition of SAMsor-0.34 was shown. **(c)** Optimization of SAMsor-0.34 by linker random mutagenesis combined with high-throughput screening. The response magnitudes of crude extracts of 2,715 linker mutants were determined in the presence of 10 μ M SAM. **(d)** Dose-response curve of SAMsor_L for increasing concentrations of SAM. The structural composition of SAMsor_L was shown. All

data shown are means $\pm$ s.d. ($n = 3$ independent experiments). (**Fig. 7a-d in the revised manuscript**)

As shown in **Fig. 10c**, a more sensitive mutant with the N-terminal linker of “Leu-Glu” and the C-terminal linker of “Thr-Pro”, denoted as SAMsor_L, was then screened from 2,715 mutants through linker random mutagenesis. Dose-response curve analysis indicated that SAMsor_L exhibited an increased ΔR_{max} of $128.5 \pm 1.1\%$, a decreased K_d of 379 ± 41 nM (~ 538 -fold lower than SAMsor), and a decreased LOD of 57.25 nM for SAM (**Fig. 10d**). Then, we prepared authentic serum and urine samples from healthy individuals and patients with lung cancer, and demonstrated the feasibility of SAMsor_L for clinical diagnostics of lung cancer. **A detailed elaboration of the diagnostic applications of SAMsor_L is provided in our response to Comment 3.**

In addition, as requested, we have now provided the corresponding live-cell imaging data for the Fig. 2m, Fig. 3m, Fig. 4m, and Fig. 6m of the previous manuscript (as shown in **Fig. 11, Fig. 12, Fig. 13, and Fig. 14** below). Specific stimulations (e.g., LAT1 overexpression or octyl-D-2-HG addition) can perturb intracellular pH, thereby affecting the fluorescence ratio of both the biosensors as well as the cpSFYFP control. The supplied images show the uncorrected original fluorescence ratio data for the four biosensors and cpSFYFP.

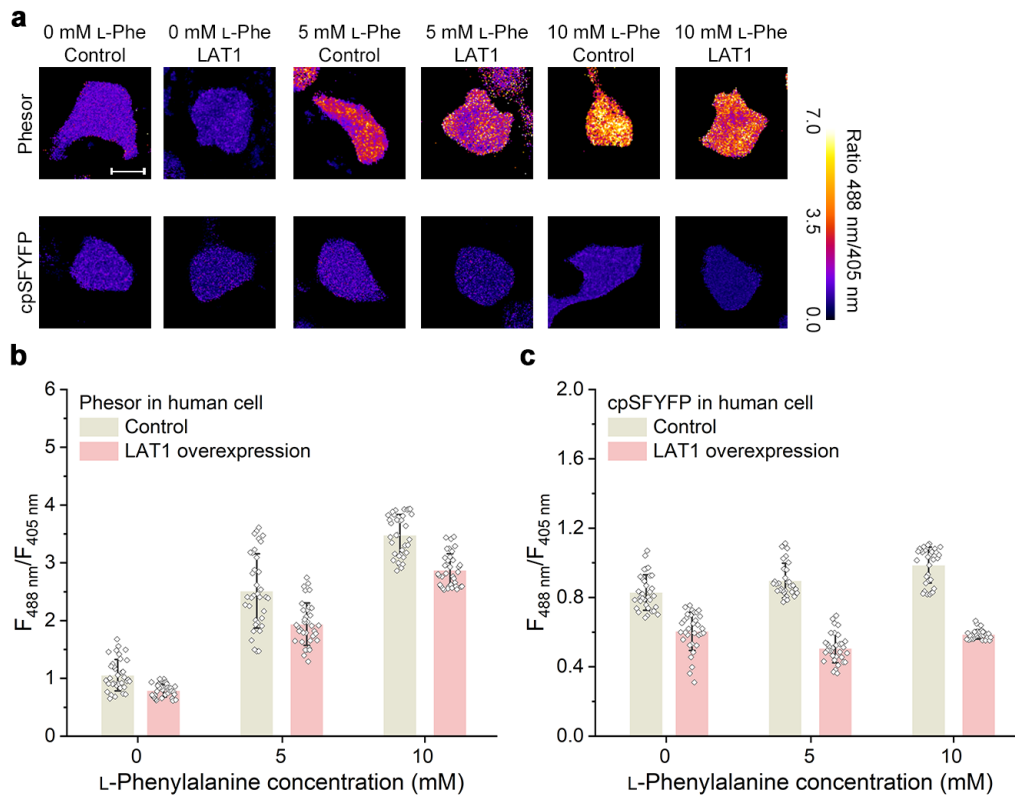


Figure 11 Analysis of the function of LAT1 in the uptake of L-phenylalanine in HEK293FT cells using Phesor. **(a)** Ratiometric images of single HEK293FT cell expressing Phesor or cpSFYFP in response to L-phenylalanine addition and LAT1 overexpression. Cells were treated with 0, 5, or 10 mM L-phenylalanine with or without LAT1 overexpression, and the fluorescence ratios were determined after 20 h. Scale bar, 10 μm . **(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 of main text. All data shown are means $\pm$ s.d. ($n = 36$ cells for **b**; $n = 33$ cells for **c**). **(Supplementary Fig. 10 in the revised manuscript)**

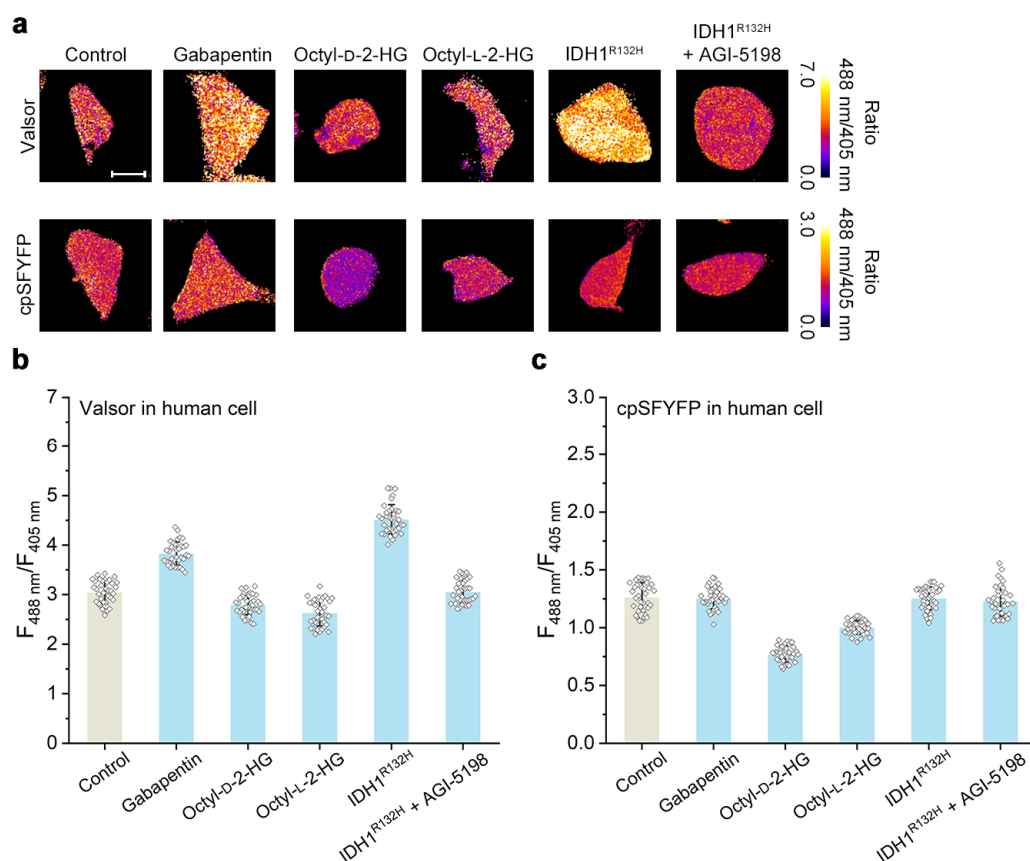


Figure 12 Analysis of the effects of different BCAT inhibitors on intracellular L-valine levels in HEK293FT cells using Valsor. **(a)** Ratiometric images of single HEK293FT cell expressing Valsor or cpSFYFP in response to different inhibitors treatment. The concentrations of gabapentin, octyl-D-2-HG (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. **(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 of main text. All data shown are means $\pm$ s.d. (n = 36 cells). **(Supplementary Fig. 23 in the revised manuscript)**

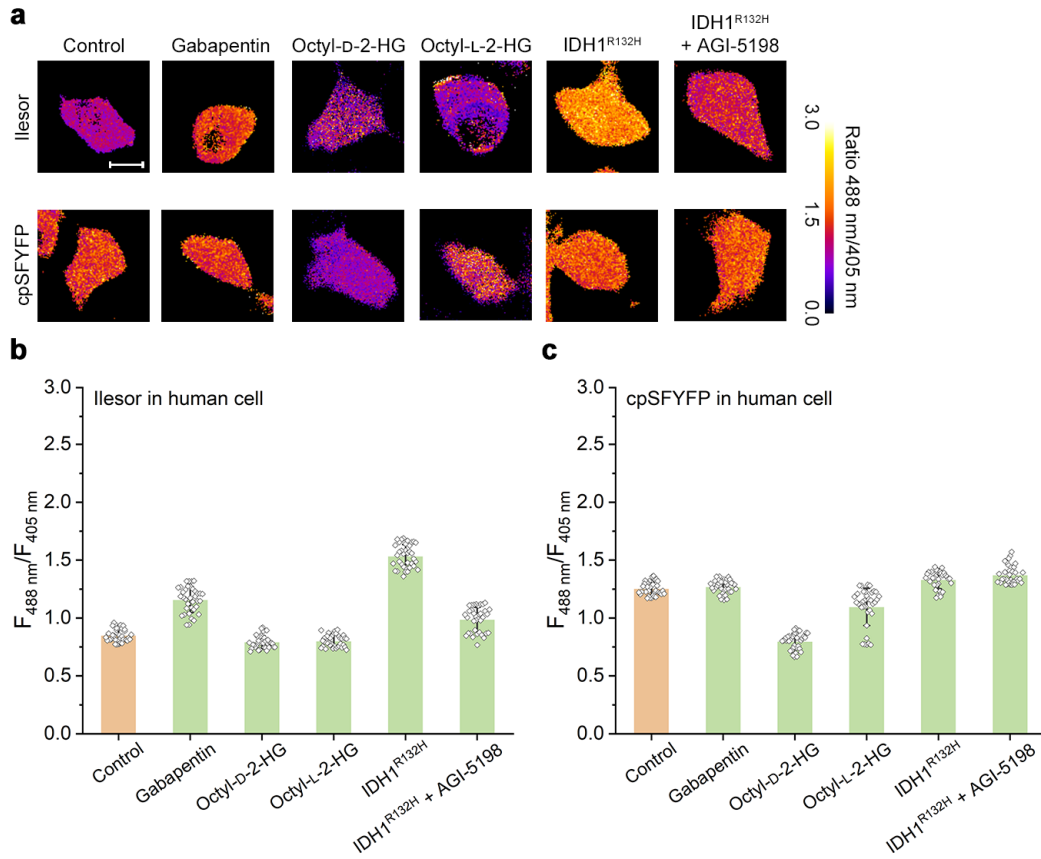


Figure 13 Analysis of the effects of different BCAT inhibitors on intracellular L-isoleucine levels in HEK293FT cells using Ilesor. **(a)** Ratiometric images of single HEK293FT cell expressing Ilesor or cpSFYFP in response to different inhibitors treatment. The concentrations of gabapentin, octyl-D-2-HG, octyl-L-2-HG, and AGI-5198 used were 2 mM, 1 mM, 1 mM, and 3 μM, respectively. The fluorescence ratios were determined 20 h after treatment with different inhibitors. Scale bar, 10 μm. **(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 of main text. All data shown are means ± s.d. (n = 36 cells). (Supplementary Fig. 33 in the revised manuscript)

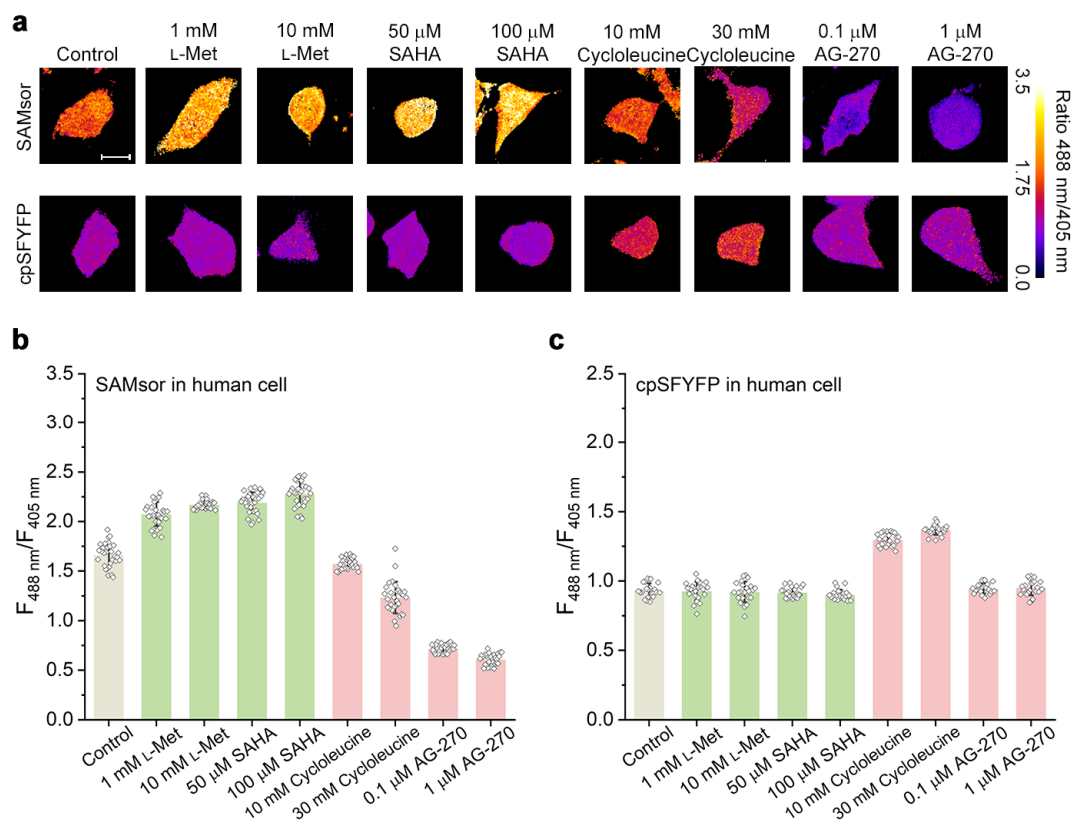


Figure 14 Analysis of the effects of different MATase activator or inhibitors on intracellular SAM levels in HEK293FT using SAMsor. **(a)** Ratiometric images of single HEK293FT cell expressing SAMsor or cpSFYFP in response to different MATase activator or inhibitors treatment. The fluorescence ratios were determined 20 h after treatment with different activator or inhibitors. Scale bar, 10 μ m. **(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 of main text. All data shown are means $\pm$ s.d. (n = 30 cells for **b**; n = 23 cells for **c**). (**Supplementary Fig. 45 in the revised manuscript**)

In addition, we have supplemented subcellular experiments for all biosensors to demonstrate their spatial resolution in living cells. [The relevant experimental data and analyses are provided in our response to Comment 3.](#)

[Collectively, these results demonstrate that the allosteric enzyme-based biosensors, Phesor, Valsor, Ilesor, and SAMsor, possess appropriate detection ranges and high sensitivity suitable for live-cell applications. Furthermore, using SAM biosensor as an example, we successfully optimized the detection range by altering the cpSFYFP insertion site, effectively addressing the limitations for SAM detection in human body fluids.](#)

Related data were presented in **Fig. 2a, c, Fig. 3b, d, Fig. 4b, d, Fig. 5e, Fig. 6c, Fig. 7a-d, Supplementary Fig. 2a, Supplementary Fig. 4, Supplementary Fig. 9, Supplementary Fig. 10, Supplementary Fig. 16, Supplementary Fig. 21, Supplementary Fig. 23, Supplementary Fig. 27a, Supplementary Fig. 28, Supplementary Fig. 32, Supplementary Fig. 33, Supplementary Fig. 37a, Supplementary Fig. 39, Supplementary Fig. 44, Supplementary Fig. 45, and Supplementary Table 2** in the revised manuscript. Related descriptions were added in the “Results” section of the revised manuscript as follows:

For Phesor:

“The 38th variant (denoted as Phesor-0.38, with cpSFYFP inserted between residues 365I/366T of the PheA_{ACT} domain) exhibited a higher maximum fluorescence ratio change (ΔR_{max}) of $67.2 \pm 2.9\%$ and an appropriate apparent dissociation constant (K_d) of $440 \pm 80 \mu\text{M}$, making it suitable for L-phenylalanine detection both in living cells and in human body fluids (**Fig. 2a and Supplementary Table 2**). Subsequently, we conducted random mutagenesis and high-throughput screening for linkers connecting the cpSFYFP and the PheA_{ACT} domain. Dose-response curve analysis indicated that the detection range of Phesor encompasses the physiological concentrations of L-phenylalanine both in bacteria and in mammalian cells (**Fig. 2c**). ...

In addition, Phesor allowed for visual detection of L-phenylalanine in human cells (Fig. 2g). Phesor expressed in human embryonic kidney (HEK293FT) cells exhibited a dramatic response to exogenous L-phenylalanine addition and endogenous L-phenylalanine depletion (maximum ratio change of $709 \pm 27\%$), with no observable fluorescence ratio changes to L-tyrosine and L-tryptophan fluctuations (**Fig. 2g, h, Supplementary Fig. 6d, e, Supplementary Fig. 8, and Supplementary Fig. 9**).”

For Valsor:

“The variant Valsor-0.03, created by inserting cpSFYFP into the middle of the two ACT domains of intact IlvH, possessed a higher ΔR_{max} of $109.2 \pm 2.1\%$ and an appropriate K_d of $414 \pm 43 \mu\text{M}$ for L-valine (**Fig. 3b**). Dose-response curve analysis indicated that Valsor achieved a wide detection range, with an increased ΔR_{max} ($875 \pm 52\%$), an appropriate K_d ($2,170 \pm 110 \mu\text{M}$; close to the value of $4.46 \pm 0.50 \text{ mM}$ determined by TSA), and a Hill slope

close to 1 for L-valine (**Fig. 3d, Supplementary Fig. 18e, and Supplementary Table 4**). ...

Valsor also allowed for real-time monitoring fluctuations of L-valine levels in HEK293FT cells with a maximum ratio change of $340 \pm 13\%$ (**Fig. 3g, h, Supplementary Fig. 19e, f, Supplementary Fig. 20, and Supplementary Fig. 21**).”

For Ilesor:

“The 23rd variant (denoted as Ilesor-0.23, with cpSFYFP inserted between residues 463K/464T of the CimA_{LA}) exhibited a higher ΔR_{max} of $97.0 \pm 2.4\%$ and an appropriate K_d of $1,700 \pm 660 \mu\text{M}$ for L-isoleucine (**Fig. 4b**). Dose-response curve analysis indicated that Ilesor achieved a suitable detection range, an increased ΔR_{max} ($1,325.3 \pm 7.1\%$), an appropriate K_d ($3,620 \pm 400 \mu\text{M}$; close to the value of $2.22 \pm 0.30 \text{ mM}$ determined by TSA), and a Hill slope close to 1 for L-isoleucine (**Fig. 4d, Supplementary Fig. 30e, and Supplementary Table 4**). ...

Ilesor could also be used for real-time monitoring of L-isoleucine fluctuations in HEK293FT cells with a maximum ratio change of $265 \pm 11\%$ (**Fig. 4g, h, Supplementary Fig. 19e, f, Supplementary Fig. 31, and Supplementary Fig. 32**).”

For SAMsor:

“Dose-response curve analysis indicated that SAMsor achieved an increased ΔR_{max} ($3,540 \pm 360\%$), an appropriate K_d ($204 \pm 21 \mu\text{M}$; close to the value of $0.295 \pm 0.061 \text{ mM}$ determined by TSA), a Hill slope close to 1, and a wide detection range suitable for detecting physiological concentrations of SAM in mammalian cells and bacteria (**Fig. 6c, Supplementary Fig. 40e, and Supplementary Table 2**). ...

SAMsor expressed in HEK293FT cells also exhibited a specific and dramatic response to fluctuations in SAM levels with a maximum ratio change of $920 \pm 39\%$ (**Fig. 6g-i, Supplementary Fig. 41d, Supplementary Fig. 43, and Supplementary Fig. 44**).”

For SAMsorL:

“SAM is also recognized as an important biomarker for evaluating human disease and health status³⁶. Elevated SAM levels in body fluids are highly associated with the occurrence of diseases including digestive cancer, lung cancer, and pneumonia (**Fig. 7a**)^{37,38}. The limit of detection (LOD) of SAMsor for SAM was calculated to be 310 nM (**Fig. 6c**), which exceeds the reported serum SAM concentrations in healthy individuals ($\sim 73.5\text{--}120 \text{ nM}$)

(**Supplementary Table 2**). Fortunately, we found that the 34th variant (denoted as SAMsor-0.34, with cpSFYFP inserted between residues 111T/112V of PH1780) exhibited higher sensitivity to SAM among the 90 insertion variants of the SAM biosensor (**Fig. 7b**, **Supplementary Fig. 37b**, and **Supplementary Fig. 39**). Dose-response curve analysis indicated that SAMsor-0.34 achieved a ΔR_{max} of $80.0 \pm 4.4\%$, a low K_d of 900 ± 110 nM, and a low LOD of 144.10 nM for SAM (**Fig. 7b**). Then, we conducted a systematic optimization of the linker in SAMsor-0.34 and identified a more sensitive biosensor SAMsor_L with the N-terminal linker of “Leu-Glu” and the C-terminal linker of “Thr-Pro” from 2,715 mutants (**Fig. 7c**). SAMsor_L exhibited an increased ΔR_{max} of $128.5 \pm 1.1\%$, a lower K_d of 379 ± 41 nM, and a decreased LOD of 57.25 nM for SAM (**Fig. 7d**).”

In addition, a brief discussion regarding the affinities of the biosensors was added in the “Discussion” section of the revised manuscript as follows:

“The methodology for developing GEFBs based on allosteric regulatory domains could be summarized in four steps: (i) selection of specific biorecognition elements, (ii) identification of optimal cpSFYFP insertion sites within loop regions to obtain a variant with obvious responsiveness and suitable affinity, (iii) optimization of linkers to maximize responsiveness, and (iv) functional testing (**Fig. 1a**). Notably, the affinities of allosteric regulatory domain-based GEFBs can be strategically modulated by shifting the insertion site of cpSFYFP. Besides SAMsor-0.34, multiple insertion variants with lower K_d values than Phesor, Valsor, and Ilesor were identified, such as Phesor-0.04 (57 ± 12 μ M), Valsor-0.01 (35 ± 29 μ M), and Ilesor-0.17 (59.4 ± 5.4 μ M) (**Supplementary Fig. 4**, **Supplementary Fig. 16**, and **Supplementary Fig. 28**). The higher affinities of these variants might be attributed to the insertion site of cpSFYFP being positioned distal to the binding pocket, which minimizes structural disruption and maintains higher architectural fidelity of the biorecognition element to its native conformation (**Supplementary Fig. 49**). This structure-function relationship provides a rational foundation for future GEFB engineering, thereby expanding the toolbox for metabolic imaging and *in vitro* diagnostics across varying concentration scales.”

We sincerely hope that the above responses have adequately addressed your concerns. While we believe the SAM biosensor optimization serves as a strong proof-of-concept for our affinity-tuning strategy, should you consider it essential to apply this strategy to the other

three amino acid biosensors, we are fully prepared to do so. We thank you again for your constructive comments.

2. Validation of biosensors for precise in vitro detection

In lines 152–158, the authors simulate phenylketonuria (PKU) conditions by adding 1 or 3 mM L-phenylalanine to healthy serum and urine samples. However, physiological upper limits for serum L-phenylalanine in PKU diagnosis are around 120 μ M. Using 1 mM greatly exceeds this range and undermines the relevance of the test. Therefore, validation experiments should be conducted within a physiologically meaningful range (e.g., ~50–200 μ M) to better reflect clinical conditions and to demonstrate the practical utility of the biosensor in realistic diagnostic scenarios. Additionally, while validation with HPLC was performed for Valsor and Ilesor, details on the sample matrices used are lacking. Were interfering substances added to the same sample or to different ones? If only a single sample was tested, the conclusion about robustness of detection may be premature. Furthermore, no standard curve information was provided for the biosensors, making it difficult to assess their quantitative agreement with HPLC measurements.

Response: Thanks for your good suggestion. In the previous manuscript, the clinical availability of Phesor was assessed using serum and urine samples from a single healthy individual spiked with 1 or 3 mM L-phenylalanine. To more rigorously evaluate the practical applicability of Phesor in clinical diagnostics and to partially address your concerns regarding the “in-depth application of one sensor (...more rigorous *in vitro* testing)” mentioned in **Comment 3**, we collected 14 authentic serum samples and 9 urine samples from healthy individuals. Then, we prepared 14 serum and 16 urine samples from simulated patients with hyperphenylalaninemia or phenylketonuria by spiking various concentrations of L-phenylalanine (30–550 μ M). As shown in **Fig. 15a-c** and **Fig. 16**, quantification of L-phenylalanine in these samples using Phesor demonstrated good correlation and consistency with HPLC measurements, and the results were unaffected by the presence of different L-phenylalanine analogs. The measured L-phenylalanine concentrations in serum and urine from healthy individuals were 72.55–102.17 μ M and 68.30–87.06 μ M, respectively, consistent with previously reported values (35–120 μ M in serum and ~62.89 μ M in urine)

(Table 1).

Dried blood spots are the primary sample type used in newborn screening for phenylketonuria. To further assess the diagnostic potential of Phesor, we collected authentic dried blood spot samples from 19 healthy children and 23 children with phenylketonuria. As shown in **Fig. 15d**, blood L-phenylalanine concentrations measured using Phesor ranged from 31.10 to 119.91 μM in healthy subjects, while those in phenylketonuria patients ranged from 393.45 to 1,821.13 μM , higher than the clinical diagnostic cut-off value of 360 μM . These results indicate that Phesor is applicable for point-of-care testing (POCT) of L-phenylalanine in human body fluids, demonstrating its potential as a reliable tool for the diagnosis and therapeutic monitoring of L-phenylalanine-related diseases.

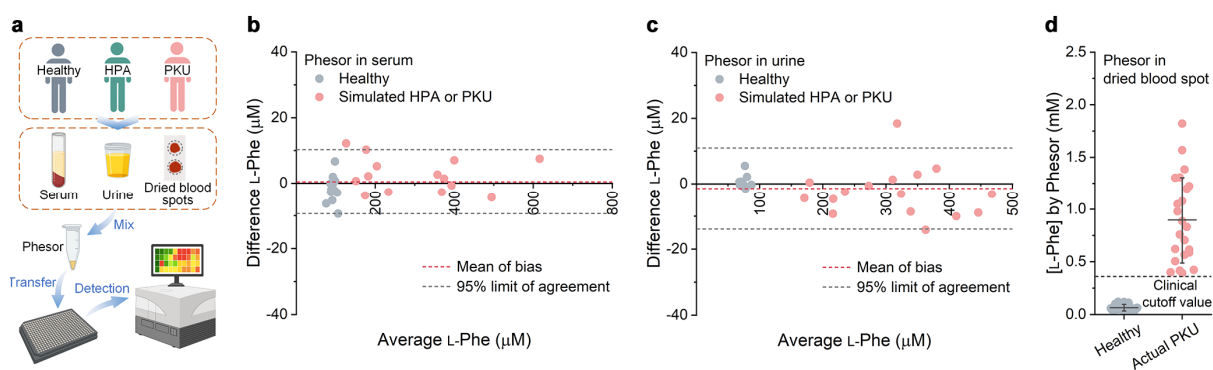


Figure 15 Application of Phesor for quantitative detection of L-phenylalanine in human body fluids. **(a)** Schematic representation of the workflow for quantitative detection of L-phenylalanine in human serum, urine, and dried blood spot samples using Phesor. **(b, c)** Bland-Altman analysis for L-phenylalanine in serum **(b)** and urine **(c)** samples from healthy adults and simulated patients with hyperphenylalaninemia (HPA) or phenylketonuria (PKU) measured by Phesor and HPLC. Bias (red dotted line) and 95% limits of agreement (gray dotted lines) were shown. **(d)** Quantitative detection of L-phenylalanine in authentic dried blood spot samples from healthy children and children with phenylketonuria (PKU) using Phesor. All data shown are means $\pm$ s.d. ($n = 3$ independent experiments). (**Fig. 2j-m in the revised manuscript**)

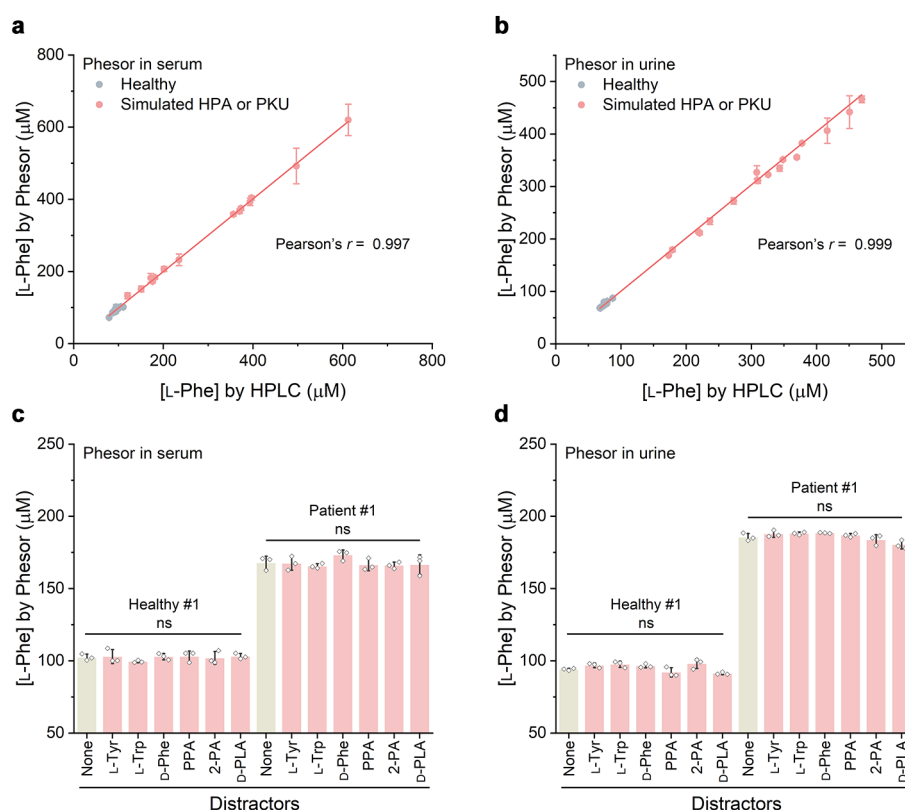


Figure 16 Analysis of the accuracy and anti-interference ability of Phesor for the quantitative detection of L-phenylalanine in serum and urine. **(a, b)** Correlation analysis between Phesor and HPLC for quantitative detection of L-phenylalanine in serum **(a)** and urine **(b)**. Quantitative results 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. ($n = 3$ independent experiments). ns, no significant difference ($P \geq 0.05$); one-way ANOVA test with Tukey's Multiple Comparison Test.

(Supplementary Fig. 13 in the revised manuscript)

Branched-chain amino acids are valuable compounds widely used in the food, cosmetic, and pharmaceutical industries, with a steadily increasing global market (e.g., the average annual increase rate of L-valine exceeding 5%). Microbial fermentation serves as the primary source of branched-chain amino acids worldwide. In the previous manuscript, the same simulated fermentation supernatant sample was used to evaluate the suitability of Valsor and Ilesor for *in vitro* applications. To more rigorously evaluate their practical applicability in bioprocess monitoring and to partially address your concern regarding the “in-depth application of one sensor (...more rigorous *in vitro* testing)” mentioned in **Comment 3**, we

conducted fed-batch fermentation using the L-valine-producing strain *Klebsiella oxytoca* VKO-9 (*Bioresour. Technol.*, 2024, 395:130403) and the L-isoleucine-producing strains *Cupriavidus necator* CnIle6 and CnIle8 (*Bioresour. Technol.*, 2024, 398:130538), respectively. Authentic bacterial fermentation medium samples were collected at specific time points and measured using Valsor/Ilesor and HPLC. As shown in **Fig. 17a, b**, Valsor exhibited a high correlation and consistency with HPLC results in the quantitative detection of L-valine in fermentation medium samples. The presence of different distractors did not affect the quantitative results obtained using Valsor (**Fig. 17c**). Similarly, Ilesor also enabled precise quantification of L-isoleucine in these samples with no interference from different distractors (**Fig. 18**). These results indicate that Valsor and Ilesor are applicable for efficient and convenient bioprocess monitoring of L-valine and L-isoleucine fermentation. These biosensors-based high-throughput screening could accelerate the construction of L-valine and L-isoleucine producing strains.

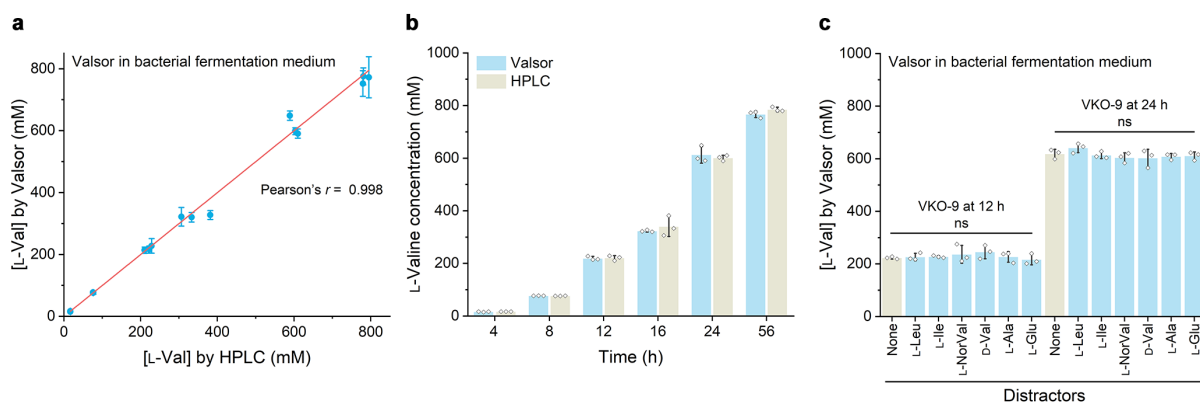


Figure 17 Application of Valsor for quantitative detection of L-valine in bacterial fermentation medium. **(a)** Correlation analysis **(a)** and consistency analysis **(b)** between Valsor and HPLC for quantitative detection of L-valine in bacterial fermentation medium. The samples were obtained from the fermentation medium of the VKO-9 strain. **(c)** Comparison of the quantitative results of Valsor for L-valine in bacterial fermentation medium in the absence or presence of 10 mM indicated L-valine analogs. All data shown are means $\pm$ s.d. ($n = 3$ independent experiments). ns, no significant difference ($P \geq 0.05$); one-way ANOVA test with Tukey's Multiple Comparison Test. **(Fig. 3j-l in the revised manuscript)**

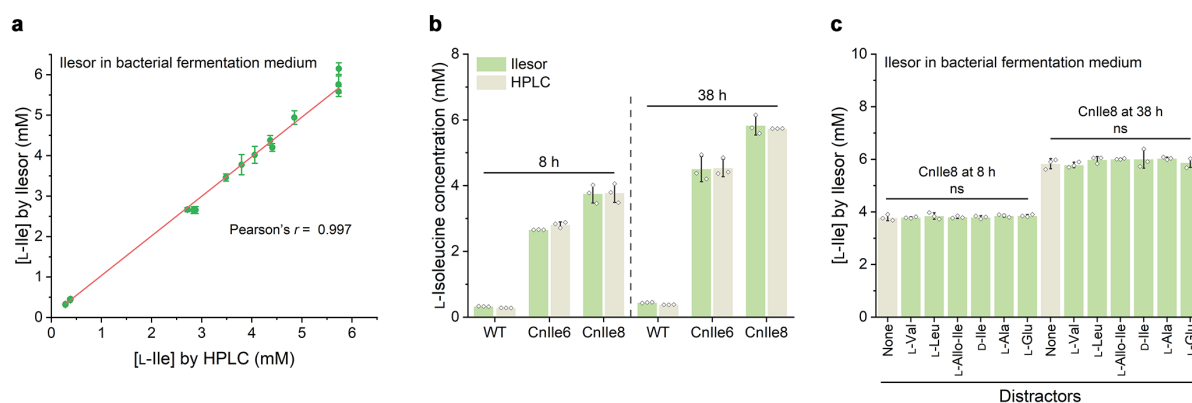


Figure 18 Application of Ilesor for quantitative detection of L-isoleucine in bacterial fermentation medium. **(a)** Correlation analysis **(a)** and consistency analysis **(b)** between Ilesor and HPLC for quantitative detection of L-isoleucine in bacterial fermentation medium. The samples were obtained from the fermentation medium of the *C. necator* H16 WT, CnIle6, and CnIle8 strains. **(c)** Comparison of the quantitative results of Ilesor for L-isoleucine in bacterial fermentation medium in the absence or presence of 10 mM indicated L-isoleucine analogs. All data shown are means $\pm$ s.d. ($n = 3$ independent experiments). ns, no significant difference ($P \geq 0.05$); one-way ANOVA test with Tukey's Multiple Comparison Test. **(Fig. 4j-l in the revised manuscript)**

Related data were presented in [Fig. 2j-m](#), [Fig. 3j-l](#), [Fig. 4j-l](#), [Supplementary Fig. 13](#), and [Supplementary Table 2](#) in the revised manuscript. Related descriptions were added in the “Results” section of the revised manuscript as follows:

For Phesor:

“L-Phenylalanine is a well-established biomarker for phenylketonuria²⁰. We collected 14 serum and 9 urine samples from healthy individuals, and prepared 14 serum and 16 urine samples from simulated patients with hyperphenylalaninemia or phenylketonuria by spiking various concentrations of L-phenylalanine (**Fig. 2j**). Quantification of L-phenylalanine in these samples using Phesor demonstrated good correlation and consistency with high-performance liquid chromatography (HPLC) measurements, and the results were unaffected by the presence of different L-phenylalanine analogs (**Fig. 2k, l and Supplementary Fig. 13**). The measured L-phenylalanine concentrations in serum and urine from healthy individuals were 72.55–102.17 μ M and 68.30–87.06 μ M, respectively, consistent with previously reported values (**Supplementary Table 2**). Furthermore, we collected authentic dried blood spot samples from 19 healthy children and 23 children with phenylketonuria. Blood L-phenylalanine concentrations measured using Phesor ranged from 31.10 to 119.91

μM in healthy subjects, while those in phenylketonuria patients ranged from 393.45 to 1,821.13 μM , higher than the clinical diagnostic cut-off value of 360 μM (**Fig. 2m**)²⁰. These results demonstrate that the GEFB Phesor based on the regulatory domain PheA_{ACT} in allosteric enzyme PheA is applicable for sensitive and precise detection of L-phenylalanine in human body fluids.”

For Valsor:

“L-Valine is a high-value bioactive compound, widely used in food, pharmaceutical, and feed industries²⁴. We conducted fed-batch fermentative production of L-valine using the *Klebsiella oxytoca* VKO-9 strain²⁴. As shown in **Fig. 3j, k**, Valsor exhibited a high correlation and consistency with HPLC results in the quantitative detection of L-valine in fermentation medium samples. The presence of different distractors did not affect the quantitative results obtained using Valsor (**Fig. 3l**). These results suggest that Valsor, developed based on the regulatory subunit IlvH in allosteric enzyme 2-acetolactate synthase, can serve as a reliable tool for precise L-valine detection both in living cells and *in vitro*.”

For Ilesor:

“In addition, we conducted fermentative production of L-isoleucine using *Cupriavidus necator* H16 and two L-isoleucine-producing strains, CnIle6 and CnIle8²⁷. Purified Ilesor enabled precise quantification of L-isoleucine in fermentation medium samples with no interference from different distractors (**Fig. 4j-l**). These results demonstrate that Ilesor, developed based on the regulatory domain CimA_{LA} in allosteric enzyme CimA, is applicable for sensitive and precise detection of L-isoleucine both in living cells and *in vitro*.”

Related experimental methods were added in the “Methods” section and “Supplementary Methods” section of the revised manuscript as follows:

“Preparation of human body fluid samples and bacterial fermentation medium samples

Serum samples from healthy individuals and patients with lung cancer were obtained by collecting venous blood in clot activator tubes, clotting for 2 h at room temperature, and then centrifuging at $2,000 \times g$ for 30 min at 4 °C. The resulting supernatant was filtered through a 0.22 μm filter and stored at $-80\text{ }^{\circ}\text{C}$ until further analysis. Urine samples from healthy individuals and patients with lung cancer were collected in sterile centrifuge tubes, filtered

through a 0.22 μm filter, and then stored at $-80\text{ }^{\circ}\text{C}$. Dried blood spot samples from healthy children and children with phenylketonuria were prepared by sorbing $\sim 25\text{--}75\text{ }\mu\text{L}$ of whole blood onto filter paper cards. To extract L-phenylalanine from the cards, the blood spot areas were intactly excised, divided into small fragments, and transferred into 500 μL Tris-HCl buffer (pH 7.4). The mixture was then sonicated for 20 min, vortexed for 2 min, and stirred overnight. The supernatant was separated by centrifugation at $12,000 \times g$ for 10 min, completely dried in a vacuum centrifuge (Concentrator Plus, Eppendorf, Germany) at 45°C , resuspended in an appropriate volume of Tris-HCl (pH 7.4), filtered through a 0.22 μm filter, and then stored at $-80\text{ }^{\circ}\text{C}$.

The L-valine-containing bacterial fermentation medium samples were generated by fed-batch fermentation of the *K. oxytoca* VKO-9 strain using glucose as the substrate²⁴. 1 mL samples were collected at specific time points, centrifuged at $4,000 \times g$ for 10 min at $4\text{ }^{\circ}\text{C}$, and then filtered through a 0.22 μm filter. The L-isoleucine-containing bacterial fermentation medium samples were generated by fed-batch fermentation of the *C. necator* H16 WT strain, CnIle6 strain, and CnIle8 strain using glucose as the substrate²⁷. 1 mL samples were collected at specific time points, centrifuged at $4,000 \times g$ for 10 min at $4\text{ }^{\circ}\text{C}$, and then filtered through a 0.22 μm filter. The prepared bacterial fermentation medium samples were stored at $-80\text{ }^{\circ}\text{C}$ until further analysis.

Determination of L-phenylalanine, L-valine, L-isoleucine, and SAM in different biological samples by Phesor, Valsor, Ilesor, and SAMsor_L

For the determination of L-phenylalanine in human serum and urine using Phesor, various concentrations of L-phenylalanine were spiked into the prepared serum and urine samples from healthy individuals to simulate body fluids from patients with hyperphenylalaninaemia or phenylketonuria. Both authentic samples from healthy subjects and simulated samples were mixed with diluted Phesor in a black 384-well plate at a volume ratio of 1:1. After incubating for 10 min at room temperature, the fluorescence ratios of Phesor were determined. The absolute quantification of L-phenylalanine was achieved by substituting the measured Phesor fluorescence ratio into the formula of the dose-response curve established based on serum or urine standards with known concentrations of L-phenylalanine. Measurement and quantification of L-phenylalanine in authentic dried blood

spot samples were performed using the same procedure.

For the determination of L-valine or L-isoleucine in bacterial fermentation medium samples, the prepared samples were diluted to an appropriate concentration using 50 mM Tris-HCl (pH 7.4). Then, 10 μ L samples were mixed with 10 μ L diluted Valsor or Ilesor in a black 384-well plate. After incubating for 20 min at room temperature, the fluorescence ratios of Valsor or Ilesor were determined. The absolute quantification of L-valine or L-isoleucine was achieved by substituting the measured fluorescence ratio of Valsor or Ilesor into the formula of the dose-response curves established based on fermentation medium standards with known concentrations of L-valine or L-isoleucine.

Measurement and quantification of SAM in authentic human serum and urine samples from healthy individuals and patients with lung cancer using SAMsor_L were performed using the same procedure as Phesor, with the urine samples required to be diluted to an appropriate concentration using 50 mM Tris-HCl (pH 7.4)."

"Determination of L-phenylalanine, L-valine, L-isoleucine, and SAM by HPLC

For the determination of L-phenylalanine in human body fluids using HPLC, the serum samples were mixed with methanol at a volume ratio of 1:1, vortexed for 2 min, centrifuged at $12,000 \times g$ for 15 min, and filtered through a 0.22 μ m filter. The urine samples and dried blood spot samples after extraction were boiled at 105 $^{\circ}$ C for 15 min, centrifuged at $12,000 \times g$ for 15 min, and filtered through a 0.22 μ m filter. The prepared samples were then analyzed by an HPLC system (Agilent 1100 series, Agilent Technologies, Germany) equipped with a ZORBAX SB-C18 column (5 μ m, 4.6 mm $\times$ 150 mm, Agilent Technologies, Germany) and a diode-array detector. The quantification was carried out using a 10 μ L injection volume, 10% acetonitrile at a flow rate of 0.6 mL min⁻¹ as mobile phase, a detection wavelength of 210 nm, and a total analysis time of 30 min.

For the determination of L-valine and L-isoleucine in bacterial fermentation medium using HPLC, the samples required pre-column derivatization by phenyl isothiocyanate (PITC)-acetonitrile and triethylamine (TEA)-acetonitrile. Briefly, 400 μ L samples were mixed with 200 μ L 0.1 M PITC-acetonitrile and 200 μ L 1 M TEA-acetonitrile, and placed for 1 h at room temperature. Then, 800 μ L *n*-hexane was added and the mixture was vortexed for 1 min. After being placed for 10 min at room temperature, the lower liquid layer was

extracted and submitted to HPLC analysis after filtration through a 0.22 μm filter. The column and detector used for HPLC analysis were consistent with L-phenylalanine detection. The quantification was carried out using a gradient elution program consisting of mobile phase A (0.76% [wt/vol] NaAc and 7% [vol/vol] acetonitrile, pH 6.5) and mobile phase B (80% [vol/vol] acetonitrile) at a flow rate of 0.6 mL min⁻¹: 0–11 min, from 0% to 7% B; 11–13.9 min, from 7% to 12% B; 13.9–14 min, from 12% to 15% B; 14–29 min, from 15% to 34% B; 29–37 min, 100% B; 37–45 min, 0% B. The injection volume was 5 μL , the column temperature was 40 °C, and the detection wavelength was 254 nm.

For the determination of SAM in human body fluids using HPLC, the serum samples were mixed with methanol at a volume ratio of 1:1, vortexed for 2 min, centrifuged at 12,000 $\times g$ for 15 min, and filtered through a 0.22 μm filter. The urine samples were boiled for 15 min at 105 °C, centrifuged at 12,000 $\times g$ for 15 min, and filtered through a 0.22 μm filter. The column and detector used for HPLC analysis were consistent with L-phenylalanine detection. The quantification was carried out using a gradient elution program consisting of mobile phase A (8 mM octanesulfonic acid sodium salt and 50 mM NaH₂PO₄, adjusted to pH 3.0 with H₃PO₄) and mobile phase B (methanol) at a flow rate of 0.5 mL min⁻¹: 0–16 min, 20% B; 16–16.9 min, from 20% to 40% B; 16.9–42 min, 40% B; 42–42.9 min, from 40% to 20% B; 42.9–63 min, 20% B. The injection volume was 20 μL , the column temperature was 25 °C, and the detection wavelength was 254 nm.”

We sincerely hope that the above responses have adequately addressed your concerns. We utilized consistency analysis (e.g., **Fig. 16a**), Bland-Altman analysis (e.g., **Fig. 15b**), and histogram comparisons (e.g., **Fig. 17b**) to determine the agreement between the results of Phesor, Valsor, and Ilesor and the HPLC measurements. If you have any further questions or concerns, we will be happy to respond and/or make further revisions.

3. Structure and logic of the manuscript

Currently, each sensor is presented as a separate, largely independent module. While this is understandable, it dilutes the central message. Since the authors emphasize that they have developed a “versatile method for constructing metabolite GEFBs using regulatory domains of allosteric enzymes,” the structure should reflect a unified workflow: (1) selection

of regulatory domain, (2) identification of insertion sites, (3) linker optimization, (4) functional testing. A schematic summarizing this generalizable design pipeline would be highly valuable. In addition, rather than offering superficial validation of all four sensors, the authors might consider presenting in-depth application of one sensor (e.g., optimization for better intracellular resolution or more rigorous in vitro testing) to highlight the method's potential.

Response: Thanks for your constructive suggestions. In accordance with your advice, a schematic diagram summarizing the generalizable design pipeline for allosteric regulatory domain-based GEFBs has been added to Fig. 1a of the revised manuscript (also shown in Fig. 19 below). The workflow comprises four steps: (1) selection of allosteric enzymes with high specificity for the target analyte; (2) identification of optimal cpSFYFP insertion sites within the loop regions of the separated regulatory domain to obtain a variant with obvious responsiveness and suitable affinity; (3) optimization of the linkers connecting cpSFYFP and the regulatory domain to maximize responsiveness; and (4) functional testing in living cells, human body fluids, etc.

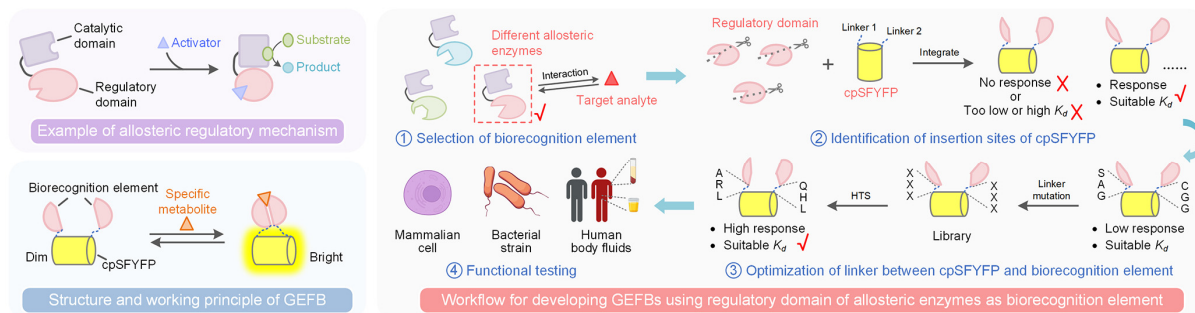


Figure 19 Schematic representations of the regulatory mechanisms of allosteric enzymes and the workflow for developing GEFBs based on allosteric regulatory domains and cpSFYFP. The construction process involves four steps: (i) selecting a specific allosteric enzyme; (ii) identifying optimal cpSFYFP insertion sites within the loop regions of the separated regulatory domain to obtain a variant with obvious responsiveness and suitable affinity; (iii) optimizing the linkers connecting cpSFYFP and regulatory domain to maximize responsiveness; and (iv) functional testing. HTS, high-throughput screening. (**Fig. 1a in the revised manuscript**)

Related data and descriptions were presented in Fig. 1a and its legend in the revised manuscript. In addition, a brief description was also added in the “Discussion” section of the revised manuscript as follows:

“... The methodology for developing GEFBs based on allosteric regulatory domains could be summarized in four steps: (i) selection of specific biorecognition elements, (ii) identification of optimal cpSFYFP insertion sites within loop regions to obtain a variant with obvious responsiveness and suitable affinity, (iii) optimization of linkers to maximize responsiveness, and (iv) functional testing (**Fig. 1a**). ...”

Furthermore, surpassing the request for rigorous *in vitro* testing of a single biosensor, we have comprehensively validated all developed biosensors to demonstrate their robust performance and practical utility. As detailed in our response to **Comment 2**, we have demonstrated the applicability of Phesor in clinical diagnostics using authentic human serum, urine, and dried blood spot samples. The utility of Valsor and Ilesor for bioprocess monitoring has also been validated using authentic bacterial fermentation samples. In addition, as detailed in **Comment 1**, we developed a more sensitive SAM biosensor, SAMsor_L, by strategically varying the cpSFYFP insertion site. SAMsor_L exhibited a lower detection range suitable for quantifying SAM in human body fluids.

Next, to assess the diagnostic potential of SAMsor_L, we prepared authentic serum and urine samples from 13 healthy individuals and 15 patients with lung cancer. Quantification of SAM in these samples using SAMsor_L exhibited good correlation and consistency with HPLC measurements, and the results were unaffected by the presence of different SAM analogs (**Fig. 20a-d and Fig. 21**). The measured SAM concentrations in serum and urine from healthy individuals were 100 ± 40 nM and 11.9 ± 1.7 μ M, respectively, consistent with previously reported values (~ 73.5 – 120 nM in serum and ~ 10.2 – 22.1 μ M in urine) (**Fig. 20e, f and Table 1**). Notably, serum SAM levels were significantly elevated in lung cancer patients, approximately 2.81-fold higher than those in healthy individuals (**Fig. 20e**). However, no significant differences were observed in urine SAM levels between the two population groups (**Fig. 20f**). Receiver operating characteristic (ROC) analysis indicated that serum SAM could effectively differentiate patients with lung cancer from healthy subjects, with an area under the curve (AUC) of 0.759, while urine SAM could not (AUC = 0.585) (**Fig. 20g, h**). These results demonstrate that serum SAM has the potential to be a clinically valuable biomarker for the diagnosis and treatment of lung cancer. Furthermore, the SAM biosensor SAMsor_L is applicable for sensitive and precise detection of SAM in human body fluids.

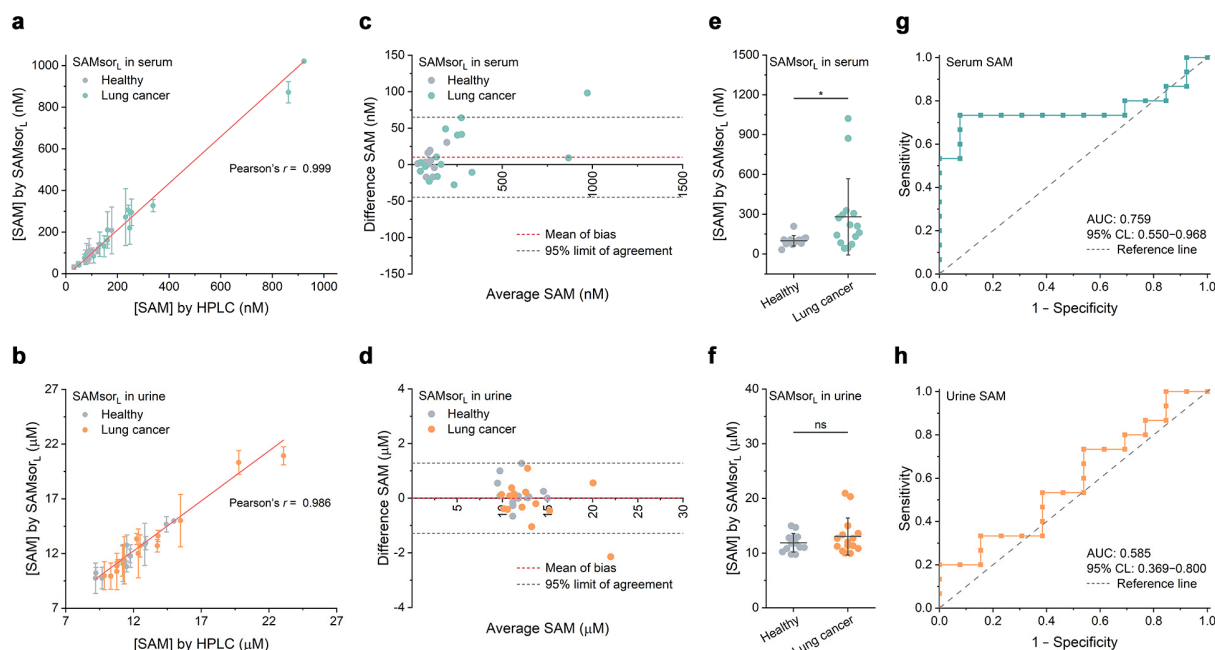


Figure 20 Application of SAMsor_L for quantitative detection of SAM in human body fluids. **(a, b)** Correlation analysis between SAMsor_L and HPLC for quantitative detection of SAM in serum **(a)** and urine **(b)** samples from healthy individuals and patients with lung cancer. Quantitative results measured by SAMsor_L were plotted against those obtained by HPLC. r , correlation coefficient. **(c, d)** Bland-Altman analysis for SAM in serum **(c)** and urine **(d)** samples from healthy individuals and patients with lung cancer measured by SAMsor_L and HPLC. Bias (red dotted line) and 95% limits of agreement (gray dotted lines) were shown. **(e, f)** Analysis of differences in serum **(e)** and urine **(f)** SAM levels between healthy individuals and patients with lung cancer measured by SAMsor_L. **(g, h)** ROC curves of patients with lung cancer compared with healthy individuals based on serum **(g)** or urine **(h)** SAM levels. The area under the curve (AUC) and 95% confidence level (CL) were shown. All data shown are means $\pm$ s.d. ($n = 3$ independent experiments). *, $P < 0.05$; ns, no significant difference ($P \geq 0.05$); two-tailed Student's t test. **(Fig. 7e-l in the revised manuscript)**

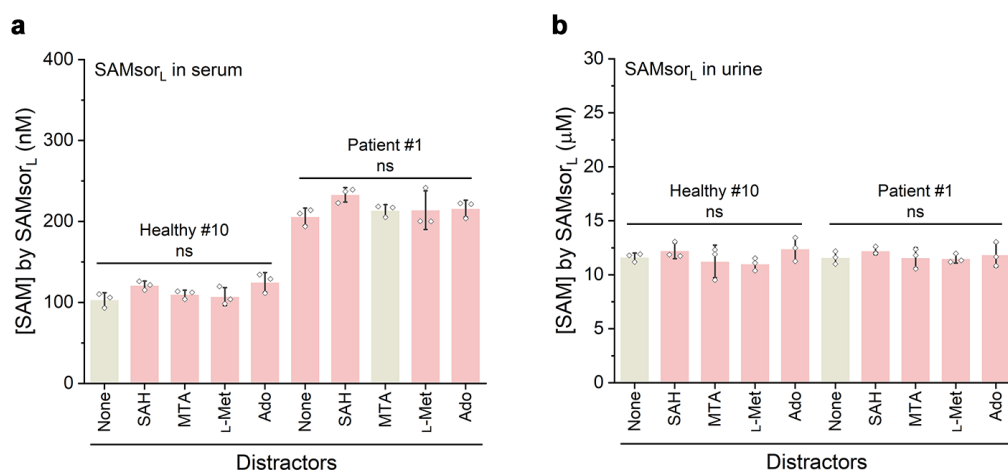


Figure 21 Analysis of the interference of different SAM analogs on the quantitative detection of SAM by

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 μ M (for SAH, MTA, adenosine) or 100 μ M (for L-methionine) depending on their physiological and pathological concentrations in serum. The concentration of distractors used in urine was set at 10 μ M (for SAH, MTA, adenosine) or 100 μ M (for L-methionine) depending on their physiological and pathological concentrations in urine. All data shown are means $\pm$ s.d. (n = 3 independent experiments). ns, no significant difference ($P \geq 0.05$); one-way ANOVA test with Tukey's Multiple Comparison Test. **(Supplementary Fig. 48 in the revised manuscript)**

Related data were presented in Fig. 7e-l and Supplementary Fig. 48 in the revised manuscript. Related descriptions were added in the “Results” section of the revised manuscript as follows:

“SAM is also recognized as an important biomarker for evaluating human disease and health status³⁶. Elevated SAM levels in body fluids are highly associated with the occurrence of diseases including digestive cancer, lung cancer, and pneumonia (Fig. 7a)^{37,38}. Fortunately, we found that the 34th variant (denoted as SAMsor-0.34, with cpSFYFP inserted between residues 111T/112V of PH1780) exhibited higher sensitivity to SAM among the 90 insertion variants of the SAM biosensor (Fig. 7b, Supplementary Fig. 37b, and Supplementary Fig. 39). Then, we conducted a systematic optimization of the linker in SAMsor-0.34 and identified a more sensitive biosensor SAMsor_L with the N-terminal linker of “Leu-Glu” and the C-terminal linker of “Thr-Pro” from 2,715 mutants (Fig. 7c). SAMsor_L exhibited an increased ΔR_{max} of $128.5 \pm 1.1\%$, a lower K_d of 379 ± 41 nM, and a decreased LOD of 57.25 nM for SAM (Fig. 7d).

We prepared authentic serum and urine samples from 13 healthy individuals and 15 patients with lung cancer. Quantification of SAM in these samples using SAMsor_L exhibited good correlation and consistency with HPLC measurements, and the results were unaffected by the presence of different SAM analogs (Fig. 7e-h and Supplementary Fig. 48). The measured SAM concentrations in serum and urine from healthy individuals were 100 ± 40 nM and 11.9 ± 1.7 μ M, respectively, consistent with previously reported values (Fig. 7i, j and Supplementary Table 2). Notably, serum SAM levels were significantly elevated in lung cancer patients, approximately 2.81-fold higher than those in healthy individuals (Fig. 7i). However, no significant differences were observed in urine SAM levels between the two

population groups (**Fig. 7j**). Receiver operating characteristic (ROC) analysis indicated that serum SAM could effectively differentiate patients with lung cancer from healthy subjects, with an area under the curve (AUC) of 0.759, while urine SAM could not (AUC = 0.585) (**Fig. 7k, l**). These results demonstrate that GEFBs with adjustable affinities can be derived from the same allosteric regulatory domain. Furthermore, the SAM biosensor SAM_{sort}_L is applicable for sensitive and precise detection of SAM in human body fluids.”

In addition to the in-depth *in vitro* applications, we have also supplemented subcellular experiments for all four biosensors to thoroughly characterize their spatial responsiveness in living cells. Taking Phesor as an example, the sequences for nuclear exclusion, mitochondrial localization, and nuclear localization were fused to the N-, N-, and C-terminus of Phesor to realize its subcellular positioning in HEK293FT cells. As shown in **Fig. 22a**, the fluorescence of Phesor was successfully restricted to the cytosol, mitochondria, and nucleus by introducing corresponding localization signals. Next, 750 μ M L-phenylalanine was added to the imaging medium of digitonin-pre-permeabilized HEK293FT cells. As shown in **Fig. 22**, Phesor expressed in different subcellular compartments exhibited identical responses to exogenous L-phenylalanine addition, whereas the fluorescence ratio of cpSFYFP remained unaffected.

Then, the subcellular distribution of L-phenylalanine was investigated by comparing the fluorescence ratios of the differentially localized Phesor in resting HEK293FT cells (**Fig. 23**). As shown in **Fig. 23c**, the cpSFYFP-corrected fluorescence ratio of Phesor was nearly identical in the cytosol and the nucleus, but was lowest in the mitochondria. This result indicates a relatively lower abundance of L-phenylalanine in the mitochondria, which was consistent with previous results obtained using complicated mass spectrometry technology (*Cell*, 2016, 166:1324–1337). In addition, different subcellular localized Phesor could also respond to endogenous L-phenylalanine fluctuations induced by L-phenylalanine addition and LAT1 overexpression (**Fig. 23c**). These results demonstrate the applicability of Phesor for spatiotemporal monitoring of L-phenylalanine fluctuations in living cells.

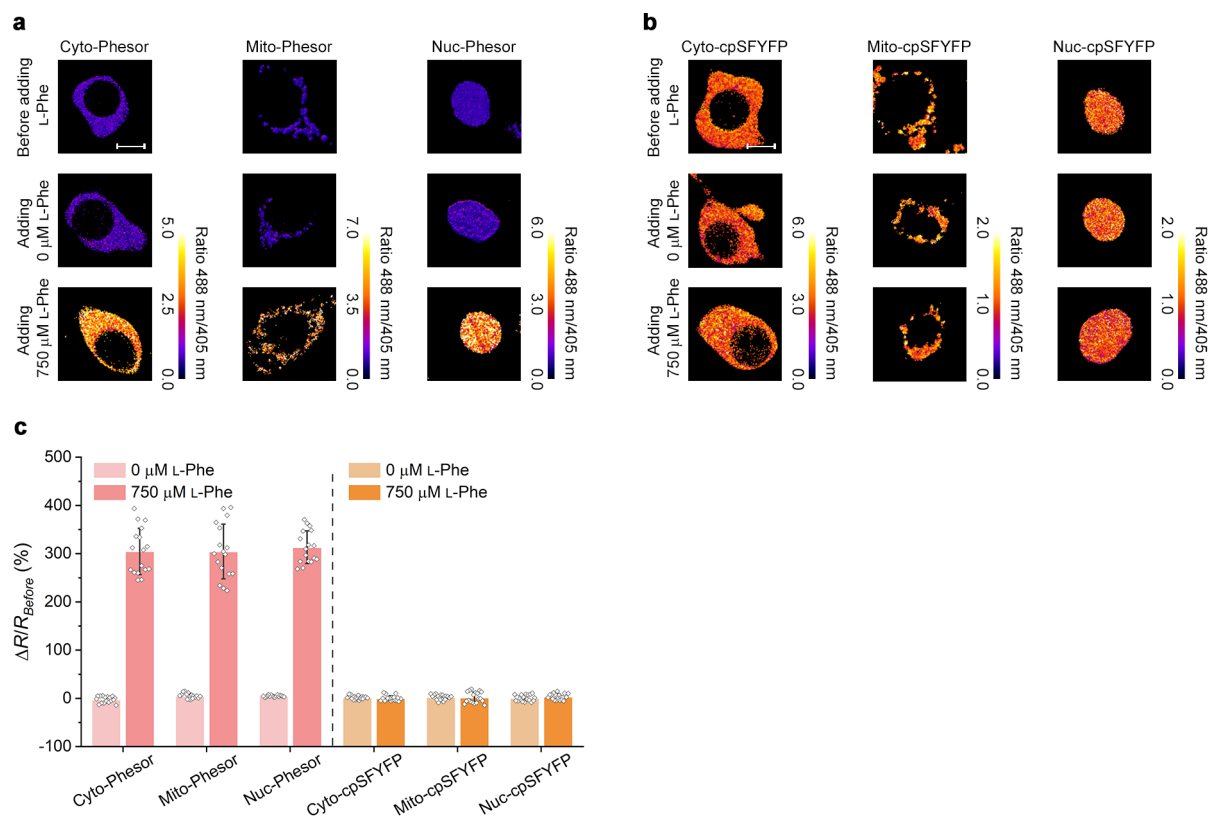


Figure 22 Characterization of different subcellular localized Phesor in HEK293FT cells. **(a, b)** Ratiometric images of single HEK293FT cell expressing Cyto-Phesor, Mito-Phesor, Nuc-Phesor **(a)**, or Cyto-cpSFYFP, Mito-cpSFYFP, Nuc-spSFYFP **(b)** before and after addition of 0 or 750 μM L-phenylalanine. Scale bar, 10 μm . **(c)** Fluorescence response of different subcellular localized Phesor or cpSFYFP to exogenous 750 μM L-phenylalanine addition. The HEK293FT cells were permeabilized with 80 μM digitonin. Data shown in panel **c** are means $\pm$ s.d. ($n = 17$ cells for different subcellular localized Phesor; $n = 19$ cells for different subcellular localized cpSFYFP). (**Supplementary Fig. 11 in the revised manuscript**)

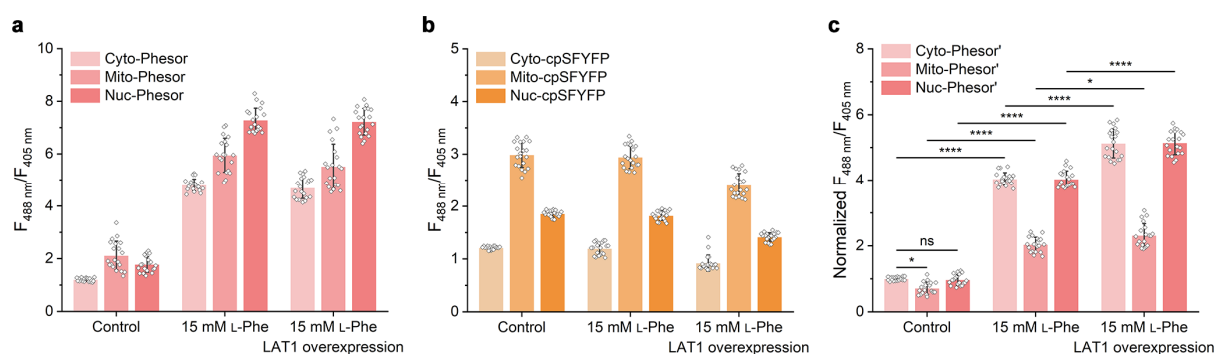


Figure 23 Analysis of subcellular distribution of L-phenylalanine using different subcellular localized Phesor. **(a, b)** Fluorescence ratios of HEK293FT cells expressing different subcellular localized Phesor **(a)** or cpSFYFP **(b)** in response to 15 mM L-phenylalanine addition and LAT1 overexpression. 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 subcellular localized Phesor required correction using cpSFYFP. **(c)** Corrected fluorescence ratios of HEK293FT cells expressing different subcellular localized Phesor by cpSFYFP. 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 test with Tukey's Multiple Comparison Test. **(Supplementary Fig. 12 in the revised manuscript)**

Similarly, all three other biosensors (Valsor, Ilesor, and SAMsor) could be correctly expressed in the cytosol, mitochondria, and nucleus of HEK293FT cells. These differentially localized biosensors exhibited identical fluorescence ratio changes in response to exogenous addition of target ligands (**Fig. 24, Fig. 26, and Fig. 28**). Notably, they also enabled *in situ* analysis of compartment-specific fluctuations in endogenous target ligands induced by treatment with various inhibitors (**Fig. 25, Fig. 27, and Fig. 29**). For example, in mammalian cells, L-valine and L-isoleucine are converted into branched-chain α -keto acids through transamination mediated by cytosolic branched-chain amino acid transaminase 1 (BCAT1) and mitochondrial branched-chain amino acid transaminase 2 (BCAT2). Treatment with the selective BCAT1 inhibitor gabapentin resulted in significant accumulation of L-valine and L-isoleucine in the cytosol and nucleus. The slightly elevated fluorescence ratios in mitochondria may be attributed to the transport of L-valine and L-isoleucine across the mitochondrial membrane (**Fig. 25c and Fig. 27c**). In addition, relatively lower abundances of L-valine, L-isoleucine, and SAM in the mitochondria were also observed, which were consistent with previously reported results (*Cell*, 2016, 166:1324–1337). Collectively, these results systematically demonstrate the robust performance of the four biosensors for compartmental monitoring of target ligands. These biosensors would pave the way for spatially resolved analysis of metabolic mechanisms and physiological functions at an unprecedented level.

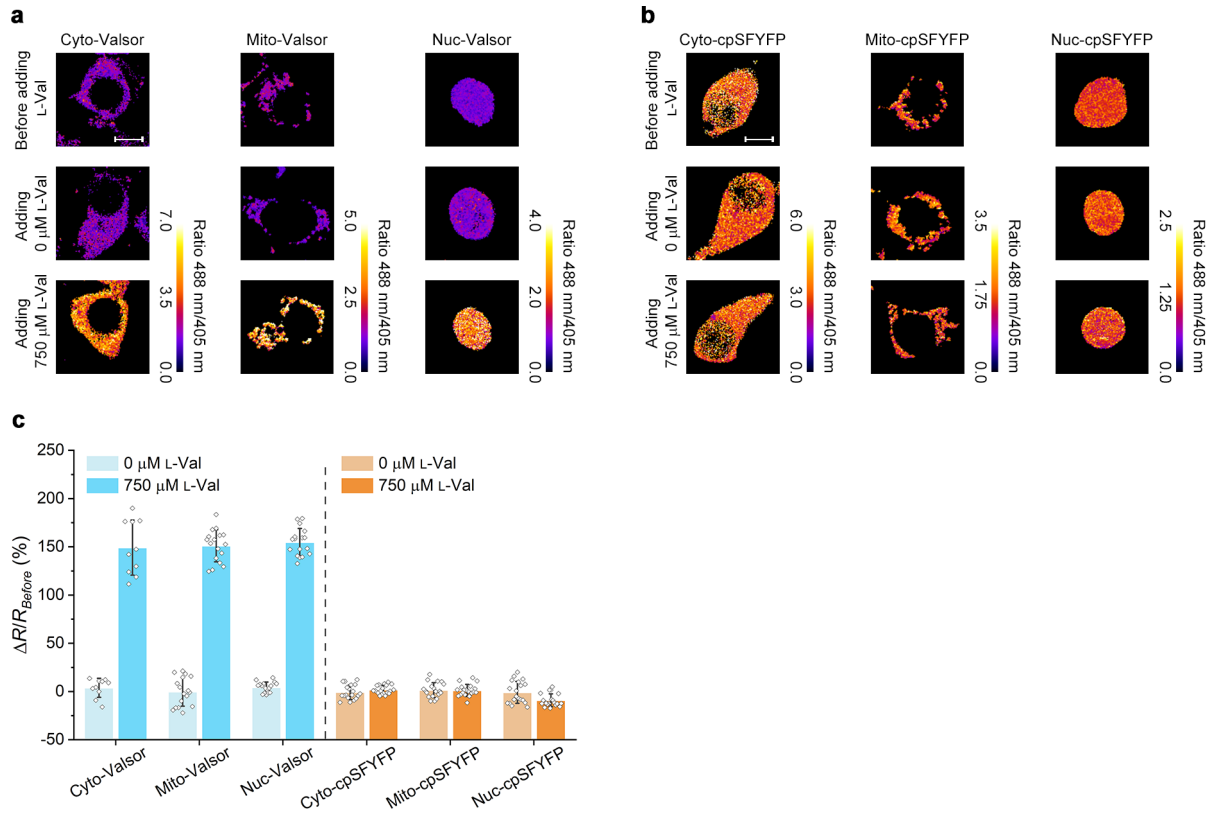


Figure 24 Characterization of different subcellular localized Valsor in HEK293FT cells. **(a, b)** Ratiometric images of single HEK293FT cell expressing Cyto-Valsor, Mito-Valsor, Nuc-Valsor **(a)**, or Cyto-cpSFYFP, Mito-cpSFYFP, Nuc-spSFYFP **(b)** before and after addition of 0 or 750 μM L-valine. Scale bar, 10 μm. **(c)** Fluorescence response of different subcellular localized Valsor or cpSFYFP to exogenous 750 μM L-valine addition. The HEK293FT cells were permeabilized with 80 μM digitonin. Data shown in panel **c** are means $\pm$ s.d. (n = 10, 10, 17, 17, 17, 17, 19, 19, 19, 19, 19 cells from left to right). **(Supplementary Fig. 24 in the revised manuscript)**

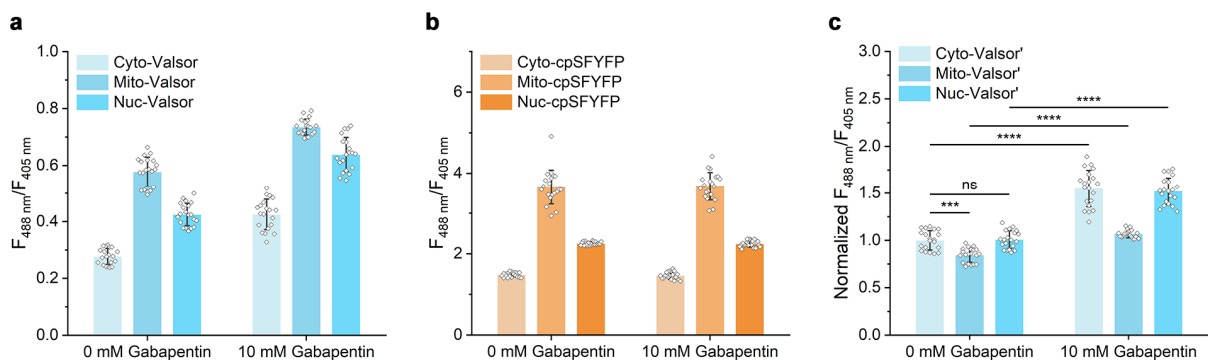


Figure 25 Analysis of subcellular distribution of L-valine using different subcellular localized Valsor. **(a, b)** Fluorescence ratios of HEK293FT cells expressing different subcellular localized Valsor **(a)** or cpSFYFP **(b)** in response to 10 mM gabapentin addition. Cells were treated with 0 or 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

subcellular localized Valsor required correction using cpSFYFP. **(c)** Corrected fluorescence ratios of HEK293FT cells expressing different subcellular localized Valsor by cpSFYFP. All data shown are means $\pm$ s.d. ($n = 20$ cells). ***, $P < 0.001$; ****, $P < 0.0001$; ns, no significant difference ($P \geq 0.05$); one-way ANOVA test with Tukey's Multiple Comparison Test. **(Supplementary Fig. 25 in the revised manuscript)**

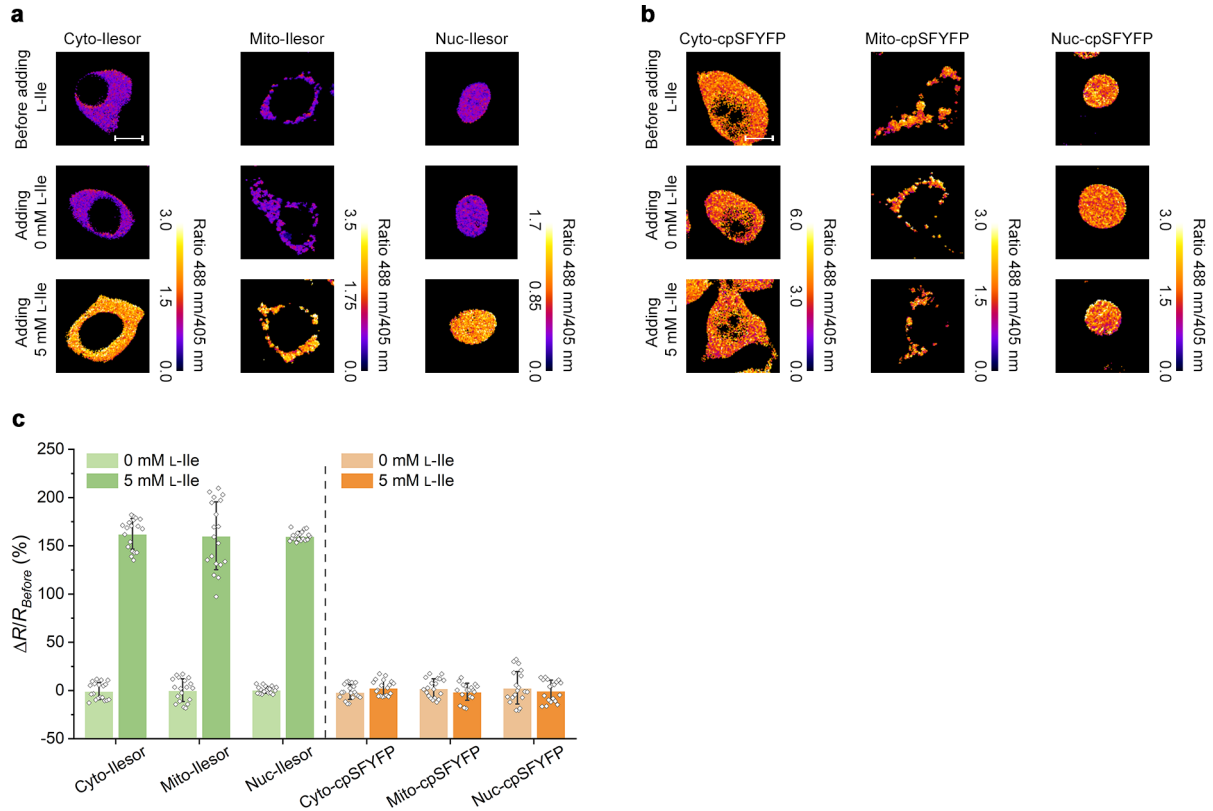


Figure 26 Characterization of different subcellular localized Ilesor in HEK293FT cells. **(a, b)** Ratiometric images of single HEK293FT cell expressing Cyto-Ilesor, Mito-Ilesor, Nuc-Ilesor **(a)**, or Cyto-cpSFYFP, Mito-cpSFYFP, Nuc-spSFYFP **(b)** before and after addition of 0 or 5 mM L-isoleucine. Scale bar, 10 μ m. **(c)** Fluorescence response of different subcellular 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). **(Supplementary Fig. 34 in the revised manuscript)**

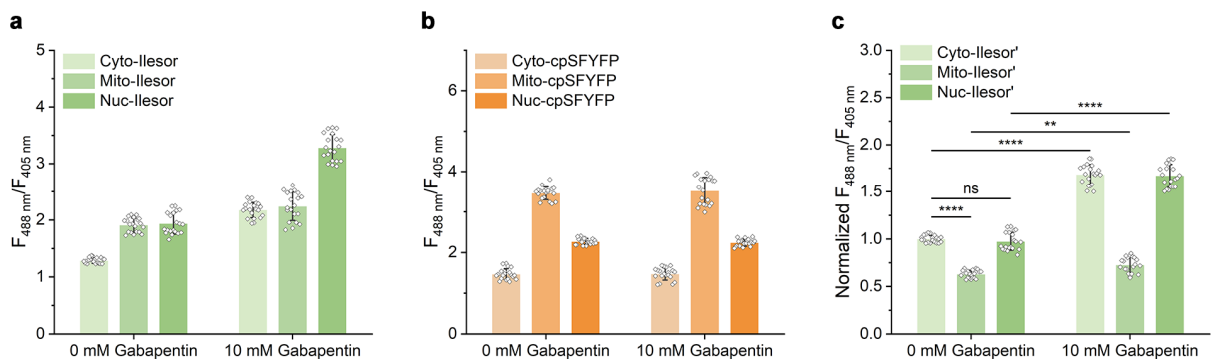


Figure 27 Analysis of subcellular distribution of L-isoleucine using different subcellular localized Ilesor.

(a, b) Fluorescence ratios of HEK293FT cells expressing different subcellular 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 subcellular localized Ilesor required correction using cpSFYFP. **(c)** Corrected fluorescence ratios of HEK293FT cells expressing different subcellular localized Ilesor by cpSFYFP. 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 test with Tukey's Multiple Comparison Test. **(Supplementary Fig. 35 in the revised manuscript)**

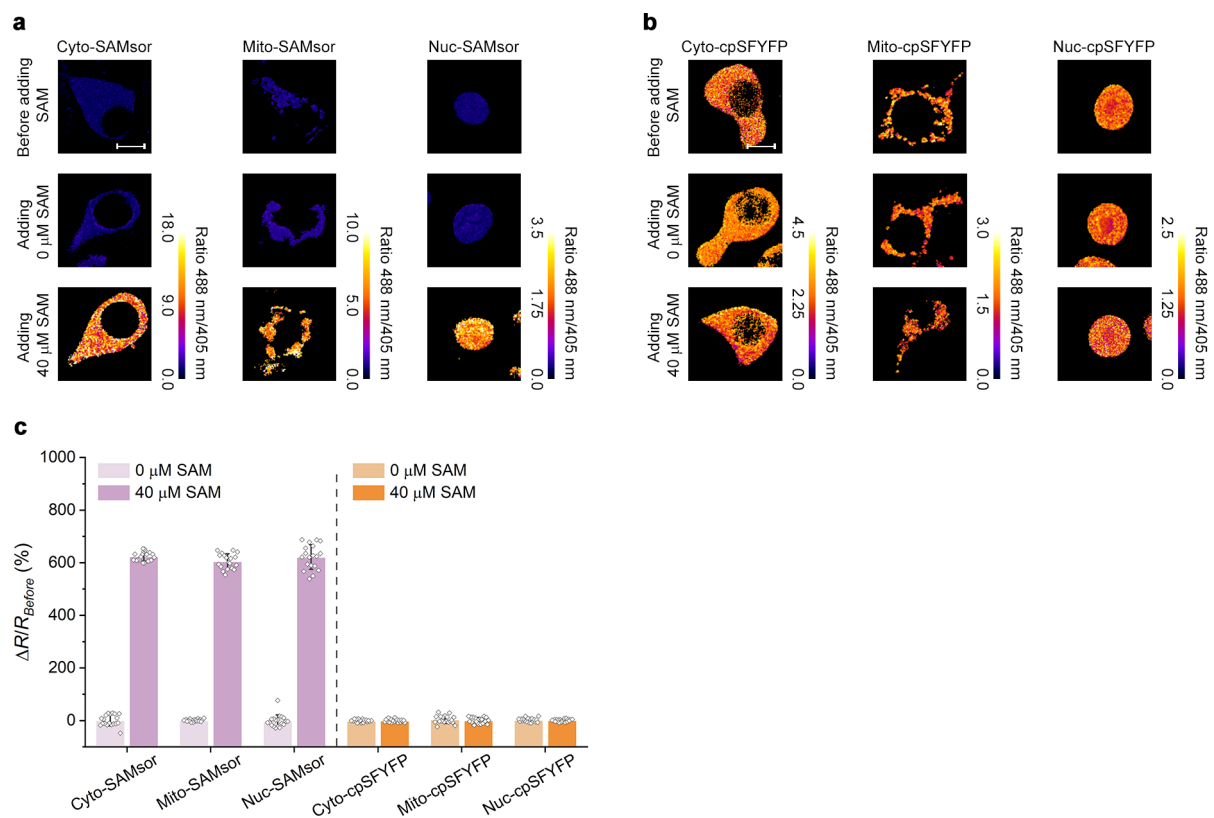


Figure 28 Characterization of different subcellular localized SAMsor in HEK293FT cells. **(a, b)** Ratiometric images of single HEK293FT cell expressing Cyto-SAMsor, Mito-SAMsor, Nuc-SAMsor **(a)**, or Cyto-cpSFYFP, Mito-cpSFYFP, Nuc-spSFYFP **(b)** before and after addition of 0 or 40 μM SAM. Scale bar, 10 μm . **(c)** Fluorescence response of different subcellular localized SAMsor or cpSFYFP to exogenous 40 μM SAM addition. The HEK293FT cells were permeabilized with 80 μM digitonin. Data shown in panel **c** are means $\pm$ s.d. ($n = 19$ cells). **(Supplementary Fig. 46 in the revised manuscript)**

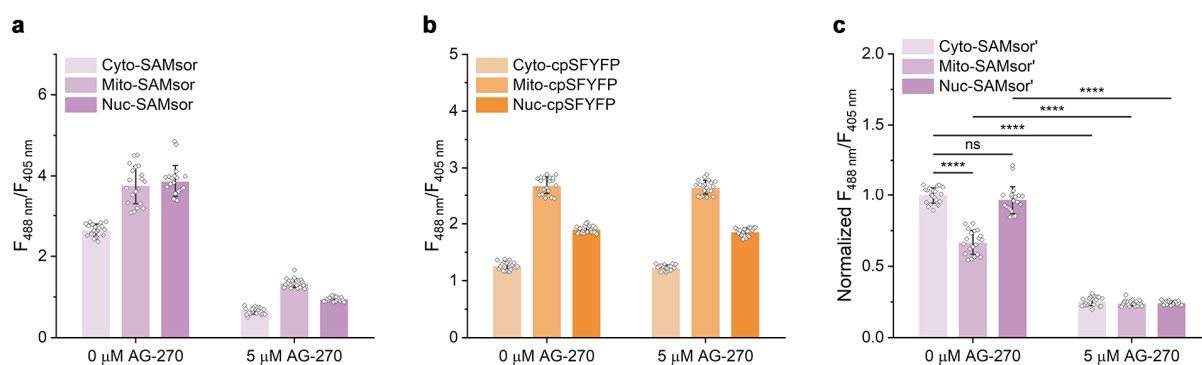


Figure 29 Analysis of subcellular distribution of SAM using different subcellular localized SAMsor. **(a, b)** Fluorescence ratios of HEK293FT cells expressing different subcellular localized SAMsor **(a)** or cpSFYFP **(b)** in response to 5 μ M AG-270 addition. Cells were treated with 0 or 5 μ M AG-270, 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 subcellular localized SAMsor required correction using cpSFYFP. **(c)** Corrected fluorescence ratios of HEK293FT cells expressing different subcellular localized SAMsor by cpSFYFP. 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 test with Tukey's Multiple Comparison Test. (**Supplementary Fig. 47 in the revised manuscript**)

Related data were presented in **Supplementary Fig. 11, Supplementary Fig. 12, Supplementary Fig. 24, Supplementary Fig. 25, Supplementary Fig. 34, Supplementary Fig. 35, Supplementary Fig. 46, and Supplementary Fig. 47** in the revised manuscript. Related descriptions were added in the “Results” section of the revised manuscript as follows:

For Phesor:

“Phesor could also be expressed in different subcellular compartments and responded to endogenous L-phenylalanine fluctuations (**Supplementary Fig. 11 and Supplementary Fig. 12**). We further investigated the subcellular distribution of L-phenylalanine in HEK293FT cells and found a relatively lower abundance of L-phenylalanine in the mitochondria than in the cytosol and nucleus (**Supplementary Fig. 12c**), which was consistent with previous results obtained using complicated mass spectrometry technology¹⁹. These results demonstrate that Phesor can monitor L-phenylalanine fluctuations in human cells with high spatiotemporal resolution.”

For Valsor:

“Valsor could be correctly expressed in the cytosol, mitochondria, and nucleus, and

responded to compartment-specific L-valine fluctuations induced by L-valine addition or gabapentin treatment (**Supplementary Fig. 24 and Supplementary Fig. 25**). Furthermore, we analyzed the subcellular distribution of L-valine and demonstrated its lower abundance in the mitochondria (**Supplementary Fig. 25c**).”

For Ilesor:

“Ilesor also allowed *in situ* detection of L-isoleucine in different subcellular compartments, and a lower abundance of mitochondrial L-isoleucine was identified (**Supplementary Fig. 34 and Supplementary Fig. 35**).”

For SAMsor:

“SAMsor expressed in different subcellular organelles also enabled compartment-specific SAM detection, with a lower mitochondrial SAM distribution observed (**Supplementary Fig. 46 and Supplementary Fig. 47**). These results suggest that SAMsor developed based on PH1780 is applicable for sensitive and precise detection of SAM in living cells.”

Experimental methods related to subcellular localization and characterization were added in the “Supplementary Methods” section of the revised manuscript as follows:

“Subcellular localization of Phesor, Valsor, Ilesor, and SAMsor

The pcDNA3.1⁽⁺⁾ plasmid was used for the subcellular expression of Phesor, Valsor, Ilesor, SAMsor, or cpSFYFP in HEK293FT cells. For cytosolic localization, two repeats of the MAPKK nuclear export signal (MALQKKLEELELDEQQRKRLEDL)₂ were fused to the N-terminus of these biosensors. For mitochondrial localization, a mitochondrial localization signal (MLSLRQSIRFFKPATRTLCSRYLL) was fused to the N-terminus of these biosensors. For nuclear localization, three repeats of the SV40 large T antigen nuclear localization signal (DPKKKRKV)₃ were fused to the C-terminus of these biosensors. The expression of subcellular localized Phesor, Valsor, Ilesor, SAMsor, or cpSFYFP in HEK293FT cells followed the same procedure as that used for the non-targeted biosensors. Functional characterization of these subcellular localized biosensors in HEK293FT cells was performed 24 h after transfection. The cells were washed twice with 37 °C pre-warmed HHBSS buffer, permeabilized with 80 μM digitonin, and then treated with 750 μM L-phenylalanine, 750 μM L-valine, 5 mM L-isoleucine, or 40 μM SAM. Fluorescence ratios

were measured before and after the addition of different target ligands using a Zeiss 900 confocal microscope. Different subcellular localized cpSFYFP were used in parallel experiments to exclude potential effects due to pH fluctuations.

Application of Phesor, Valsor, Ilesor, and SAMsor for subcellular detection of target ligands in human cells

For detection of endogenous L-phenylalanine fluctuations at subcellular resolution using Phesor, 0 or 15 mM L-phenylalanine was added into the medium of HEK293FT cells transfected with different subcellular localized Phesor or co-transfected with different subcellular localized Phesor and LAT1. Changes in the fluorescence ratio of different subcellular localized Phesor were imaged 20 h after L-phenylalanine addition. For detection of endogenous L-valine or L-isoleucine fluctuations at subcellular resolution using Valsor or Ilesor, 0 or 10 mM gabapentin was added into the medium of HEK293FT cells transfected with different subcellular localized Valsor or Ilesor. Changes in the fluorescence ratio of different subcellular localized Valsor or Ilesor were imaged 20 h after gabapentin treatment. For detection of endogenous SAM fluctuations at subcellular resolution using SAMsor, 0 or 5 μ M AG-270 was added into the medium of HEK293FT cells transfected with different subcellular localized SAMsor. Changes in the fluorescence ratio of different subcellular localized SAMsor were imaged 20 h after gabapentin treatment. Different subcellular localized cpSFYFP were used in parallel experiments to exclude potential effects due to pH fluctuations.”

We sincerely thank you again for your constructive comments. If you consider any further revisions necessary, we are fully prepared to undertake the additional work to improve our manuscript.

4. cpSFYFP insertion strategy

It would be helpful to know whether any general rules emerged regarding cpSFYFP insertion sites in different regulatory domains. Can these be clustered in conserved motifs or loops? A summary of insertion-site mapping would be valuable for guiding future efforts.

Response: Thanks for your valuable and insightful comments. We fully agree that establishing general design rules is essential for guiding future engineering efforts. **Firstly,**

we analyzed the correlation between the cpSFYFP insertion sites and the responsiveness of the resulting biosensor variants. In this study, allosteric regulatory domains from distinct protein families (ACT domain, Pfam ID: PF01842; Cim_{ALA} domain, PF08502; CBS domain, PF00571) were utilized to demonstrate the feasibility and versatility of developing GEFBs. We systematically targeted all loop regions within these domains for cpSFYFP insertion to screen for responsive variants. As shown in **Fig. 6b**, **Fig. 8b**, and **Fig. 9b**, we mapped the distribution of responsive insertion sites relative to the secondary structures of the biorecognition elements. While responsive insertion variants could be identified across different loop regions, they tended to cluster within specific loops. For example, in the L-phenylalanine biosensor, variants with cpSFYFP inserted into the loop connecting the second α -helix and the fourth β -sheet exhibited broad responsiveness (**Fig. 6b**).

However, regarding “conserved motifs or loops”, we were unable to identify a universal rule applicable across the distinct families of regulatory domains used in this study. This is because these domains are evolutionarily divergent (belonging to distinct clans in the Pfam database) and exhibit distinct topological folds. Specifically, the ACT domain adopts a ferredoxin-like fold ($\beta\alpha\beta\beta\alpha\beta$), the Cim_{ALA} domain features a $(\beta\beta\beta\alpha)_2$ fold, and the CBS domain typically forms a Bateman module composed of tandem CBS motifs ($\alpha\beta\alpha\beta\beta\alpha$) (*Curr. Opin. Struct. Biol.*, 2001, 11:694–700; *Biochem. J.*, 2009, 421:133–143; *Arch. Biochem. Biophys.*, 2013, 540:70–81) (**Fig. 30b**, **Fig. 31b**, **Fig. 32b**, **Fig. 33b**).

To quantify this structural divergence, we performed structural alignments. The analysis revealed high root mean square deviation (RMSD) values: 3.67 Å between Phe_{ACT} and Cim_{ALA}, 12.26 Å between Phe_{ACT} and PH1780_{CBS4}, and 4.18 Å between Cim_{ALA} and PH1780_{CBS4}. These values significantly exceed the empirical threshold of ~3.0 Å typically used to define structural homology (*EMBO J.*, 1986, 5:823–826; *Fold. Des.*, 1998, 3:141–147). Given these structural differences, identifying universal responsive insertion sites is challenging across distinct families of regulatory domains. The selection of responsive loop regions should be guided by the conserved structure within the same family of regulatory domains.

Next, we analyzed the correlation between the cpSFYFP insertion sites and the affinities of the resulting biosensor variants. As detailed in our response to **Comment 1**,

we discovered that the cpSFYFP insertion site significantly influences the affinity of the resulting biosensors. Taking the L-phenylalanine biosensor as an example, the reported native dissociation constant of the recognition element PheA_{ACT} for L-phenylalanine is ~20 μ M (**Fig. 30**). This value is close to the K_d value of ~57 μ M for Phesor-0.04 (with cpSFYFP inserted at the N-terminus of PheA_{ACT}), but is significantly lower than the K_d value of ~440 μ M for Phesor-0.38 (with cpSFYFP inserted in the interior of PheA_{ACT}) (**Fig. 6 and Fig. 30**). We speculated that the distinct cpSFYFP insertion sites caused varying structural perturbations in the biorecognition elements, which may account for the observed affinity differences.

Therefore, we performed structural modeling using AlphaFold 3 for PheA, Phesor-0.04, and Phesor-0.38, and compared the structural similarity of their PheA_{ACT} domains. As shown in **Fig. 30**, a higher structural similarity, indicated by a lower RMSD of **0.196 Å**, was observed between the PheA_{ACT} domains of Phesor-0.04 and PheA compared to that between Phesor-0.38 and PheA (RMSD of **0.429 Å**). This result suggests that the PheA_{ACT} structure in Phesor-0.04 more closely resembles its native conformation, providing a structural basis for its higher affinity.

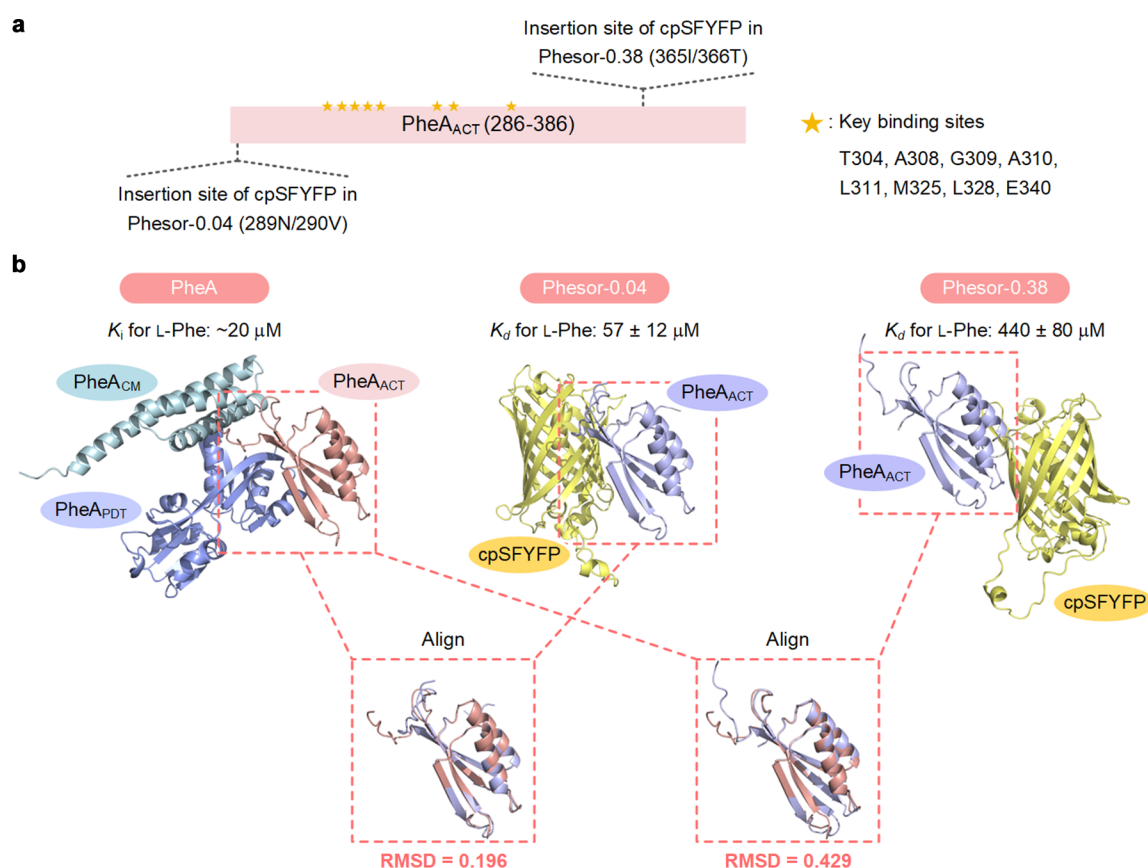


Figure 30 Analysis of the effect of cpSFYFP insertion sites on the affinity of the resulting biosensor variants. **(a)** Schematic representation of the cpSFYFP insertion sites in the PheA_{ACT} domain of Phesor-0.04 (a low- K_d L-phenylalanine biosensor variant) and Phesor-0.38 (a high- K_d variant). Key L-phenylalanine binding sites were shown. **(b)** Structure 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.

(Supplementary Fig. 49a, b in the revised manuscript)

Notably, consistent with the observations in Phesor, multiple insertion variants spanning a range of K_d values were also identified during the development of Valsor (K_d from ~ 35 to $\sim 414 \mu\text{M}$), Ilesor (K_d from ~ 59 to $\sim 17,000 \mu\text{M}$), and SAMsor (K_d from ~ 0.9 to $\sim 8.3 \mu\text{M}$) (Fig. 7, Fig. 8, and Fig. 9). Importantly, in the low- K_d variants, the cpSFYFP insertion sites were consistently positioned distal to the ligand-binding pockets. Consequently, the allosteric regulatory domains in these variants retained higher structural similarity (lower RMSD) to the native conformations (Fig. 31, Fig. 32, and Fig. 33).

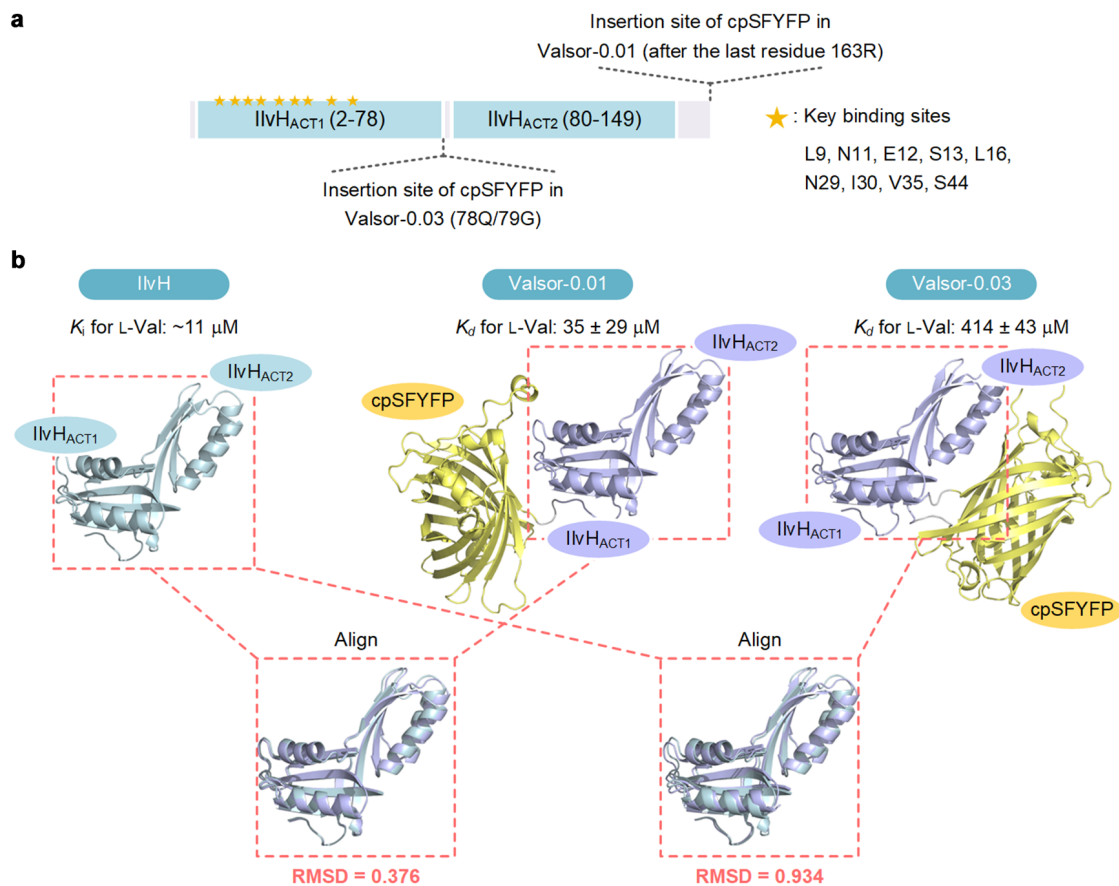


Figure 31 Analysis of the effect of cpSFYFP insertion sites on the affinity of the resulting biosensor variants. **(a)** Schematic representation of the cpSFYFP insertion sites in the IlvH domain of Valsor-0.01 (a low- K_d L-valine biosensor variant) and Valsor-0.03 (a high- K_d variant). Key L-valine binding sites were shown. **(b)** Structure 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. (Supplementary Fig. 49c, d in the revised manuscript)

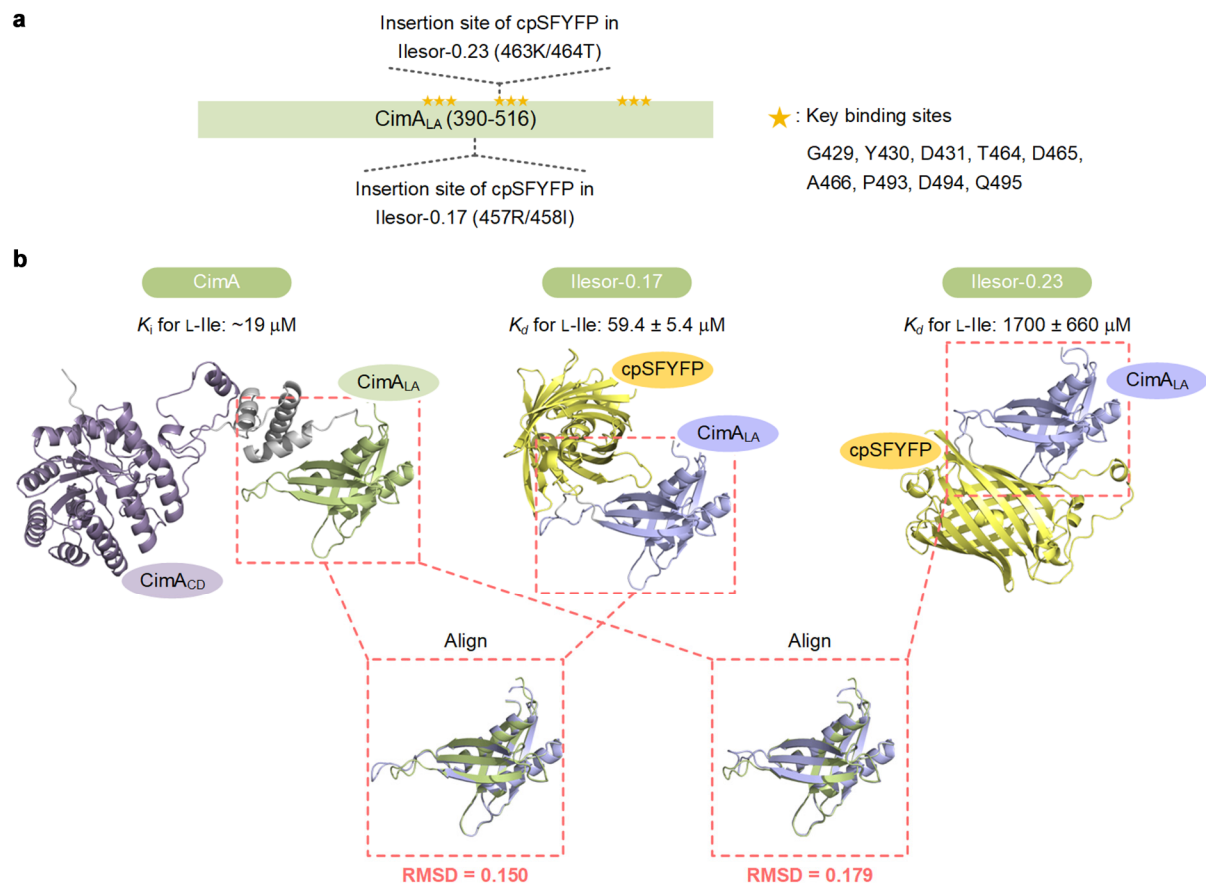


Figure 32 Analysis of the effect of cpSFYFP insertion sites on the affinity of the resulting biosensor variants. **(a)** Schematic representation of the cpSFYFP insertion sites in the CimA_{LA} domain of Ilesor-0.17 (a low- K_d L-isoleucine biosensor variant) and Ilesor-0.23 (a high- K_d variant). Key L-isoleucine binding sites were shown. **(b)** Structure 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. (**Supplementary Fig. 49e, f in the revised manuscript**)

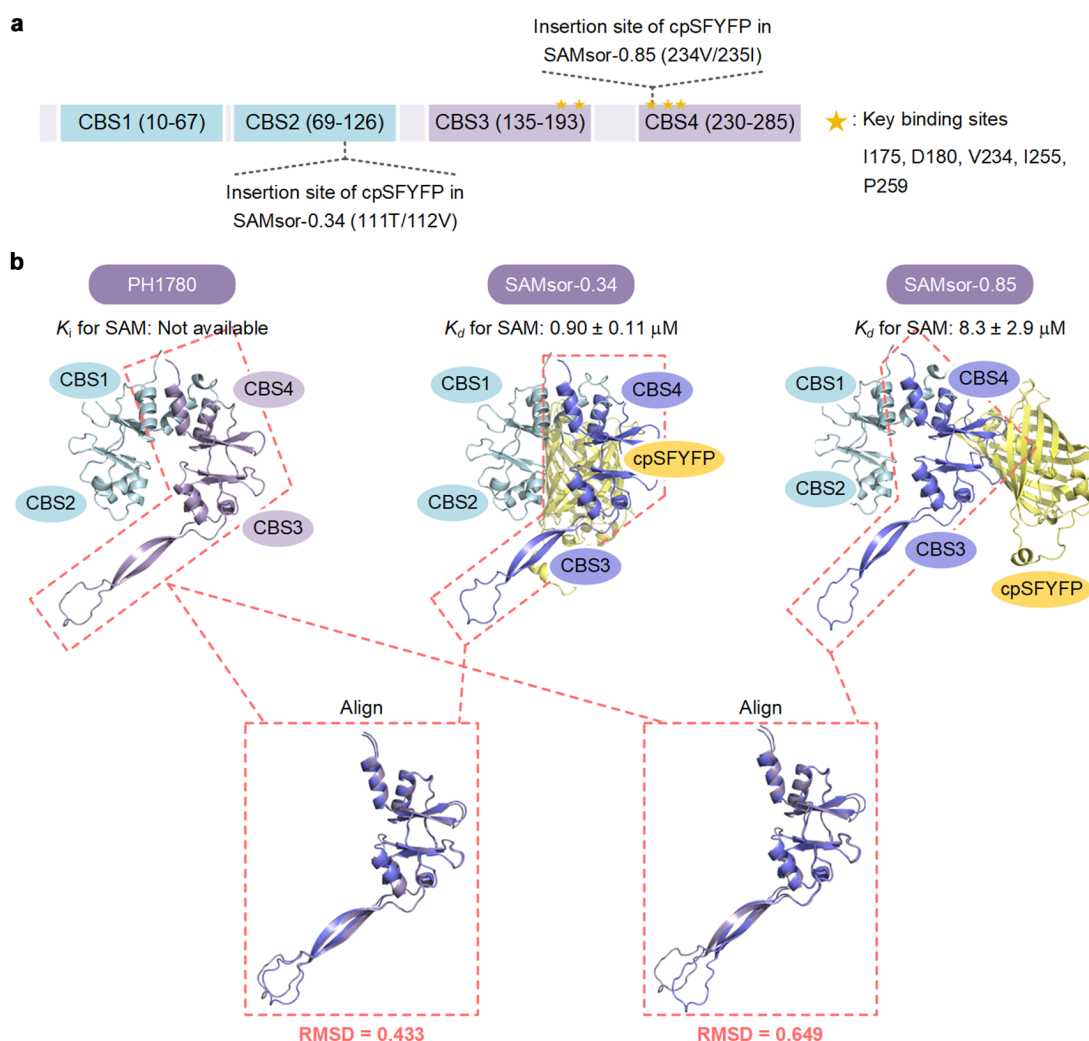


Figure 33 Analysis of the effect of cpSFYFP insertion sites on the affinity of the resulting biosensor variants. **(a)** Schematic representation of the cpSFYFP insertion sites in the PH1780 domain of SAMsor-0.34 (a low- K_d SAM biosensor variant) and SAMsor-0.85 (a high- K_d variant). Key SAM binding sites were shown. **(b)** Structure 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. (**Supplementary Fig. 49g, h in the revised manuscript**)

In conclusion, the affinities of allosteric regulatory domain-based GEFBs can be strategically modulated by positioning cpSFYFP either near to or distal from the ligand-binding pocket. This structure-function relationship provides a rational foundation for the future engineering of GEFBs with predictable affinities, thereby expanding the toolbox for metabolic imaging and *in vitro* diagnostics across varying concentration scales.

Related data were presented in **Supplementary Fig. 49** in the revised manuscript.

Related descriptions were added in the “Discussion” section of the revised manuscript as follows:

“In this study, we demonstrated the feasibility of utilizing regulatory domains of allosteric enzymes as biorecognition elements for GEFBs development. By coupling the ACT domain from homomultimeric enzyme (PheA_{ACT}), ACT-containing regulatory subunit from heteromultimeric enzyme (IlvH), or LA domain from homomultimeric enzyme (CimA_{LA}) with cpSFYFP, we developed three biosensors for detection of L-phenylalanine, L-valine, and L-isoleucine, respectively. We also confirmed that the multi-ligand-binding PH1780 can serve as a biorecognition element to derive diverse specific biosensors, and then constructed two SAM-specific biosensors with complementary detection ranges based on PH1780 and cpSFYFP. These biosensors assembled into proper quaternary structures consistent with those of the native PheA_{ACT} domain, IlvH, CimA_{LA} domain, and CBS domain^{13,15,21,25,29}. Each ligand molecule bound independently to individual monomers within the multimeric biosensors (hill slope close to 1). These results demonstrate that the developed biosensors preserved the intrinsic ligand-binding characteristics of their native allosteric regulatory domains. The methodology for developing GEFBs based on allosteric regulatory domains could be summarized in four steps: (i) selection of specific biorecognition elements, (ii) identification of optimal cpSFYFP insertion sites within loop regions to obtain a variant with obvious responsiveness and suitable affinity, (iii) optimization of linkers to maximize responsiveness, and (iv) functional testing (**Fig. 1a**). Notably, the affinities of allosteric regulatory domain-based GEFBs can be strategically modulated by shifting the insertion site of cpSFYFP. Besides SAMsor-0.34, multiple insertion variants with lower K_d values than Phesor, Valsor, and Ilesor were identified, such as Phesor-0.04 ($57 \pm 12 \mu\text{M}$), Valsor-0.01 ($35 \pm 29 \mu\text{M}$), and Ilesor-0.17 ($59.4 \pm 5.4 \mu\text{M}$) (**Supplementary Fig. 4, Supplementary Fig. 16, and Supplementary Fig. 28**). The higher affinities of these variants might be attributed to the insertion site of cpSFYFP being positioned distal to the binding pocket, which minimizes structural disruption and maintains higher architectural fidelity of the biorecognition element to its native conformation (**Supplementary Fig. 49**). This structure-function relationship provides a rational foundation for future GEFB engineering, thereby expanding the toolbox for metabolic imaging and *in vitro* diagnostics across varying concentration scales.”

Minor Points:

1. Figure 1i and Figure 2d show redundant ligand specificity data for Phesor. One could be moved to the supplementary materials.

Response: Thanks for your good suggestion. As recommended, we have relocated the redundant specificity data previously shown in Fig. 2d to the supplementary materials, where it is now presented as **Supplementary Fig. 5g**.

2. Line 267: Please spell out CBS domain in full on first mention.

Response: Thanks for your good suggestion. The term “CBS domain” has now been spelled out in full at its first mention in both the “Introduction” section and the “Results” section (corresponding to Line 267 in the previous manuscript). The related changes are described in the revised manuscript as follows:

“... Two *S*-adenosyl-L-methionine (SAM, the second most chemically active cofactor in cells¹²)-specific biosensors with complementary detection ranges and an *S*-methyl-5'-thioadenosine (MTA)-specific biosensor can be developed based on PH1780 with multi-ligand-binding cystathionine β -synthase (CBS) domains. ...”

“Cystathionine β -synthase (CBS) domain as biorecognition element for SAM biosensor and MTA biosensor

Many allosteric enzymes possess multiple ligand binding sites and thus respond to diverse ligands (**Supplementary Table 1**)^{28,29}. ...”

3. Lines 74-75 state “two specific biosensors for the previously unavailable distinction of L-isoleucine and L-valine.” However, Wang et al. (Nat Commun, 2023, 14:6680) had already reported a biosensor for L-isoleucine using a similar approach. This prior work should be acknowledged.

Response: Thanks for your constructive comment. As you correctly indicated, a recent study reported a method for developing GEFBs using the ANL superfamily enzymes as biorecognition elements (*Nat. Commun.*, 2023, 14:6680). As a result of this method, several GEFBs have been developed for specific detection of various amino acids *in vitro*, including

L-phenylalanine and L-valine. However, these ANL superfamily enzyme-based GEFBs rely on the adenylation reactions between the analytes and ATP to achieve their responsiveness. For example, the L-phenylalanine biosensor based on ANL superfamily enzyme requires enzymatic ATP depletion and phenylalanyl-AMP generation to realize L-phenylalanine detection. Due to the lack of reversibility and potential metabolic disturbances induced by enzymatic activity, these biosensors have limitations in real-time and *in situ* imaging of target ligands in living cells. In contrast, the GEFBs designed in our study utilize the regulatory domains of allosteric enzymes as biorecognition elements, avoiding interference with cellular metabolism and thus being compatible with intracellular assays. L-Phenylalanine, L-valine, and L-isoleucine are important endogenous metabolites in living organisms. The GEFBs developed in this manuscript enabled previously unavailable reversible and specific detection of these amino acids both *in vitro* and in living cells.

To enhance the precision of our description, we have modified the relevant statement in the revised manuscript as follows:

“Then, we also use the LeuA regulatory (LA) domain in the homomultimeric allosteric enzyme D-citramalate synthase (CimA) and the regulatory domain (existing in the form of the regulatory subunit IlvH) in the heteromultimeric allosteric enzyme 2-acetolactate synthase as the biorecognition elements to construct two specific biosensors for the previously unavailable distinction of L-isoleucine and L-valine both *in vitro* and in living cells.”

In addition, a short discussion about the ANL superfamily enzymes-based GEFBs have been added in the “Discussion” section of the revised manuscript as follows:

“The biosynthesis of many structurally similar metabolites often involves a common precursor and is co-regulated at the expression level. Due to their structural similarities and the promiscuity of the related transcriptional regulators, the distinction and specific tracking of these metabolites through transcriptional regulator-based biosensors are technically unavailable. For example, AAAs biosensor can be constructed based on TyrR, but it is unable to distinguish L-phenylalanine from the other two AAAs, L-tyrosine and L-tryptophan^{14,39}. In addition, a method for developing GEFBs using ANL superfamily enzymes as biorecognition elements has been reported to achieve specific detection of L-phenylalanine and L-isoleucine *in vitro*. However, these GEFBs rely on adenylation reactions between analytes and ATP. The

potential metabolic disturbances may limit their applications in living systems⁴⁰. In this study, we successfully used the allosteric regulatory domain of PheA, the key enzyme involved in L-phenylalanine biosynthesis, to construct a specific biosensor Phesor. Phesor allowed for previously unavailable high spatiotemporal resolution detection of L-phenylalanine in living cells. Traditional screening of inhibitors of LAT1, a cancer therapeutic target involved in L-phenylalanine transport, was achieved using cumbersome radioisotope technology¹⁸. The *in situ* assay of intracellular L-phenylalanine through Phesor may also support high-throughput screening of LAT1 inhibitors. In addition, L-phenylalanine is a critical biomarker for phenylketonuria diagnosis and therapeutic monitoring²⁰. Compared to traditional HPLC- or mass spectrometry-based methods, Phesor is easy to handle, cost-effective, rapid, and compatible with high-throughput assays (**Supplementary Table 5**), making it an ideal tool for point-of-care diagnostics of L-phenylalanine-related diseases.”

In summary, while the conceptual framework of using allosteric regulatory domains as sensing modules for GEFBs is valuable, the practical implementation in this manuscript requires more rigorous validation and clearer focus to meet the novelty and impact expectations of Nature Communications.

Response: We sincerely thank you again for your valuable comments and constructive suggestions. In response to your recommendations, we have demonstrated the applicability of the exemplified biosensors for real-time and *in situ* tracking of target ligands in living cells. Furthermore, we have supplemented rigorous *in vitro* testing for these biosensors (particularly developing a new, high-affinity SAM biosensor), demonstrating their potential applications in bioprocess monitoring and clinical diagnostics. Additionally, we have elucidated the correlation between the cpSFYFP insertion sites and biosensor affinity, establishing a generalized strategy for predictable affinity tuning based on structure-function relationships.

The key innovations of our revised manuscript are highlighted as follows:

(1) Traditional biorecognition elements for GEFBs include G protein-coupled receptors (GPCRs), bacterial periplasmic binding proteins (PBPs), and bacterial transcriptional regulators. A versatile method for developing neuropeptide GEFBs based on GPCRs was

recently published in *Science* (*Science*, 2023, 382:eabq8173). GPCR-based biosensors specialize in detecting signal transduction but not intracellular metabolite concentrations due to their insolubility, while PBP-based biosensors typically require extensive affinity screening due to their poor selectivity (*Cell*, 2020, 183:1986–2002). Our group has previously focused on identifying novel transcriptional regulators from bacterial metabolic pathways and developing corresponding metabolite GEFBs (*Nat. Commun.*, 2025, 16:6913; *Nat. Commun.*, 2021, 12:3619; *Nat. Commun.*, 2021, 12:7108). However, most transcriptional regulator-based GEFBs prefer sensing exogenous metabolites or metabolites at non-physiological levels. Allosteric regulation of proteins is regarded as the “second secret of life”. The allosteric regulatory domain-based GEFBs designed in this study exhibited soluble expression and good specificity, making them suitable for intracellular applications. Importantly, more than 30,000 chemicals, including many key endogenous metabolites, have been identified as allosteric ligands of allosteric enzymes. The method devised here thus provides new opportunities for developing many previously unavailable metabolite GEFBs.

(2) Numerous studies have reported the development of GEFBs targeting various cofactors, such as ATP (*Nat. Commun.*, 2024, 15:5299; *Proc. Natl. Acad. Sci. USA*, 2024, 121:e2314604121; *Neuron*, 2022, 110:770–782; *Nat. Commun.*, 2019, 10:711; *Nat. Protoc.*, 2017, 12:1542–1562), NADPH/NADP⁺ (*Nat. Commun.*, 2024, 15:10704; *Nat. Commun.*, 2023, 14:3277), and coenzyme A (*Nat. Chem. Biol.*, 2023, 19:346–355). SAM is considered the second most commonly used enzyme cofactor after ATP. However, there were previously no assays available for specifically tracking the fluctuations of SAM in living cells. By utilizing the allosteric regulatory domain as biorecognition element, we successfully developed the first SAM biosensor with high specificity and spatiotemporal resolution. The anabolic and catabolic mechanisms of SAM in bacteria and human cells were further identified. This biosensor will provide deeper insights into the metabolism, physiology, and pathology of SAM. For example, it can support high-throughput screening of SAM-dependent methyltransferases and facilitate the development of efficient inhibitors targeting methyltransferases related to various diseases.

(3) Many GEFBs have been developed for selective imaging of various amino acids in living cells, such as L-arginine (*Cell Metab.*, 2025, 37:291–304), L-glutamate (*Nat. Methods*,

2023, 20:925–934), and L-aspartate (*Cell Chem. Biol.*, 2024, 31:1529–1541). Recently, a method for development of GEFBs for amino acids using the ANL superfamily enzymes as biorecognition elements was published in *Nat. Commun.* (as you mentioned in **Minor Comment 3**; *Nat. Commun.*, 2023, 14:6680). **However, the ANL superfamily enzyme-based GEFBs rely on the adenylation reactions between the analytes and ATP to achieve their responsiveness.** Due to the lack of reversibility and potential metabolic disturbances induced by enzymatic activity, these biosensors are not capable of real-time and *in situ* imaging in living cells. **In contrast, the GEFBs designed in our study utilize the regulatory domains of allosteric enzymes as biorecognition elements, avoiding interference with cellular metabolism and thus being compatible with intracellular assays.** L-Phenylalanine, L-valine, and L-isoleucine are important endogenous metabolites in living organisms. The GEFBs developed in this manuscript enabled previously unavailable reversible and specific detection of these amino acids both *in vitro* and in living cells.

(4) L-Phenylalanine and SAM are key biomarkers for the diagnosis and therapeutic monitoring of various human diseases (*Nat. Rev. Dis. Primers*, 2021, 7:36; *Physiol. Rev.*, 2012, 92:1515–1542). Currently, the clinical detection of these metabolites relies on complicated chromatography- or mass spectrometry-based methods, which are time-consuming, expensive to perform, and require specialized personnel. In this study, we demonstrated the feasibility of the allosteric enzyme-based GEFBs for point-of-care testing (POCT) of L-phenylalanine and SAM in authentic body fluid samples from patients with phenylketonuria and lung cancer, respectively. These biosensors represent simplified and accessible tools that could advance the clinical diagnostics for these diseases. In addition, we demonstrated the performance of the L-valine biosensor and the L-isoleucine biosensor for continuous bioprocess monitoring in authentic bacterial fermentation samples. Such biosensor-based high-throughput screening may accelerate the engineering of the L-valine and L-isoleucine producing strains.

Overall, we believe that our study will be a starting point for the systematic utilization of regulatory domains of allosteric enzymes for developing metabolite GEFBs and will be of great interest to researchers in the field of biosensing technology. To emphasize this methodological advance, we have revised the title of the manuscript to: “**A versatile method**

for designing biosensors via regulatory domains of allosteric enzymes". In addition, the biosensors developed in this study will have far-reaching implications for physiological and pathological studies of target metabolites. We trust the quality of the revised manuscript has significantly improved and hope it meets your satisfaction.

To Reviewer #2:

Kang et al. report the development of genetically encoded fluorescent biosensors for L-phenylalanine, L-valine, L-isoleucine, and S-adenosyl-L-methionine (SAM), named Phesor, Valsor, Ilesor, and SAMsor, respectively. The authors use the regulatory domains of enzymes as sensing domains to engineer these biosensors. They develop and characterize the biosensors, demonstrating their capability to detect changes in target concentrations both in vitro and in living mammalian cells. The authors suggest that these biosensors may significantly contribute to the field by providing a novel platform for biosensor development.

The manuscript is well-written and contributes valuable insights into biosensor technology, making it a strong candidate for publication in Nature Communications. However, I recommend addressing the following major and minor concerns prior to publication:

Response: We sincerely appreciate your positive evaluation of our work. We have thoroughly revised the manuscript based on your constructive suggestions and hope the updated version meets your satisfaction.

Major Issues:

1. Lines 111–112: the authors note that the PheA_{ACT}, used as a sensing domain in Phesor, forms a dimer. This raises questions about whether L-phenylalanine binding to one Phesor monomer might affect the other. Clarifying whether this interaction influences the binding dynamics and reporting the Hill coefficient for L-phenylalanine would be informative. An analysis of the oligomeric state of each biosensor would enhance the manuscript.

Response: Thanks for your constructive comments, which are important for elucidating the working mechanism of the allosteric regulatory domain-based GEFBs. Following your suggestions, we characterized the oligomeric state of Phesor using gel filtration chromatography. As shown in **Fig. 34a**, Phesor exists as a dimer both in the absence and presence of 1 mM L-phenylalanine, consistent with the known dimeric conformation of the PheA_{ACT} domain (*J. Biol Chem.*, 1998, 273:6248–6253). Furthermore, we derived the Hill coefficient from the dose-response curve of Phesor to L-phenylalanine. As summarized in

Table 2, a Hill coefficient close to 1 indicates that L-phenylalanine binds to Phesor in a non-cooperative manner. Each L-phenylalanine molecule binds independently to individual monomers within the dimeric Phesor.

Similarly, we analyzed the oligomeric states of Valsor, Ilesor, and SAMsor. As shown in **Fig. 34b-d**, Valsor and SAMsor exist as dimers, whereas Ilesor forms a mixture of dimers and tetramers, both in the apo and ligand-bound states. These results align closely with the native quaternary structures of their respective biorecognition elements (*J. Mol. Biol.*, 2006, 357:951–963; *Biochem. J.*, 2009, 421:133–143; *Arch. Biochem. Biophys.*, 2013, 540:70–81). Hill coefficient analysis revealed values close to 1 for all three biosensors (**Table 2**), supporting a model of independent and non-cooperative ligand binding within each functional unit.

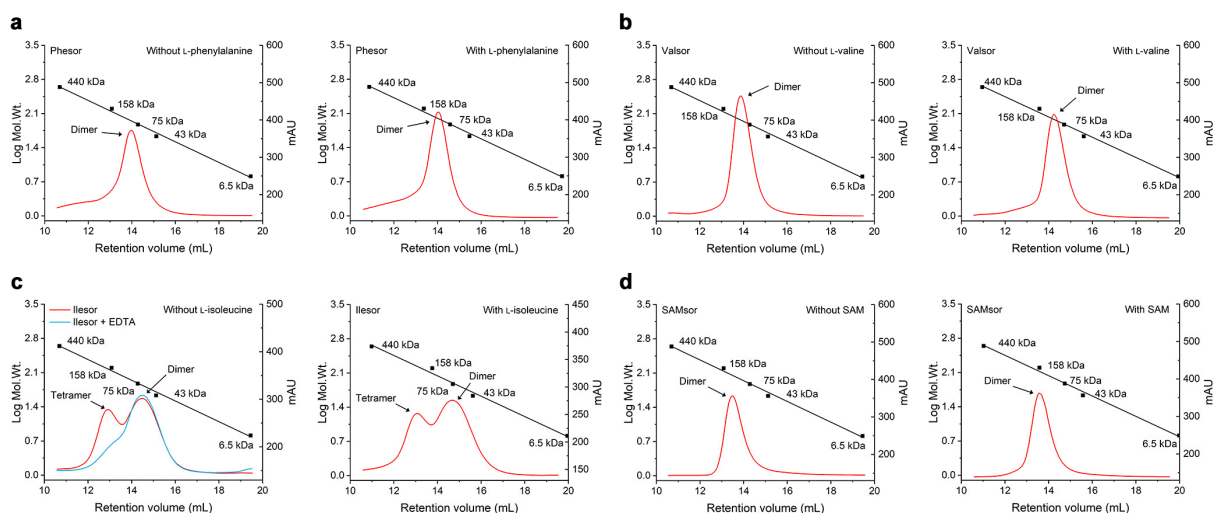


Figure 34 Characterization of Phesor, Valsor, Ilesor, and SAMsor *in vitro*. **(a-d)** Analysis of oligomeric state of Phesor **(a)**, Valsor **(b)**, Ilesor **(c)**, and SAMsor **(d)** in the absence or presence of 1 mM L-phenylalanine, 1 mM L-valine, 1 mM L-isoleucine, and 100 μ M SAM by gel filtration chromatography. 10 mM EDTA was added for chelating Zn^{2+} ions at the side-by-side dimer interface of the Ilesor tetramer. Red curve, chromatogram of purified Phesor; black line, standard curve for protein molecular mass standards. **(Supplementary Fig. 5a, b, Supplementary Fig. 18a, b, Supplementary Fig. 30a, b, and Supplementary Fig. 40a, b in the revised manuscript)**

Table 2 Detection properties of Phesor, Valsor, Ilesor, SAMsor, SAMsor_L, and cpSFYFP. (Supplementary Table 4 in the revised manuscript)

Properties	Phesor	Valsor	Ilesor	SAMsor	SAMsor _L	cpSFYFP
ΔR_{max}	4,030 $\pm$ 40%	875 $\pm$ 52%	1,325.3 $\pm$ 7.1%	3,540 $\pm$ 360%	128.5 $\pm$ 1.1%	/
K_d	1,760 $\pm$ 40 μ M	2,170 $\pm$ 110 μ M	3,620 $\pm$ 400 μ M	204 $\pm$ 21 μ M	0.379 $\pm$ 0.041 μ M	/
Hill slope	1.13 $\pm$ 0.03	1.03 $\pm$ 0.04	0.98 $\pm$ 0.05	1.15 $\pm$ 0.04	0.91 $\pm$ 0.07	/
LOD (<i>in vitro</i>) ^a	48.52 μ M	48.39 μ M	26.41 μ M	0.31 μ M	0.057 μ M	/
LOD (in human cells) ^a	67.39 μ M	75.17 μ M	47.93 μ M	0.45 μ M	/	/
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)	9.03	8.68	9.08	8.87	/	8.54
pK _a (with ligand) ^b	8.23	8.50	8.72	7.72	/	8.49 (L-phenylalanine) 8.49 (L-valine) 8.69 (L-isoleucine) 8.60 (SAM)

^aThe limit of detection (LOD) is calculated by interpolating the average background counts + (or -) 3 $\times$ standard deviation value (*Nat. Commun.*, 2021, 12:3619).

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

Related data were presented in **Supplementary Fig. 5a, b, Supplementary Fig. 18a, b, Supplementary Fig. 30a, b, Supplementary Fig. 40a, b, and Supplementary Table 4** in the revised manuscript. Related descriptions were added in the “Results” section of the revised manuscript as follows:

For Phesor:

“Purified Phesor existed as a dimer both in the absence and presence of L-phenylalanine (**Supplementary Fig. 5a, b**). Dose-response curve analysis indicated that the detection range of Phesor encompasses the physiological concentrations of L-phenylalanine both in bacteria and in mammalian cells (**Fig. 2c**). Furthermore, it achieved a high ΔR_{max} ($4,030 \pm 40\%$), an appropriate K_d ($1,760 \pm 40 \mu\text{M}$; close to the value of $2.69 \pm 0.13 \text{ mM}$ determined by thermal shift assay [TSA]), and a Hill slope close to 1 for L-phenylalanine (**Fig. 2c, Supplementary Fig. 5e, and Supplementary Table 4**).”

For Valsor:

“Valsor existed as a dimer and had two excitation peaks around 426 and 504 nm, with a single emission peak around 509 nm (405 nm excitation) or 518 nm (488 nm excitation) (**Supplementary Fig. 18a-d**). Dose-response curve analysis indicated that Valsor achieved a wide detection range, with an increased ΔR_{max} ($875 \pm 52\%$), an appropriate K_d ($2,170 \pm 110 \mu\text{M}$; close to the value of $4.46 \pm 0.50 \text{ mM}$ determined by TSA), and a Hill slope close to 1 for L-valine (**Fig. 3d, Supplementary Fig. 18e, and Supplementary Table 4**).”

For Ilesor:

“Consistent with the quaternary structure of CimA_{LA}²⁵, Ilesor exists as a mixture of dimers and tetramers both in the absence and presence of L-isoleucine (**Supplementary Fig. 30a, b**). Dose-response curve analysis indicated that Ilesor achieved a suitable detection range, an increased ΔR_{max} ($1,325.3 \pm 7.1\%$), an appropriate K_d ($3,620 \pm 400 \mu\text{M}$; close to the value of $2.22 \pm 0.30 \text{ mM}$ determined by TSA), and a Hill slope close to 1 for L-isoleucine (**Fig. 4d, Supplementary Fig. 30e, and Supplementary Table 4**).”

For SAMsor:

“SAMsor existed as a dimer and had two excitation peaks around 420 and 504 nm, with a single emission peak around 514 nm (405 nm excitation) or 518 nm (488 nm excitation) (**Supplementary Fig. 40a-d**). Dose-response curve analysis indicated that SAMsor achieved

an increased ΔR_{max} ($3,540 \pm 360\%$), an appropriate K_d ($204 \pm 21 \mu\text{M}$; close to the value of $0.295 \pm 0.061 \text{ mM}$ determined by TSA), a Hill slope close to 1, and a wide detection range suitable for detecting physiological concentrations of SAM in mammalian cells and bacteria (Fig. 6c, Supplementary Fig. 40e, and Supplementary Table 2).”

In addition, a short discussion regarding the interaction modes between ligands and their biorecognition elements was added in the “Discussion” section of the revised manuscript as follows:

“... These biosensors assembled into proper quaternary structures consistent with those of the native Phe_{ACT} domain, IlvH, Cim_{ALA} domain, and CBS domain^{13,15,21,25,29}. Each ligand molecule bound independently to individual monomers within the multimeric biosensors (hill slope close to 1). These results demonstrate that the developed biosensors preserved the intrinsic ligand-binding characteristics of their native allosteric regulatory domains. ...”

2. Lines 137–138: Discrepancies in the reported excitation (405/488 nm) and emission (528 nm) peaks of Phesor compared to Supplementary Fig. 3a need correction. Furthermore, similar spectral data for Valsor, Ilesor, and SAMsor should also be included as it is fundamental for characterizing these biosensors.

Response: Thanks for your good suggestion. We have corrected the excitation and emission peak values for Phesor in the “Results” section and have supplemented the complete spectral characterization for Valsor, Ilesor, and SAMsor. As shown in Fig. 35a, b, Phesor exhibits two excitation peaks around 416 and 490 nm, with a single emission peak around 508 nm (405 nm excitation) or 516 nm (488 nm excitation). As shown in Fig. 35c, d, Valsor exhibits two excitation peaks around 426 and 504 nm, with a single emission peak around 509 nm (405 nm excitation) or 518 nm (488 nm excitation). As shown in Fig. 35e, f, Ilesor exhibits two excitation peaks around 420 and 504 nm, with a single emission peak around 507 nm (405 nm excitation) or 517 nm (488 nm excitation). As shown in Fig. 35g, h, SAMsor exhibits two excitation peaks around 420 and 504 nm, with a single emission peak around 514 nm (405 nm excitation) or 518 nm (488 nm excitation).

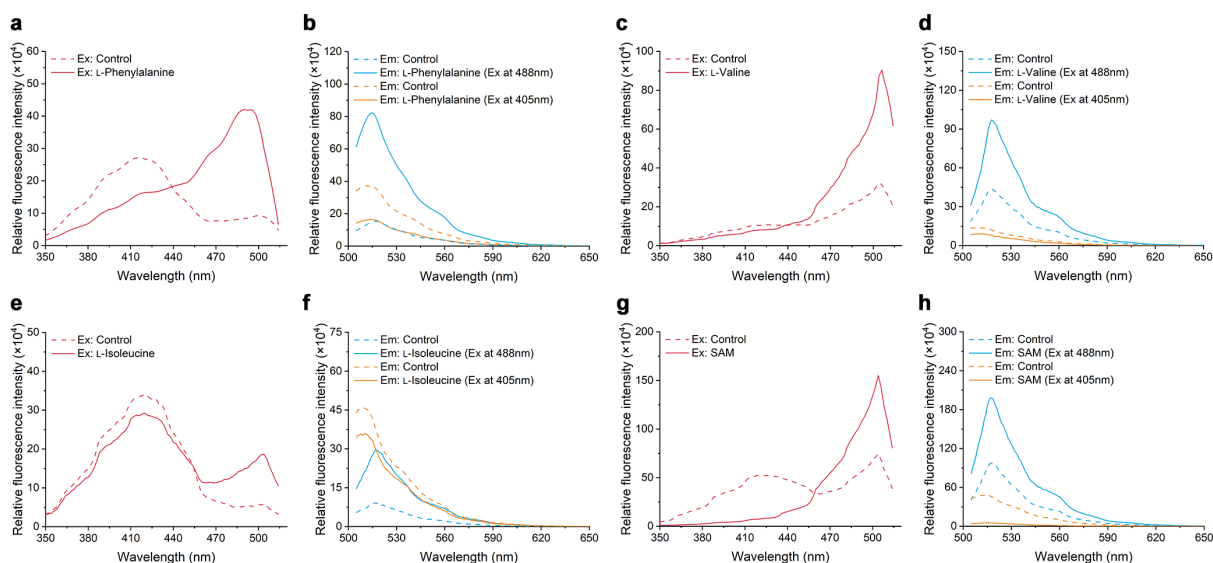


Figure 35 Characterization of Phesor, Valsor, Ilesor, and SAMsor *in vitro*. **(a, b)** Fluorescence excitation **(a)** and emission **(b)** spectra of Phesor in the absence or presence of 1 mM L-phenylalanine. **(c, d)** Fluorescence excitation **(c)** and emission **(d)** spectra of Valsor in the absence or presence of 1 mM L-valine. **(e, f)** Fluorescence excitation **(e)** and emission **(f)** spectra of Ilesor in the absence or presence of 1 mM L-isoleucine. **(g, h)** Fluorescence excitation **(g)** and emission **(h)** spectra of SAMsor in the absence or presence of 100 μ M SAM. (Supplementary Fig. 5c, d, Supplementary Fig. 18c, d, Supplementary Fig. 30c, d, and Supplementary Fig. 40c, d in the revised manuscript)

Related data were presented in [Supplementary Fig. 5c, d](#), [Supplementary Fig. 18c, d](#), [Supplementary Fig. 30c, d](#), and [Supplementary Fig. 40c, d](#) in the revised manuscript. Related descriptions were added in the “Results” section of the revised manuscript as follows:

For Phesor:

“Purified Phesor existed as a dimer both in the absence and presence of L-phenylalanine (Supplementary Fig. 5a, b). It had two excitation peaks around 416 and 490 nm, with a single emission peak around 508 nm (405 nm excitation) or 516 nm (488 nm excitation) (Supplementary Fig. 5c, d).”

For Valsor:

“Valsor existed as a dimer and had two excitation peaks around 426 and 504 nm, with a single emission peak around 509 nm (405 nm excitation) or 518 nm (488 nm excitation) (Supplementary Fig. 18a-d).”

For Ilesor:

“Ilesor exists as a mixture of dimers and tetramers both in the absence and presence of L-isoleucine (**Supplementary Fig. 30a, b**). It had two excitation peaks around 420 and 504 nm, with a single emission peak around 507 nm (405 nm excitation) or 517 nm (488 nm excitation) (**Supplementary Fig. 30c, d**).”

For SAMsor:

“SAMsor existed as a dimer and had two excitation peaks around 420 and 504 nm, with a single emission peak around 514 nm (405 nm excitation) or 518 nm (488 nm excitation) (**Supplementary Fig. 40a-d**).”

3. The manuscript presents fluorescence response and kinetics data upon target concentration increase but lacks corresponding data for decreases. Wash-out method used in this study lacks temporal information for turn-off response. Demonstrating reversible behavior through additional kinetic studies for the turn-off response would provide a more comprehensive understanding of the biosensors' functionality.

Response: Thanks for your good suggestion. In accordance with your advice, we conducted kinetic studies to characterize the turn-off responses of Phesor, Valsor, Ilesor, and SAMsor through enzymatic depletion of their target ligands. Specifically, the following enzyme systems were employed: aromatic aldehyde synthase from *Petroselinum crispum* (AAS) for L-phenylalanine depletion; L-leucine dehydrogenase from *Bacillus subtilis* 168 (LeuDH) for L-valine and L-isoleucine depletion; and catechol-*O*-methyltransferase from *Solanum lycopersicum* (COMT) for SAM depletion. Each reaction system was supplemented with the appropriate cofactors and co-substrates. As shown in **Fig. 36**, the elevated fluorescence ratios of the four biosensors induced by ligand binding rapidly returned to basal levels following the initiation of the enzymatic reactions. These results demonstrate the rapid off-kinetics and good reversibility of the four biosensors.

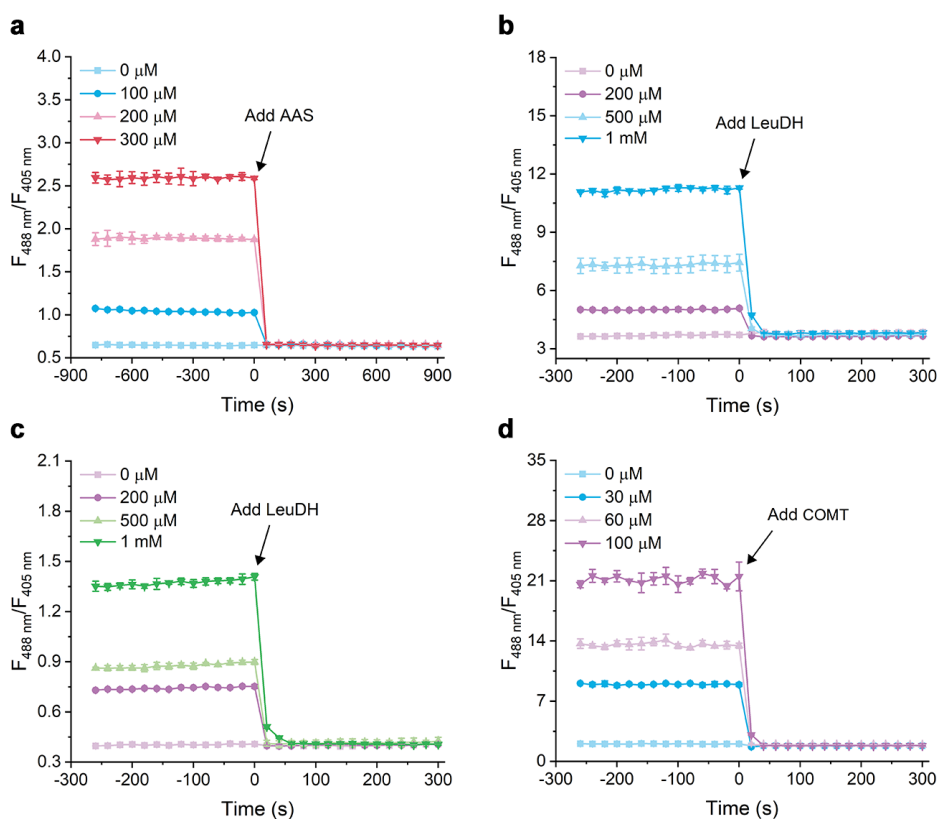


Figure 36 Kinetics for turn-off response of Phesor, Valsor, Ilesor, and SAMsor. **(a-d)** Kinetic analysis of the fluorescence response of Phesor **(a)**, Valsor **(b)**, Ilesor **(c)**, and SAMsor **(d)** to L-phenylalanine, L-valine, L-isoleucine, and SAM depletion. For Phesor, indicated concentrations of L-phenylalanine were added prior to testing, and 25 mg mL⁻¹ AAS together with 50 μM pyridoxal 5'-phosphate (PLP) were added at time point zero. For Valsor, indicated concentrations of L-valine were added prior to testing, and 30 mg mL⁻¹ LeuDH together with 3 mM NAD⁺ were added at time point zero. For Ilesor, 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. For SAMsor, indicated concentrations of SAM were added prior to testing, and 25 mg mL⁻¹ COMT together with 100 mM Mg²⁺, 2 mM dithiothreitol, and 500 μM catechol were added at time point zero. All data shown are means ± s.d. (n = 3 independent experiments). (Supplementary Fig. 5i, Supplementary Fig. 18i, Supplementary Fig. 30i, and Supplementary Fig. 40i in the revised manuscript)

Related data were presented in [Supplementary Fig. 5i](#), [Supplementary Fig. 18i](#), [Supplementary Fig. 30i](#), and [Supplementary Fig. 40i](#) in the revised manuscript. Related descriptions were elaborated in the “Results” section of the revised manuscript as follows:

For Phesor:

“In addition, Phesor showed high specificity for L-phenylalanine It also responded rapidly to variations in L-phenylalanine concentration within the assay system

(**Supplementary Fig. 5h, i**). The increased fluorescence ratios induced by L-phenylalanine addition could return to basal levels upon the removal of L-phenylalanine from the system (**Fig. 2d and Supplementary Fig. 5i**).”

For Valsor:

“In addition, Valsor demonstrated a robust response independent of its own concentration, high specificity, good reversibility, and correctable pH-stability from pH 5.0 to 10.0 for L-valine detection (**Supplementary Fig. 17d-f, Supplementary Fig. 18f-j, and Supplementary Fig. 19a**).”

For Ilesor:

“In addition, Ilesor demonstrated a robust response independent of its own concentration, high specificity, good reversibility, and correctable pH-stability from pH 5.0 to 8.2 for L-isoleucine detection (**Supplementary Fig. 19a, Supplementary Fig. 29d-f, and Supplementary Fig. 30f-j**).”

For SAMsor:

“In addition, SAMsor demonstrated good reversibility and correctable pH-stability from pH 5.0 to 10.0 for SAM detection (**Supplementary Fig. 40k-m and Supplementary Fig. 41a**).”

Related experimental methods were added in the “Supplementary Methods” section of the revised manuscript as follows:

“Supplementary Methods

Characterization of Phesor, Valsor, Ilesor, and SAMsor *in vitro*

Kinetic analysis for ligand dissociation through enzymatic reactions. Purified Phesor, Valsor, Ilesor, and SAMsor were initially incubated with different concentrations of target ligands. Subsequently, specific enzymes along with corresponding cofactors and co-substrates were introduced to catalyze ligand decomposition. For Phesor, 25 mg mL⁻¹ aromatic aldehyde synthase from *Petroselinum crispum* (AAS) and 50 μM pyridoxal 5'-phosphate (PLP) was used. For Valsor and Ilesor, 30 mg mL⁻¹ L-leucine dehydrogenase from *Bacillus subtilis* 168 (LeuDH) and 3 mM NAD⁺ was used. For SAMsor, the system included 25 mg mL⁻¹ catechol-*O*-methyltransferase from *Solanum lycopersicum* (COMT), 100 mM Mg²⁺, 2 mM dithiothreitol, and 500 μM catechol. The kinetic responses of these biosensors

upon ligand dissociation were assessed by continuously monitoring the changes in fluorescence ratios before and after enzyme addition using an EnSight microplate reader.”

4. Figure 6e indicates that SAMsor does not fully recover its initial fluorescence intensity in the absence of SAM, suggesting it is not fully reversible. The implications of this for practical applications should be discussed.

Response: Thanks for your good suggestion. Off-kinetic analysis using an enzymatic depletion system confirmed that SAMsor exhibits good reversibility for SAM (**Fig. 36d**). Dose-response curve analysis indicated that SAMsor has a significantly lower limit of detection (LOD) (0.31 μM) compared to Phesor (48.52 μM), Valsor (48.39 μM), and Ilesor (26.41 μM) (**Table 2**). We speculated that the incomplete reversibility of SAMsor observed in Fig. 6e of the previous manuscript might be attributed to the residual detectable SAM in the assay system when using the same elution procedure as for the other three biosensors. Therefore, we performed two additional rounds of elution procedures for SAMsor. As shown in **Fig. 37**, the SAM-induced elevated fluorescence ratio of SAMsor returned completely to basal levels after SAM elution, indicating the full reversibility of SAMsor.

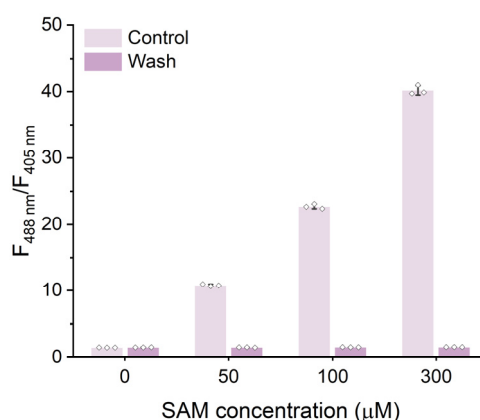


Figure 37 Reversibility analysis of SAMsor. The fluorescence ratios of SAMsor after SAM addition and subsequent removal were recorded. All data shown are means $\pm$ s.d. (n = 3 independent experiments).

(Supplementary Fig. 40k in the revised manuscript)

Related data were presented in **Supplementary Fig. 40k** in the revised manuscript. Related descriptions were elaborated in the “Results” section of the revised manuscript as follows:

“In addition, SAMsor demonstrated good reversibility and correctable pH-stability from

pH 5.0 to 10.0 for SAM detection (Supplementary Fig. 40k-m and Supplementary Fig. 41a).”

Related experimental methods were added in the “Supplementary Methods” section of the revised manuscript as follows:

“Supplementary Methods

Characterization of Phesor, Valsor, Ilesor, and SAMsor *in vitro*

Reversibility analysis. Purified Phesor, Valsor, and Ilesor were first mixed with different concentrations of target ligands, after which the ligands were eluted by centrifugation at $3,800 \times g$ for 30 min at 4 °C using a 10-kDa ultrafiltration centrifuge tube. The volume of the assay system was then replenished to the initial level using ligand-free 50 mM Tris-HCl (pH 7.4). For SAMsor, the same elution process was performed three times to completely remove SAM from the assay system. The reversibility of the biosensors was analyzed by detecting the changes in fluorescence ratio before and after ligand elution.”

5. The claimed stability of biosensor fluorescence across pH changes is based on a limited range (6.6–8.2). Expanding this range (e.g., 5–10) and reporting the pK_a values of each biosensor (and cpSFYFP control) would substantiate the authors’ claims more robustly.

Response: Thanks for your good suggestion. According to your advice, we have re-tested the pH stability of Phesor, Valsor, Ilesor, and SAMsor across an extended pH range of 5.0 to 10.0. As shown in **Fig. 38**, the fluorescence ratios of the four biosensors, when corrected by cpSFYFP, remained stable within the pH ranges of 5.0–8.2, 5.0–10.0, 5.0–8.2, and 5.0–10.0, respectively. In addition, we have determined the pK_a values for each biosensor and the cpSFYFP control in the absence or presence of target ligands. As summarized in **Table 2**, the pK_a values of Phesor and cpSFYFP were determined as 9.03 and 8.54 (without L-phenylalanine) or 8.23 and 8.49 (with 1 mM L-phenylalanine), respectively. The pK_a values of Valsor and cpSFYFP were determined as 8.68 and 8.54 (without L-valine) or 8.50 and 8.49 (with 1 mM L-valine), respectively. The pK_a values of Ilesor and cpSFYFP were determined as 9.08 and 8.54 (without L-isoleucine) or 8.72 and 8.69 (with 1 mM L-isoleucine), respectively. The pK_a values of SAMsor and cpSFYFP were determined as 8.87 and 8.54 (without SAM) or 7.72 and 8.60 (with 200 μ M SAM), respectively.

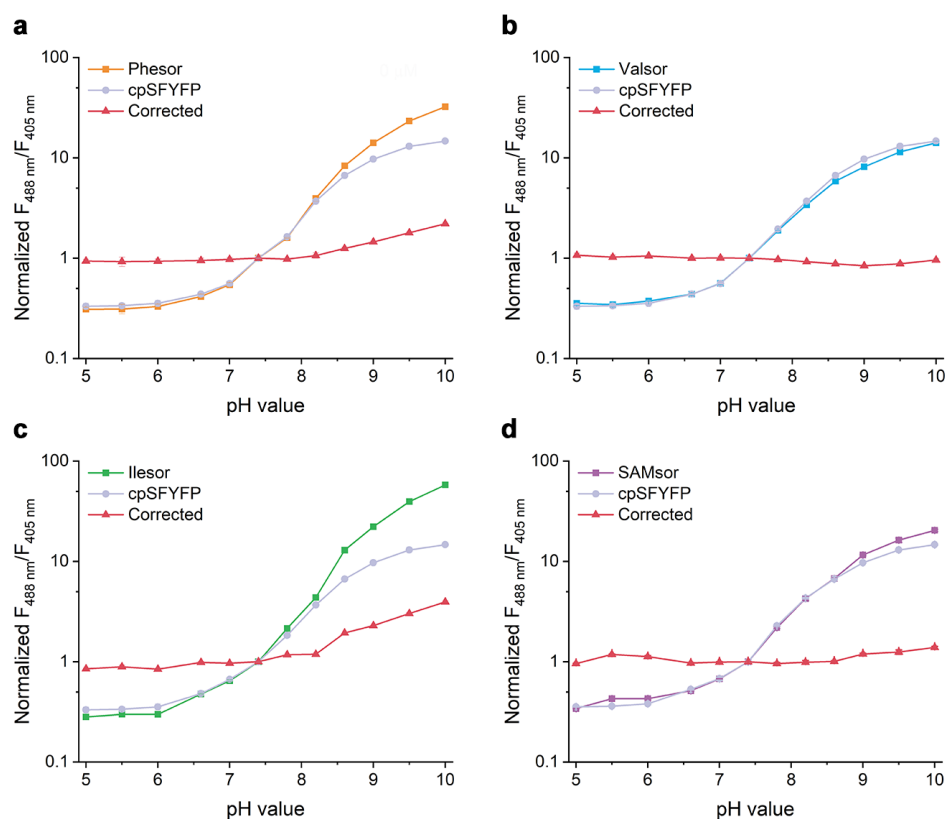


Figure 38 pH-stability analysis of Phesor, Valsor, Ilesor, and SAMsor. **(a-d)** pH-correction of the fluorescence ratio of Phesor **(a)**, Valsor **(b)**, Ilesor **(c)**, and SAMsor **(d)** by cpSFYFP. Data were normalized to the ratio at pH 7.4. All data shown are means $\pm$ s.d. ($n = 3$ independent experiments). (**Supplementary Fig. 5k**, **Supplementary Fig. 18j**, **Supplementary Fig. 30j**, and **Supplementary Fig. 40m** in the revised manuscript)

Related data were presented in **Supplementary Fig. 5k**, **Supplementary Fig. 18j**, **Supplementary Fig. 30j**, **Supplementary Fig. 40m**, and **Supplementary Table 4** in the revised manuscript. Related descriptions were added in the “Results” section of the revised manuscript as follows:

For Phesor:

“Consistent with other cpFP-based biosensors^{6,7}, the ratiometric response of Phesor was pH-dependent, with the pK_a varying from 9.03 to 8.23 upon L-phenylalanine binding (**Supplementary Fig. 5j** and **Supplementary Table 4**). Thus, an L-phenylalanine-unresponsive control biosensor with similar pH-sensitive properties, cpSFYFP, was introduced for parallel experiments (**Supplementary Fig. 6a** and **Supplementary Table 4**)⁷. As shown in **Supplementary Fig. 5k**, the fluorescence ratio of Phesor corrected by cpSFYFP remained stable between pH 5.0 and 8.2.”

For Valsor:

“The pK_a value of Valsor was determined as 8.68, and decreased to 8.50 upon L-valine binding (**Supplementary Table 4**). In addition, Valsor demonstrated a robust response independent of its own concentration, high specificity, good reversibility, and correctable pH-stability from pH 5.0 to 10.0 for L-valine detection (**Supplementary Fig. 17d-f, Supplementary Fig. 18f-j, and Supplementary Fig. 19a**).”

For Ilesor:

“The pK_a value of Ilesor was determined as 9.08, and decreased to 8.72 upon L-isoleucine binding (**Supplementary Table 4**). In addition, Ilesor demonstrated a robust response independent of its own concentration, high specificity, good reversibility, and correctable pH-stability from pH 5.0 to 8.2 for L-isoleucine detection (**Supplementary Fig. 19a, Supplementary Fig. 29d-f, and Supplementary Fig. 30f-j**).”

For SAMsor:

“The pK_a value of SAMsor was determined as 8.87, and decreased to 7.72 upon SAM binding (**Supplementary Table 4**). In addition, SAMsor demonstrated good reversibility and correctable pH-stability from pH 5.0 to 10.0 for SAM detection (**Supplementary Fig. 40k-m and Supplementary Fig. 41a**).”

6. The authors apply each biosensor in live HEK293FT cells: Phesor (Fig. 2m), Valsor (Fig. 3m), Ilesor (Fig. 4m), and SAMsor (Fig. 6m). Pharmacological stimulations can perturb intracellular pH. To more rigorously demonstrate that these biosensors specifically enable monitoring the change in target concentrations in living mammalian cells, the authors should show the same data set using cpSFYFP control as well.

Response: Thanks for your good suggestion. As you correctly noted, various pharmacological stimuli could perturb intracellular pH in HEK293FT cells, thereby influencing the fluorescence ratios of both the biosensors and the cpSFYFP control. In Fig. 2m, Fig. 3m, Fig. 4m, and Fig. 6m of the previous manuscript, we presented fluorescence ratios corrected by the cpSFYFP control. To ensure rigorous data presentation, we have now supplemented the uncorrected, raw fluorescence ratio data for all four biosensors, alongside the corresponding cpSFYFP control data, in the revised manuscript.

As shown in **Fig. 39**, **Fig. 41**, **Fig. 43**, and **Fig. 45**, treatments such as LAT1 overexpression and the administration of octyl-D-2-hydroxyglutarate, octyl-L-2-hydroxyglutarate, or cycloleucine may induce intracellular pH fluctuations, which affected the fluorescence ratios of the four biosensors and the cpSFYFP control. The cpSFYFP-corrected fluorescence ratios for Phesor, Valsor, Ilesor, and SAMsor were shown in **Fig. 40**, **Fig. 42**, **Fig. 44**, and **Fig. 46**, respectively.

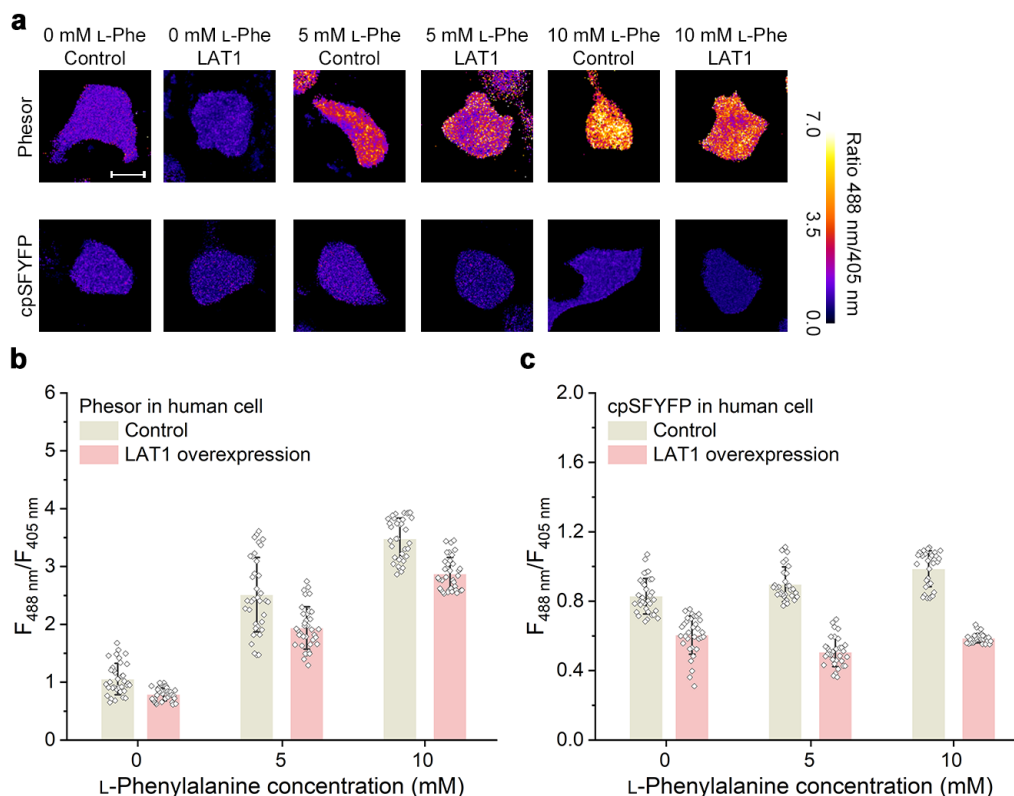


Figure 39 Analysis of the function of LAT1 in the uptake of L-phenylalanine in HEK293FT cells using Phesor. **(a)** Ratiometric images of single HEK293FT cell expressing Phesor or cpSFYFP in response to L-phenylalanine addition and LAT1 overexpression. Cells were treated with 0, 5, or 10 mM L-phenylalanine with or without LAT1 overexpression, and the fluorescence ratios were determined after 20 h. Scale bar, 10 μm . **(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. 40**. All data shown are means $\pm$ s.d. (n = 36 cells for **b**; n = 33 cells for **c**). (Supplementary Fig. 10 in the revised manuscript)

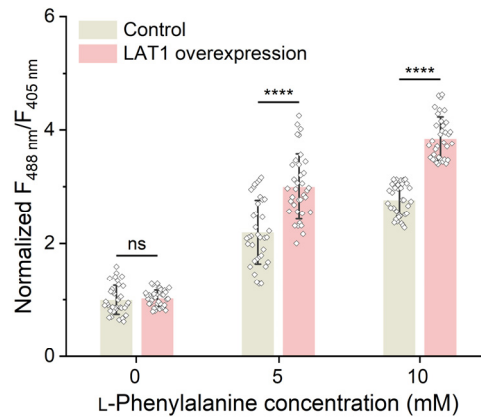


Figure 40 Analysis of the function of LAT1 in the uptake of L-phenylalanine in HEK293FT cells using Phesor. Data were corrected by cpSFYFP. All data shown are means $\pm$ s.d. ($n = 36$ cells). ****, $P < 0.0001$; ns, no significant difference ($P \geq 0.05$); one-way ANOVA test with Tukey's Multiple Comparison Test. (Fig. 2i in the revised manuscript)

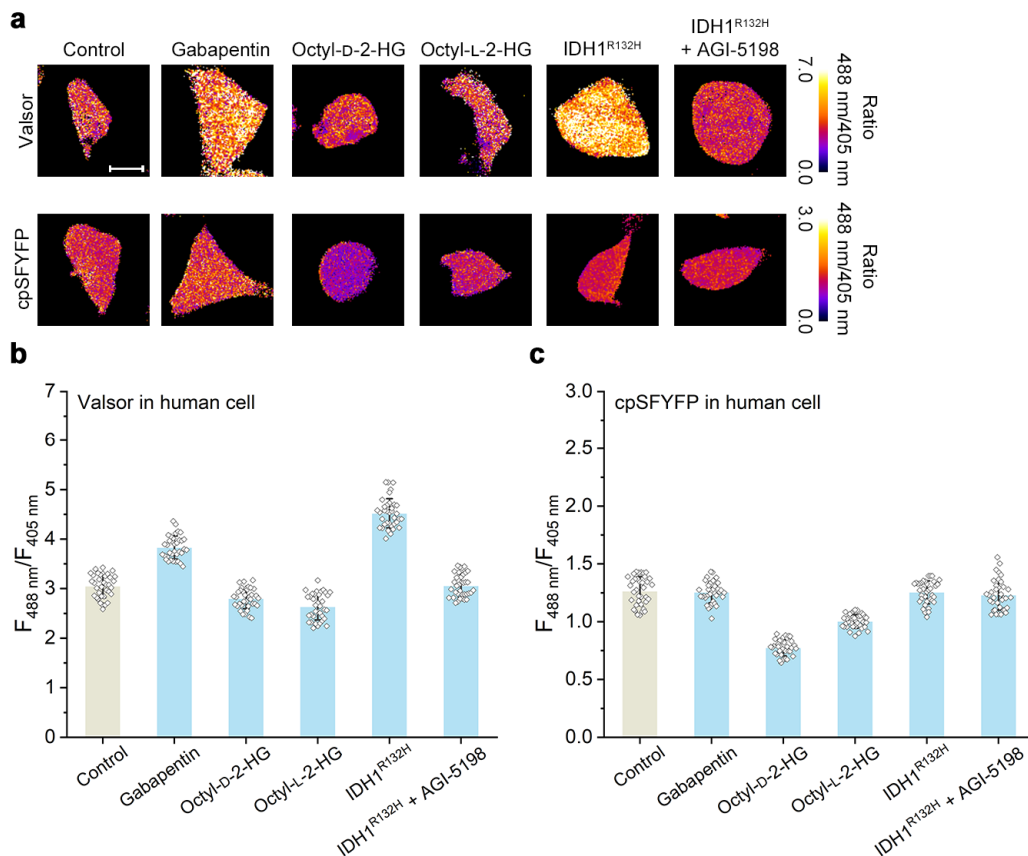


Figure 41 Analysis of the effects of different BCAT inhibitors on intracellular L-valine levels in HEK293FT cells using Valsor. (a) Ratiometric images of single HEK293FT cell expressing Valsor or cpSFYFP in response to different inhibitors treatment. The concentrations of gabapentin, octyl-D-2-HG (octyl-D-2-hydroxyglutarate), 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. (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. 42**. All data shown are means $\pm$ s.d. ($n = 36$ cells). (**Supplementary Fig. 23 in the revised manuscript**)

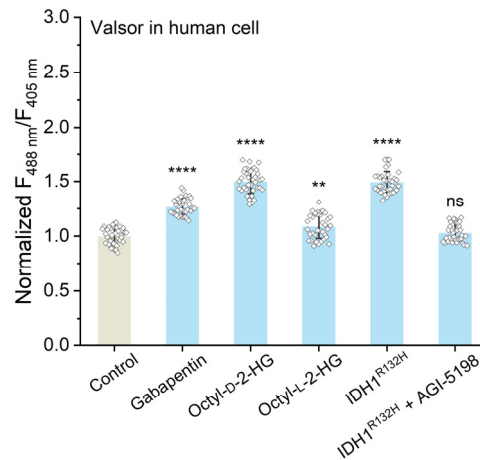


Figure 42 Analysis of the effects of different BCAT inhibitors on intracellular L-valine levels in HEK293FT cells using Valsor. The concentrations of gabapentin, octyl-D-2-HG (octyl-D-2-hydroxyglutarate), octyl-L-2-HG (octyl-L-2-hydroxyglutarate), and AGI-5198 used were 2 mM, 1 mM, 1 mM, and 3 μ M, respectively. Data were corrected by cpSFYFP. All data shown are means $\pm$ s.d. ($n = 36$ cells). **, $P < 0.01$; ****, $P < 0.0001$; ns, no significant difference ($P \geq 0.05$); one-way ANOVA test with Tukey's Multiple Comparison Test. (**Fig. 3i in the revised manuscript**)

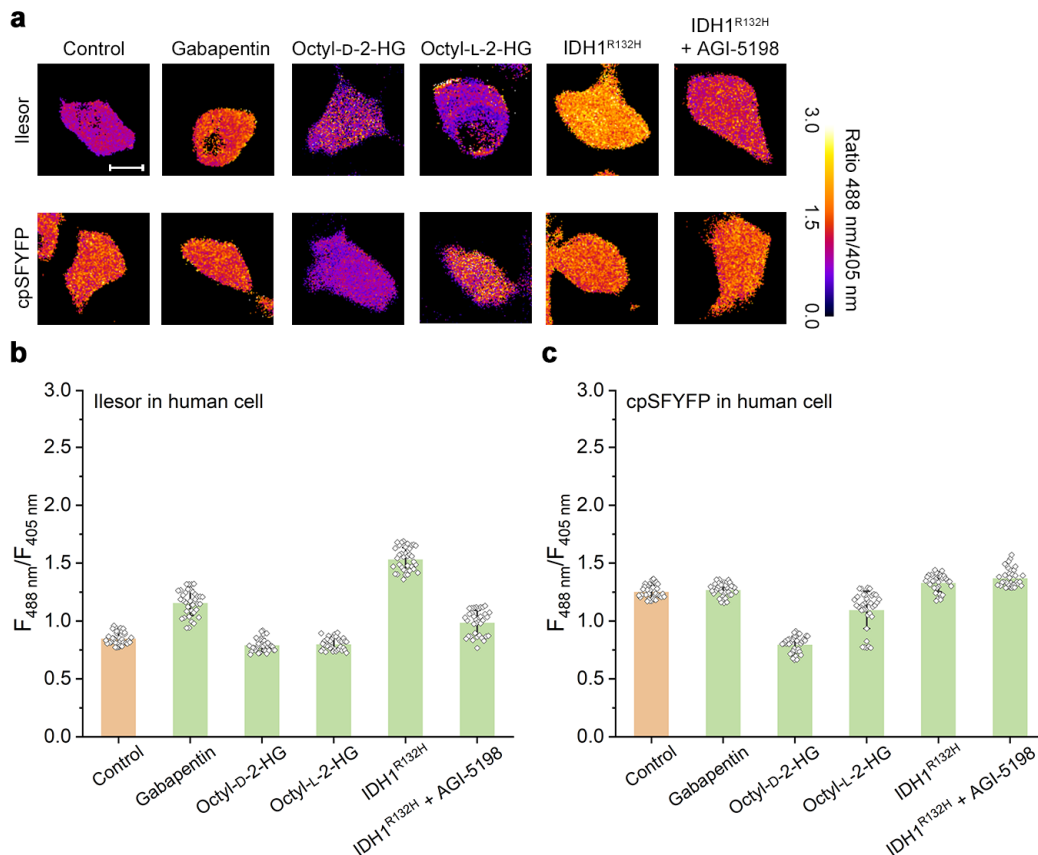


Figure 43 Analysis of the effects of different BCAT inhibitors on intracellular L-isoleucine levels in

HEK293FT cells using Ilesor. **(a)** Ratiometric images of single HEK293FT cell 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. **(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. 44**. All data shown are means $\pm$ s.d. (n = 36 cells).

(Supplementary Fig. 33 in the revised manuscript)

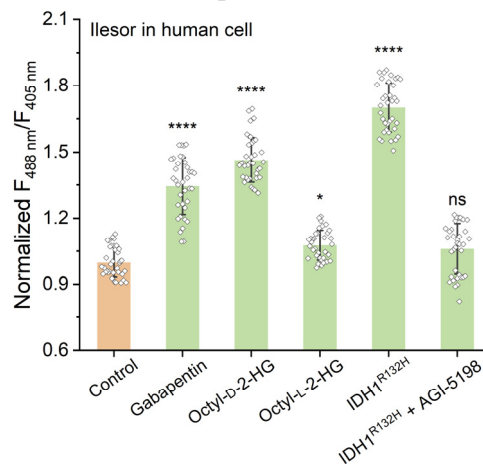


Figure 44 Analysis of the effects of different BCAT inhibitors on intracellular L-isoleucine levels in HEK293FT cells using Ilesor. Data were corrected by cpSFYFP. All data shown are means $\pm$ s.d. (n = 36 cells). **, $P < 0.01$; ****, $P < 0.0001$; ns, no significant difference ($P \geq 0.05$); one-way ANOVA test with Tukey's Multiple Comparison Test. **(Fig. 4i in the revised manuscript)**

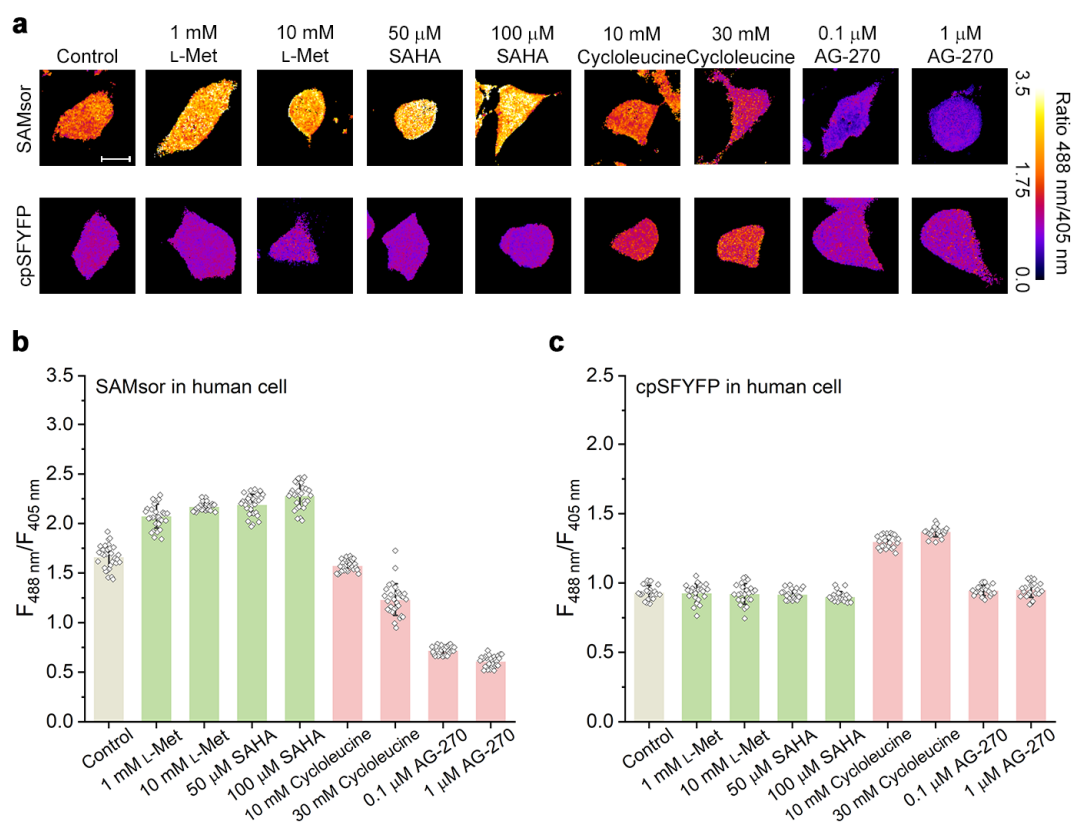


Figure 45 Analysis of the effects of different MATase activator or inhibitors on intracellular SAM levels in HEK293FT using SAMsor. **(a)** Ratiometric images of single HEK293FT cell expressing SAMsor or cpSFYFP in response to different MATase activator or inhibitors treatment. The fluorescence ratios were determined 20 h after treatment with different activator or inhibitors. Scale bar, 10 μ m. **(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. 46**. All data shown are means $\pm$ s.d. (n = 30 cells for **b**; n = 23 cells for **c**). **(Supplementary Fig. 45 in the revised manuscript)**

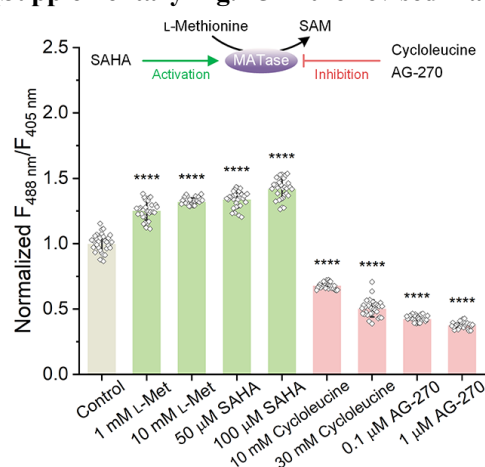


Figure 46 Analysis of the effects of different MATase activator or inhibitors on intracellular SAM levels in HEK293FT using SAMsor. Data were corrected by cpSFYFP. All data shown are means $\pm$ s.d. (n = 36

cells). ****, $P < 0.0001$; one-way ANOVA test with Tukey's Multiple Comparison Test. (Fig. 6j in the revised manuscript)

Furthermore, we have provided the cpSFYFP control data for the applications of Phesor, Valsor, Ilesor, and SAMsor in living bacteria. As shown in Fig. 47, Fig. 48, Fig. 49, and Fig. 50, none of the tested conditions interfered with the fluorescence ratios of cpSFYFP expressed in *E. coli* BL21 (DE3) and its derivatives.

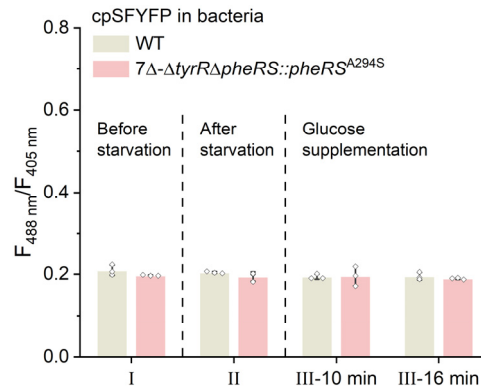


Figure 47 Fluorescence ratios of *E. coli* BL21(DE3) WT and *E. coli* BL21(DE3) $7\Delta\text{-}\Delta\text{tyrR}\Delta\text{pheRS}::\text{pheRS}^{\text{A294S}}$ expressing cpSFYFP before carbon starvation (I), 8 h after carbon starvation (II), and 10 min and 20 min after glucose supplementation (III). All data shown are means $\pm$ s.d. ($n = 3$ independent experiments). (Supplementary Fig. 6c in the revised manuscript)

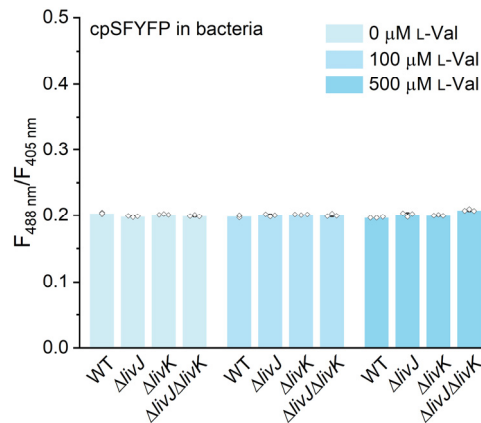


Figure 48 Fluorescence ratios of cpSFYFP expressed in *E. coli* BL21(DE3) WT, *E. coli* BL21(DE3) ΔlivJ , *E. coli* BL21(DE3) ΔlivK , and *E. coli* BL21(DE3) $\Delta\text{livJ}\Delta\text{livK}$ in response to exogenous gradient concentrations of L-valine. All data shown are means $\pm$ s.d. ($n = 3$ independent experiments). (Supplementary Fig. 19c in the revised manuscript)

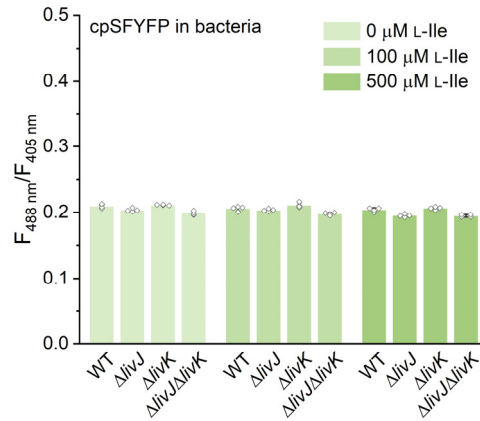


Figure 49 Fluorescence ratios of cpSFYFP expressed in *E. coli* BL21(DE3) WT, *E. coli* BL21(DE3) $\Delta livJ$, *E. coli* BL21(DE3) $\Delta livK$, and *E. coli* BL21(DE3) $\Delta livJ\Delta livK$ in response to exogenous gradient concentrations of L-isoleucine. All data shown are means $\pm$ s.d. (n = 4 independent experiments).

(Supplementary Fig. 19d in the revised manuscript)

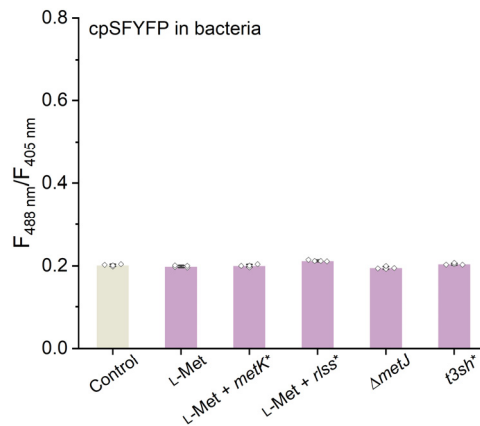


Figure 50 Fluorescence ratios of *E. coli* BL21(DE3) expressing cpSFYFP cultured under the indicated conditions. All data shown are means $\pm$ s.d. (n = 4 independent experiments). (Supplementary Fig. 41c in the revised manuscript)

Related data were presented in [Fig. 2i](#), [Fig. 3i](#), [Fig. 4i](#), [Fig. 6j](#), [Supplementary Fig. 6c](#), [Supplementary Fig. 10](#), [Supplementary Fig. 19c, d](#), [Supplementary Fig. 23](#), [Supplementary Fig. 33](#), [Supplementary Fig. 41c](#), and [Supplementary Fig. 45](#) in the revised manuscript. Related descriptions were elaborated in the “Results” section of the revised manuscript as follows:

For Phesor:

“TyrR recognizes L-phenylalanine, L-tyrosine, and L-tryptophan, and inhibits the expression of 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase (AroF), the initial enzyme in the biosynthesis of three aromatic amino acids (AAAs)¹⁴. We deleted *tyrR* in *E.*

coli BL21(DE3) $\Delta lacZ \Delta frdA \Delta ldhA \Delta poxB \Delta pflB \Delta pta \Delta ackA$ and introduced the A294S mutation into phenylalanyl-tRNA synthetase (**Supplementary Fig. 7**). The A294S mutation of phenylalanyl-tRNA synthetase exhibited an increased K_m for L-phenylalanine¹⁷, while the deletion of *tyrR* would result in increased L-phenylalanine synthesis. As expected, higher levels of intracellular L-phenylalanine in the mutant strain were detected using Phesor. Interestingly, the accumulated L-phenylalanine was catabolized during carbon starvation and was re-accumulated after 10 min of glucose supplementation (**Fig. 2f and Supplementary Fig. 6c**). ...

... The L-type amino acid transporter 1 (LAT1) was reported to function in the uptake of L-phenylalanine in human cells¹⁸. As expected, the overexpression of LAT1 in HEK293FT cells enhanced the fluorescence ratio of Phesor under conditions of L-phenylalanine addition (**Fig. 2i and Supplementary Fig. 10**).”

For Valsor:

“LivJ and LivK are two periplasmic proteins involved in the BCAA transport of *E. coli*²². The knock-out of LivJ and LivK individually or in combination diminished the response of Valsor expressed in *E. coli* BL21(DE3) to exogenous L-valine addition, suggesting both of them function in the uptake of L-valine in *E. coli* BL21(DE3) (**Fig. 3f and Supplementary Fig. 19c**). ...

... BCAA transaminases (BCAT1 and BCAT2) catalyze the conversion of BCAAs to the corresponding branched-chain 2-ketoacids (**Supplementary Fig. 22**)²³. Treatment with pharmacological inhibitors of BCATs, such as gabapentin, octyl-D-2-hydroxyglutarate, and octyl-L-2-hydroxyglutarate, increased the fluorescence ratio of Valsor-expressing HEK293FT cells (**Fig. 3i and Supplementary Fig. 23**). The overexpression of IDH1^{R132H}, a mutant isocitrate dehydrogenase 1 that causes supraphysiologic accumulation of endogenous D-2-hydroxyglutarate, also led to a $49.5 \pm 9.8\%$ increase in the fluorescence ratio of Valsor. In addition, co-administration of the IDH1^{R132H} inhibitor, AGI-5198, reduced the elevated fluorescence ratio of Valsor to near basal levels (**Fig. 3i and Supplementary Fig. 23**).”

For Ilesor:

“Based on the Ilesor-supported bacterial endogenous L-isoleucine assay, we confirmed that LivJ, but not LivK, functions in L-isoleucine uptake of *E. coli* BL21(DE3) (**Fig. 4f and**

Supplementary Fig. 19d). Treatment with different BCAT inhibitors or overexpression of IDH1^{R132H} significantly increased the fluorescence ratio of Ilesor-expressing HEK293FT cells (**Fig. 4i and Supplementary Fig. 33**).”

For SAMsor:

“In *E. coli*, SAM is produced by SAM synthetase (MetK)-catalyzed L-methionine adenylation (**Supplementary Fig. 42**)³³. The addition of 10 mM L-methionine to the medium of SAMsor-expressing *E. coli* BL21(DE3) failed to enhance the fluorescence ratio of SAMsor, whereas overexpression of MetK under the same condition resulted in a $24.0 \pm 1.3\%$ ratio increase (**Fig. 6f and Supplementary Fig. 41c**). The synthesis of L-methionine, the precursor of SAM, is repressed by the transcriptional regulator MetJ (**Supplementary Fig. 42**)³³. The knock-out of MetJ in *E. coli* BL21(DE3) also led to an $18.3 \pm 1.5\%$ increase in the fluorescence ratio of SAMsor. The rat liver SAM synthetase (RLSS) and the bacteriophage T3 SAM hydrolase (T3SH) were previously reported to be capable of modulating endogenous SAM levels in *E. coli*³⁴. As expected, overexpression of RLSS increased the ratio of SAMsor-expressing *E. coli* BL21(DE3) by $31.7 \pm 3.1\%$, while overexpression of T3SH decreased the ratio by $43.5 \pm 1.1\%$ (**Fig. 6f**). ...

... In HEK293FT cells, methionine adenosyltransferase (MATase) catalyzes the conversion of L-methionine to SAM (**Fig. 6j**)³⁵. The exogenous addition of L-methionine or suberoylanilide hydroxamic acid (SAHA), an up-regulator of MATase transcription³⁵, to the medium of SAMsor-expressing HEK293FT cells significantly increased the fluorescence ratio of SAMsor (**Fig. 6j and Supplementary Fig. 45**). Treatment with the pharmacological inhibitors of MATase, such as cycloleucine and AG-270, led to a substantial decrease in the fluorescence ratio of SAMsor-expressing HEK293FT cells (**Fig. 6j**)³⁵.”

7. The statistical methods currently applied need reconsideration. For the multiple comparisons made in Figs. 1e,f,g,h,i, 2m, 3i,m, 4i,m, 5c, 6i,m, analysis of variance (ANOVA) followed by appropriate post hoc tests should be used to ensure robust statistical validity.

Response: Thanks for your good suggestion. We have performed one-way ANOVA test with Tukey’s post-hoc test for the indicated figures. This same statistical method has been applied to all newly supplemented data in the revised manuscript where multiple comparisons

were required.

8. While the dissociation constants (K_{ds}) of each biosensor are reported, a discussion relating these values to the physiological and pathological concentrations of the metabolites in bacteria and mammalian cells would contextualize the biosensors' applicability.

Response: Thanks for your constructive comment. In accordance with your advice, we have compiled the reported physiological and pathological concentrations of L-phenylalanine, L-valine, L-isoleucine, and SAM in both bacteria and mammalian cells (**Table 3**). These concentration ranges, along with the calculated limits of detection (LOD) for Phesor, Valsor, Ilesor, and SAMsor, have been incorporated into their respective dose-response curves. As shown in **Fig. 51**, all four biosensors exhibit detection ranges that appropriately encompass the physiological concentrations of the target ligands in mammalian cells, as well as the majority of concentration ranges in bacteria.

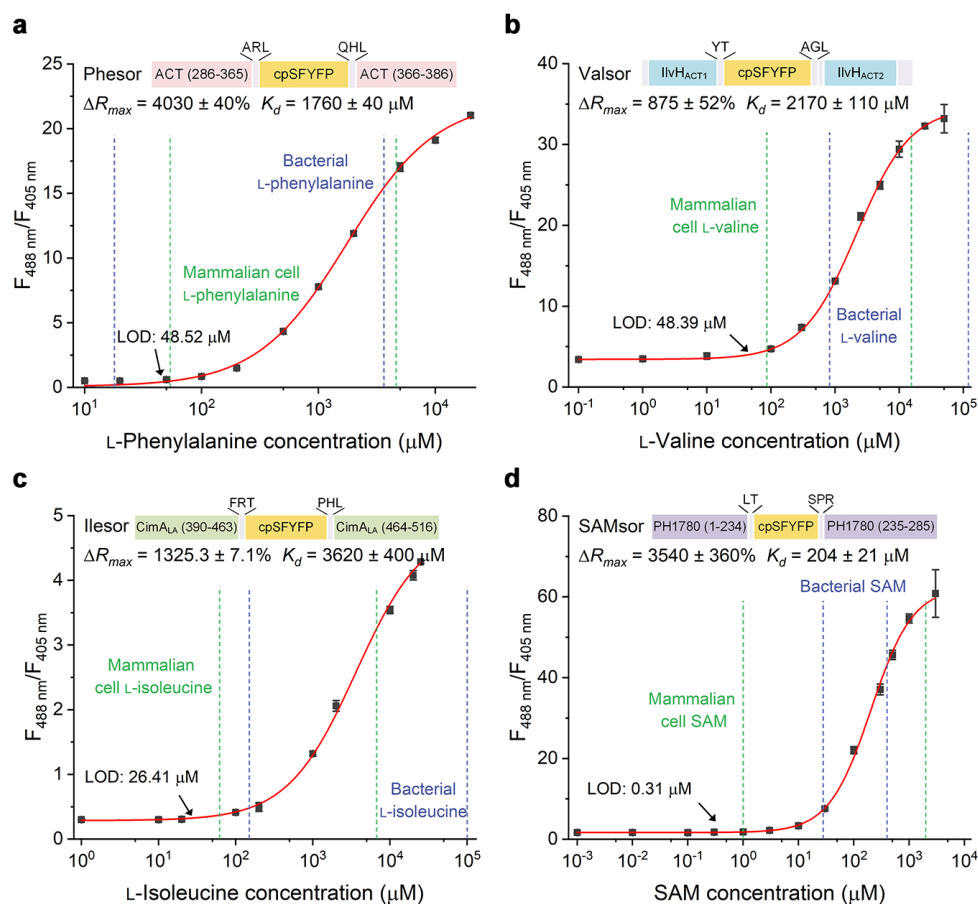


Figure 51 Characterization of Phesor, Valsor, Ilesor, and SAMsor *in vitro*. (a-d) Dose-response curve of Phesor (a), Valsor (b), Ilesor (c), and SAMsor (d) for increasing concentrations of L-phenylalanine, L-

valine, L-isoleucine, and SAM. The structural composition of the four biosensors and physiological concentrations of target ligands in bacteria and mammalian cell were shown. All data shown are means $\pm$ s.d. (n = 3 independent experiments). (**Fig. 2c, Fig. 3d, Fig. 4d, and Fig. 6c in the revised manuscript**)

Table 3 Reported concentrations of L-phenylalanine, L-valine, L-isoleucine, and SAM in bacteria, mammalian cells, and human body fluids.**(Supplementary Table 2 in the revised manuscript)**

Metabolites	Bacteria	Mammalian cells	Human body fluids
L-Phenylalanine	0.02–3.63 mM (<i>Escherichia coli</i>) ^{[1][2]}	0.05–0.28 mM (HeLa cell) ^[3] ~0.50–4.62 mM (A549 cell and U87-MG cell) ^[4] ~0.84 mM (Mouse kidney epithelial cells) ^[5] ~1.50 mM (A549 cell) ^[6] 0.34–0.81 mM (Hybridoma and CHO cell) ^[7] 0.07–0.56 mM (T cells) ^[8]	35–120 µM (Healthy serum) ^[9] 120–360 µM (Hyperphenylalaninaemia serum) ^[9] > 360 µM (Phenylketonuria serum) ^[9] ~62.89 µM (Healthy urine) ^[10]
L-Valine	~0.82–4.58 mM (<i>Escherichia coli</i>) ^[11] ~50–250 mM (<i>Corynebacterium glutamicum</i>) ^[11]	0.09–0.62 mM (HeLa cell) ^[3] ~0.62–2.44 mM (A549 cell and U87-MG cell) ^[4] ~1.51 mM (Mouse kidney epithelial cells) ^[5] ~2.50 mM (A549 cell) ^[6] 1.55–3.36 mM (Hybridoma and CHO cell) ^[7] 3.38–15.41 mM (T cells) ^[8] 1.36–10.29 mM (Fibroblasts) ^[12]	/
L-Isoleucine	~0.15–0.27 mM (<i>Escherichia coli</i>) ^[11] ~17–100 mM (<i>Corynebacterium glutamicum</i>) ^[13]	0.06–0.55 mM (HeLa cell) ^[3] ~0.58–2.09 mM (A549 cell and U87-MG cell) ^[4] ~1.76 mM (Mouse kidney epithelial cells) ^[5] ~2.10 mM (A549 cell) ^[6] 0.70–1.75 mM (Hybridoma and CHO cell) ^[7] 0.75–6.71 mM (T cells) ^[8]	/
SAM	28–400 µM (<i>Escherichia coli</i>) ^{[14][15]}	~62.99 µM (T cells) ^[8] 354 µM (Fibroblasts) ^[16] 1–37 µM (HEK293T cell) ^[17] 10–2,000 µM (Lymphoid cell, HTC116 cell, HEK293 cell) ^[18]	~73.5–120 nM (Serum) ^[19] ~22.1 µM (Urine) ^[19] ~10.2 µM (Urine) ^[20] ~12.1 µM (Urine) ^[21]

1. Bennett, B.D. *et al.* Absolute metabolite concentrations and implied enzyme active site occupancy in *Escherichia coli*. *Nat. Chem. Biol.* **5**, 593–599 (2009).
2. Liu, Y. *et al.* Genetic engineering of *Escherichia coli* to improve L-phenylalanine production. *BMC Biotechnol.* **18**, 5 (2018).
3. Yoshida, T., Nakajima, H., Takahashi, S., Kakizuka, A. & Imamura, H. OLIVE: a genetically encoded fluorescent biosensor for quantitative imaging of branched-chain amino acid levels inside single living cells. *ACS Sens.* **4**, 3333–3342 (2019).
4. Gauthier-Coles, G. *et al.* Quantitative modelling of amino acid transport and homeostasis in mammalian cells. *Nat. Commun.* **12**, 5282 (2021).
5. Park, J.O. *et al.* Metabolite concentrations, fluxes and free energies imply efficient enzyme usage. *Nat. Chem. Biol.* **12**, 482–489 (2016).
6. Bröer, S. & Gauthier-Coles, G. Amino acid homeostasis in mammalian cells with a focus on amino acid transport. *J. Nutr.* **152**, 16–28 (2022).
7. Heidemann, R., Lütkemeyer, D., Büntemeyer, H. & Lehmann, J. Effects of dissolved oxygen levels and the role of extra- and intracellular amino acid concentrations upon the metabolism of mammalian cell lines during batch and continuous cultures. *Cytotechnology* **26**, 185–197 (1998).
8. O’Keeffe, S., Sim, H., McCue, E. & Park, J.O. Ensemble quantitation of absolute metabolite concentrations in T cells reveals conserved features of immunometabolism. *bioRxiv* (2025).
9. van Spronsen, F.J. *et al.* Phenylketonuria. *Nat. Rev. Dis. Primers* **7**, 36 (2021).
10. Xie, G. *et al.* A metabolite array technology for precision medicine. *Anal. Chem.* **93**, 5709–5717 (2021).
11. Ruklisha, M., Paegle, L. & Denina, I. L-Valine biosynthesis during batch and fed-batch cultivations of *Corynebacterium glutamicum*:: relationship between changes in bacterial growth rate and intracellular metabolism. *Process Biochem.* **42**, 634–640 (2007).
12. Kok, G. *et al.* Isoleucine-to-valine substitutions support cellular physiology during isoleucine deprivation. *Nucleic Acids Res.* **53**, gkae1184 (2025).
13. Morbach, S., Sahm, H. & Eggeling, L. L-Isoleucine production with *Corynebacterium glutamicum*: further flux increase and limitation of export. *Appl. Environ. Microbiol.* **62**, 4345–4351 (1996).
14. Halliday, N.M. *et al.* Quantitative liquid chromatography-tandem mass spectrometry profiling of activated methyl cycle metabolites involved in LuxS-dependent quorum sensing in *Escherichia coli*. *Anal. Biochem.* **403**, 20–29 (2010).
15. Posnick L.M. & Samson, L.D. Influence of S-adenosylmethionine pool size on spontaneous mutation, dam methylation, and cell growth of *Escherichia coli*. *J. Bacteriol.* **181**, 6756–6762 (1999).
16. Fukumoto, K. *et al.* Excess S-adenosylmethionine inhibits methylation via catabolism to adenine. *Commun. Biol.* **5**, 313 (2022).
17. Gu, X. *et al.* SAMTOR is an S-adenosylmethionine sensor for the mTORC1 pathway. *Science* **358**, 813–818 (2017).
18. Ye, C. & Tu, B.P. Sink into the epigenome: histones as repositories that influence cellular metabolism. *Trends Endocrinol. Metab.* **29**, 626–637 (2018).
19. Bravo, A.C. *et al.* Analysis of S-adenosylmethionine and S-adenosylhomocysteine: method optimisation and profiling in healthy adults upon short-term dietary

intervention. *Metabolites* **12**, 373 (2022).

20. Stabler, S.P. & Allen, R.H. Quantification of serum and urinary S-adenosylmethionine and S-adenosylhomocysteine by stable-isotope-dilution liquid chromatography-mass spectrometry. *Clin. Chem.* 50, 365–372 (2004).
21. Ivanov, A.V. *et al.* Determination of S-adenosylmethionine, S-adenosylhomocysteine, and methylthioadenosine in urine using solvent-modified micellar electrokinetic chromatography. *Electrophoresis* **41**, 209–214 (2020).

Related data were presented in **Fig. 2c, Fig. 3d, Fig. 4d, Fig. 6c, and Supplementary Table 2** in the revised manuscript. Related descriptions were added in the “Results” section of the revised manuscript as follows:

For Phesor:

“Dose-response curve analysis indicated that the detection range of Phesor encompasses the physiological concentrations of L-phenylalanine both in bacteria and in mammalian cells (**Fig. 2c**). Furthermore, it achieved a high ΔR_{max} ($4,030 \pm 40\%$), an appropriate K_d ($1,760 \pm 40 \mu\text{M}$; close to the value of $2.69 \pm 0.13 \text{ mM}$ determined by thermal shift assay [TSA]), and a Hill slope close to 1 for L-phenylalanine (**Fig. 2c, Supplementary Fig. 5e, and Supplementary Table 4**).”

For Valsor:

“Dose-response curve analysis indicated that Valsor achieved a wide detection range, with an increased ΔR_{max} ($875 \pm 52\%$), an appropriate K_d ($2,170 \pm 110 \mu\text{M}$; close to the value of $4.46 \pm 0.50 \text{ mM}$ determined by TSA), and a Hill slope close to 1 for L-valine (**Fig. 3d, Supplementary Fig. 18e, and Supplementary Table 4**).”

For Ilesor:

“Dose-response curve analysis indicated that Ilesor achieved a suitable detection range, an increased ΔR_{max} ($1,325.3 \pm 7.1\%$), an appropriate K_d ($3,620 \pm 400 \mu\text{M}$; close to the value of $2.22 \pm 0.30 \text{ mM}$ determined by TSA), and a Hill slope close to 1 for L-isoleucine (**Fig. 4d, Supplementary Fig. 30e, and Supplementary Table 4**).”

For SAMsor:

“Dose-response curve analysis indicated that SAMsor achieved an increased ΔR_{max} ($3,540 \pm 360\%$), an appropriate K_d ($204 \pm 21 \mu\text{M}$; close to the value of $0.295 \pm 0.061 \text{ mM}$ determined by TSA), a Hill slope close to 1, and a wide detection range suitable for detecting physiological concentrations of SAM in mammalian cells and bacteria (**Fig. 6c, Supplementary Fig. 40e, and Supplementary Table 2**).”

9. Regarding the specificity, Figs. 2d, 3f, and 4f are based on 1 mM metabolites. However, Phesor, Valsor, and Ilesor are not saturated in 1 mM L-phenylalanine, L-valine, L-isoleucine, respectively. Thus, the authors should show the data of 10 mM metabolites for the

characterization of specificity of these biosensors. Especially, Fig. 4f indicates that L-valine and L-leucine induce substantial fluorescence response of Ilesor. This fact raises concern on the specificity of Ilesor for the BCAAs. The authors should show the titration data of Ilesor for L-valine and L-leucine. Unexpectedly, Fig. 6d is based on different concentrations of each metabolite. SAM at 200 μ M is not enough to saturate the SAMsor's fluorescence response. The authors should show the data of 1 mM SAM and other metabolites. If there are substantial fluorescence response of SAMsor upon 1 mM of MTA, SAH, and Ado, the authors should show the titration data of SAMsor for these metabolites as well.

Response: Thanks for your good suggestion. According to your advice, we have re-evaluated the specificity of Phesor, Valsor, Ilesor, and SAMsor using higher concentrations of potential interfering metabolites. As shown in **Fig. 52** and **Fig. 53**, both Phesor and Valsor exhibited high specificity for L-phenylalanine and L-valine, respectively. No significant fluorescence responses were observed in the presence of 10 mM of the other 19 amino acids (except L-tyrosine, which was determined at a concentration of 1 mM due to solubility limitations) or various analogs of L-phenylalanine or L-valine.

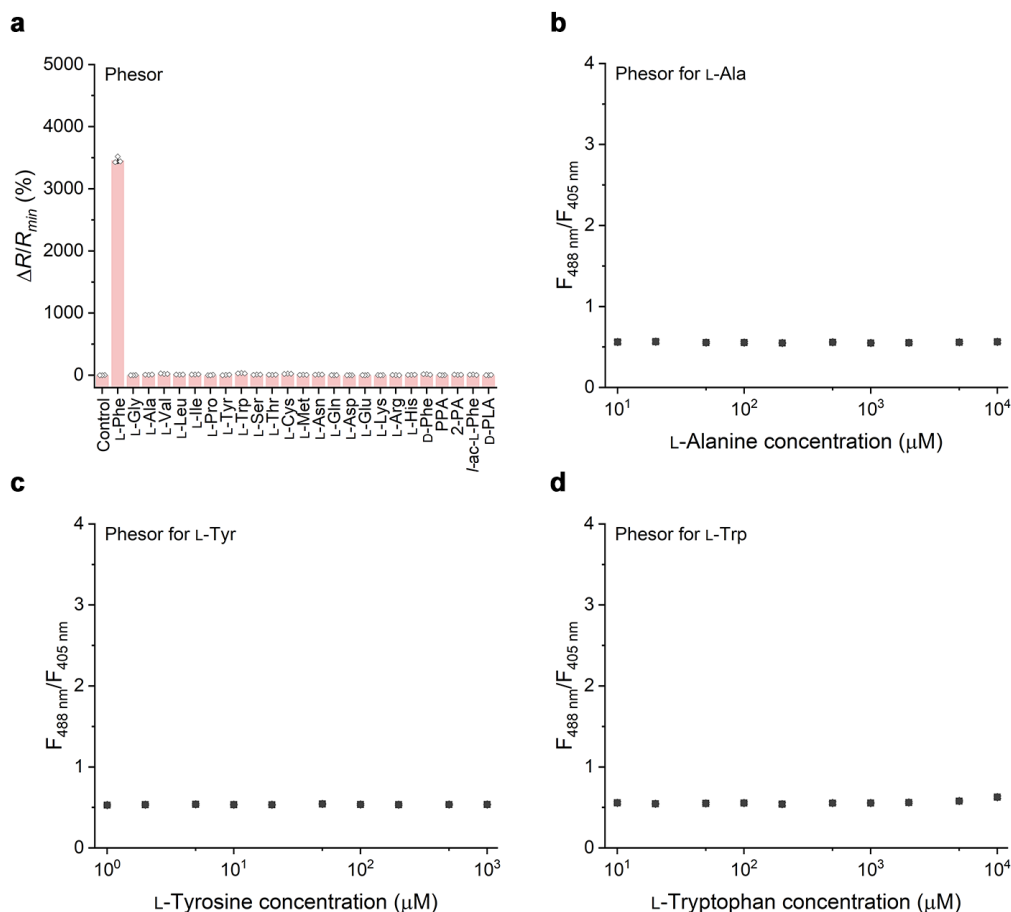


Figure 52 Specificity analysis of Phesor. **(a)** Response of Phesor to 10 mM indicated metabolites (except L-tyrosine, which was determined at a concentration of 1 mM due to solubility limitations). PPA, phenylpyruvate; 2-PA, 2-phenylacetamide; *N*-ac-L-Phe, *N*-acetyl-L-phenylalanine; D-PLA, D-phenyllactate. **(b-c)** Dose-response curves of Phesor for increasing concentrations of L-alanine **(b)**, L-tyrosine **(c)**, and L-tryptophan **(d)**. All data shown are means $\pm$ s.d. ($n \geq 3$ independent experiments). (Supplementary Fig. 3g-i and Supplementary Fig. 5g in the revised manuscript)

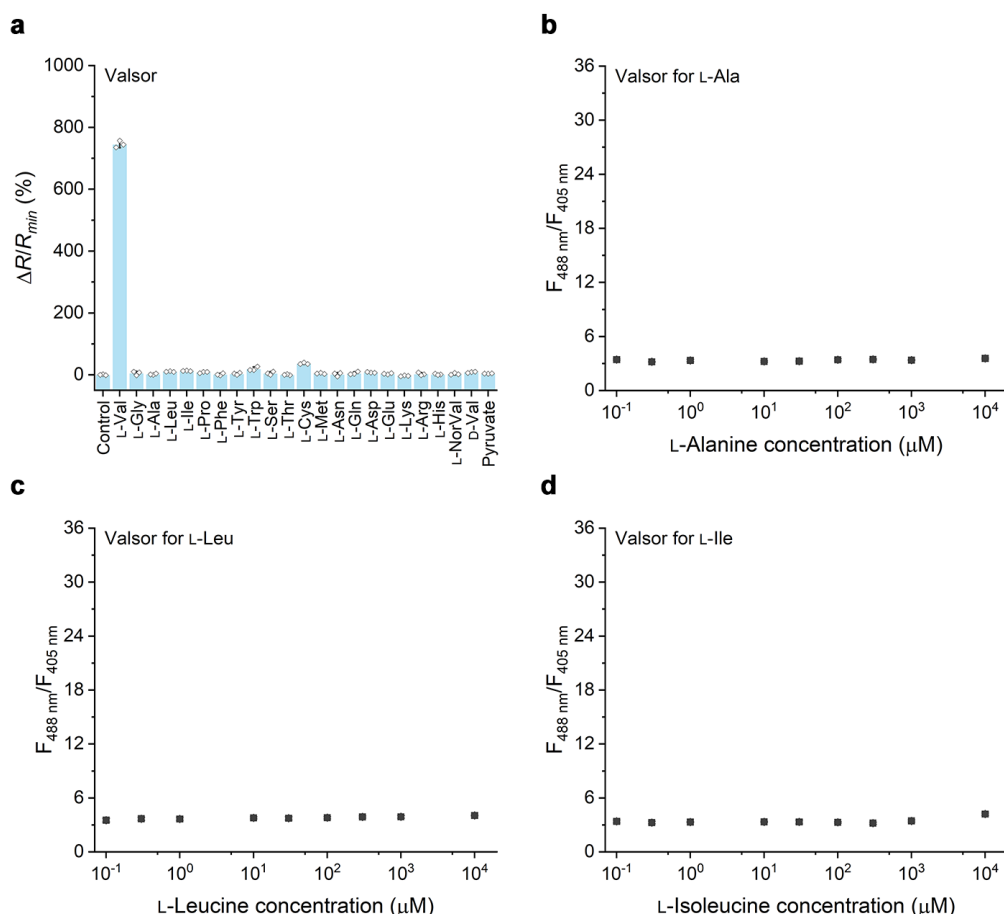


Figure 53 Specificity analysis of Valsor. **(a)** Response of Valsor to 10 mM indicated metabolites (except L-tyrosine, which was determined at a concentration of 1 mM due to solubility limitations). **(b-c)** Dose-response curves of Valsor for increasing concentrations of L-alanine **(b)**, L-leucine **(c)**, and L-isoleucine **(d)**. All data shown are means $\pm$ s.d. ($n = 3$ independent experiments). (Supplementary Fig. 17d-f and Supplementary Fig. 18g in the revised manuscript)

For Ilesor, no detectable responses were observed to physiological concentrations of L-valine and L-leucine (2 mM). Even at the limiting concentration of 10 mM, the changes in the fluorescence ratio for L-valine and L-leucine remained below 100%. In contrast, the responses to L-isoleucine reached $\sim 585\%$ and $\sim 1,070\%$ at 2 mM and 10 mM, respectively (Fig. 54). In addition, none of the other 17 amino acids as well as various L-isoleucine analogs can induce fluorescence ratio changes of Ilesor (Fig. 54). These results demonstrate that Ilesor possesses

good specificity for L-isoleucine under physiological conditions.

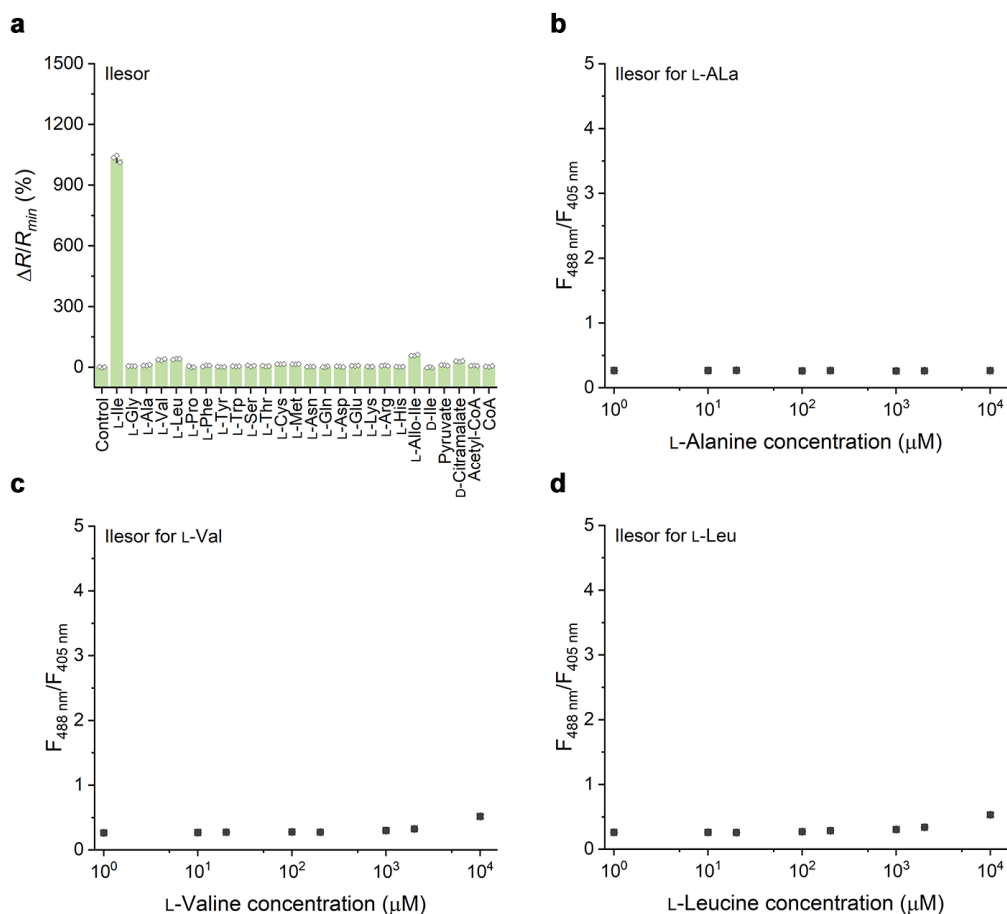


Figure 54 Specificity analysis of Ilesor. **(a)** Response of Ilesor to 10 mM indicated metabolites (except L-tyrosine, which was determined at a concentration of 1 mM due to solubility limitations). **(b-c)** Dose-response curves of Ilesor for increasing concentrations of L-alanine **(b)**, L-valine **(c)**, and L-leucine **(d)**. All data shown are means $\pm$ s.d. ($n \geq 3$ independent experiments). **(Supplementary Fig. 29d-f and Supplementary Fig. 30g in the revised manuscript)**

For SAMsor, notable fluorescence changes were observed in the presence of 1 mM *S*-methyl-5'-thioadenosine (MTA), *S*-adenosyl-L-homocysteine (SAH), or adenosine (**Fig. 55a**). Notably, we note that the physiological concentrations of these metabolites in mammalian cells typically range from nanomolar to low micromolar levels (*BMC Cancer*, 2010, 10:265; *Science*, 2017, 358:813–818; *Circ. Res.*, 1997, 81:154–164). Dose-response curve analysis indicated that SAMsor did not respond to MTA, SAH, or adenosine at concentrations up to 100 μM , confirming its selectivity for SAM under physiological conditions (**Fig. 55b-d**).

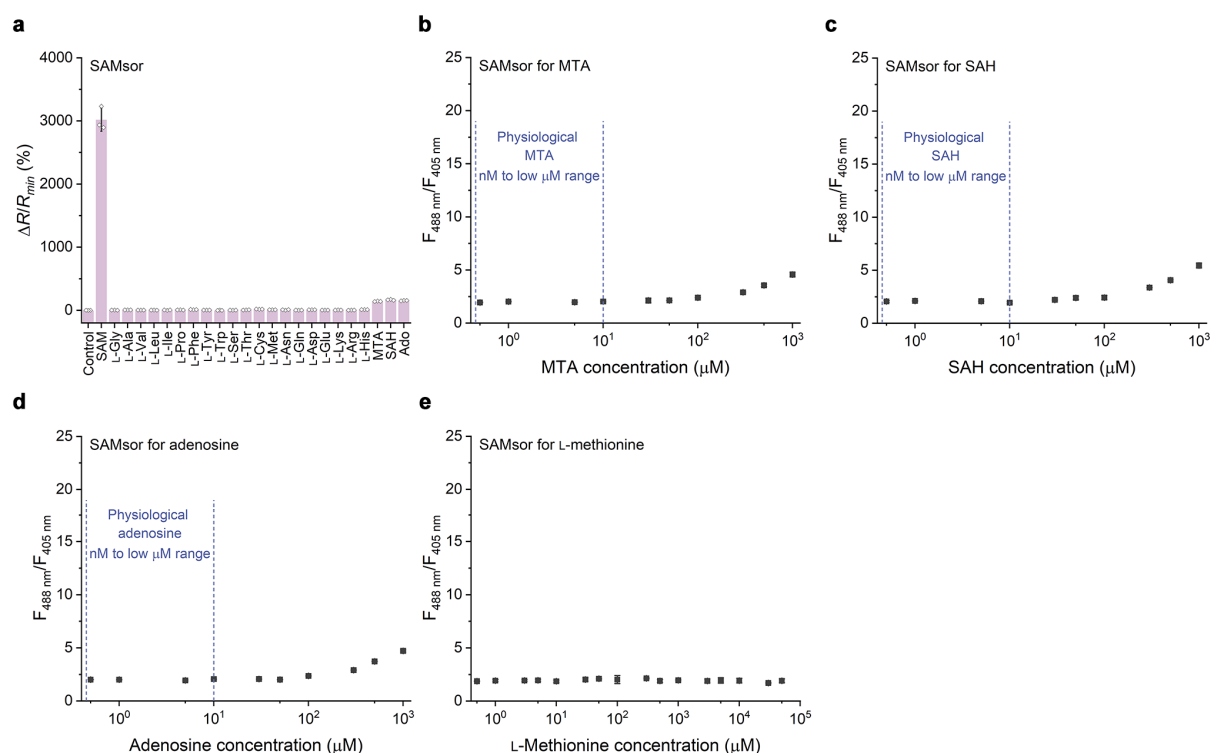


Figure 55 Specificity analysis of SAMsor. **(a)** Response of SAMsor to 1 mM indicated metabolites. Ado, adenosine. **(b-e)** Dose-response curves of SAMsor for increasing concentrations of MTA **(b)**, SAH **(c)**, adenosine **(d)**, and L-methionine **(e)**. All data shown are means $\pm$ s.d. ($n = 3$ independent experiments). **(Fig. 6d and Supplementary Fig. 40g-j in the revised manuscript)**

Related data were presented in **Fig. 6d, Supplementary Fig. 3g-i, Supplementary Fig. 5g, Supplementary Fig. 17d-f, Supplementary Fig. 18g, Supplementary Fig. 29d-f, Supplementary Fig. 30g, and Supplementary Fig. 40g-j** in the revised manuscript. Related descriptions were elaborated in the “Results” section of the revised manuscript as follows:

For Phesor:

“In addition, Phesor showed high specificity for L-phenylalanine, with no significant fluorescence changes in response to the other 19 amino acids, as well as D-phenylalanine, phenylpyruvate, 2-phenylacetamide, *N*-acetyl-L-phenylalanine, and D-phenyllactate **(Supplementary Fig. 3 and Supplementary Fig. 5g).**”

For Valsor:

“In addition, Valsor demonstrated a robust response independent of its own concentration, high specificity, good reversibility, and correctable pH-stability from pH 5.0 to 10.0 for L-valine detection **(Supplementary Fig. 17d-f, Supplementary Fig. 18f-j, and Supplementary Fig. 19a).**”

For Ilesor:

“In addition, Ilesor demonstrated a robust response independent of its own concentration, high specificity, good reversibility, and correctable pH-stability from pH 5.0 to 8.2 for L-isoleucine detection (**Supplementary Fig. 19a, Supplementary Fig. 29d-f, and Supplementary Fig. 30f-j**).”

For SAMsor:

“None of the 20 amino acids, along with physiologic concentrations of MTA, SAH, or adenosine could induce fluorescence ratio changes of SAMsor (**Fig. 6d and Supplementary Fig. 40g-j**).”

Minor Issues:

1. Line 430–431: The authors mentions “The regulatory domains of allosteric enzymes can also be integrated with signal transduction systems like Förster resonance energy transfer (FRET) or bioluminescence resonance energy transfer (BRET) to develop other types of biosensors”. This claim is not experimentally validated in this study and should be moved from the summary to a more speculative section in the Discussion.

Response: Thanks for your good suggestion. The relevant statement has been moved from the summary paragraph of the “Discussion” section to a more speculative section in the penultimate paragraph. The related descriptions in the revised manuscript are as follows:

“Allosteric regulation of proteins is regarded as the “second secret of life”¹⁰. More than 30,000 chemicals, including many key intracellular metabolites for which GEFBs have yet to be developed, have been identified as allosteric ligands of allosteric enzymes (Supplementary Table 1)¹¹. The corresponding allosteric regulatory domains may serve as promising biorecognition elements for the development of GEFBs. In addition, a single metabolite may participate in different metabolic processes and allosterically regulate multiple allosteric enzymes. For example, SAM has been demonstrated to allosterically regulate PH1780, L-threonine synthase in *Arabidopsis thaliana*⁴⁶, and cystathionine β -synthase in humans⁴⁷. The diversity of allosteric enzymes responding to one specific metabolite provides opportunities for the development of GEFBs with distinct properties, such as affinity, response magnitude, and applicable species. Beyond cpFP-based platforms, the regulatory domains of allosteric

enzymes can also be integrated with signal transduction systems, such as Förster resonance energy transfer (FRET) or bioluminescence resonance energy transfer (BRET) to develop other types of biosensors. In addition, the metabolite-responsive allosteric regulatory domains may also contribute to the design of controllable biological processes^{48,49}. For example, incorporating an allosteric regulatory domain of interest into the interior of Cas9 proteins may enable the construction of specific metabolite-dependent allosteric CRISPR-Cas9 switches⁴⁸. Fusing allosteric regulatory domains with a split ferredoxin, an electron carrier participating in various redox reactions, may allow for specific metabolite-induced production of reduced metabolites⁴⁹.”

2. Line 583: The title of the method section is “Characterization of Phesor, Valsor, Ilesor, and SAMsor in living bacteria”. However, in this method, the bacteria are not live due to permeabilization process. The sample prepared in this protocol should be just called crude extract protein. The authors should revise the main text and Methods accordingly.

Response: Thanks for your good suggestion. We have clarified the related description in the “Methods” section of the revised manuscript as follows:

“Characterization of Phesor, Valsor, Ilesor, and SAMsor in permeabilized bacteria”

Corresponding descriptions have also been updated in the “Results” section of the revised manuscript:

For Phesor:

“Phesor expressed in permeabilized *Escherichia coli* BL21 (DE3) specifically responded to the exogenous gradient of L-phenylalanine addition in a dose-dependent manner (**Fig. 2e and Supplementary Fig. 6b**).”

For Valsor:

“Gradient concentrations of L-valine induced a dose-dependent increase in the fluorescence ratio of Valsor expressed in permeabilized *E. coli* BL21(DE3), while the other two BCAAs, L-leucine and L-isoleucine, could not (**Fig. 3e and Supplementary Fig. 19b**).”

For Ilesor:

“Ilesor expressed in permeabilized *E. coli* BL21 (DE3) specifically responded to the exogenous L-isoleucine addition in a dose-dependent manner (**Fig. 4e and Supplementary**

Fig. 19b).”

For SAMsor:

“Exogenous gradient concentrations of SAM induced a dose-dependent increase in the fluorescence ratio of SAMsor expressed in permeabilized *E. coli* BL21(DE3), while MTA, SAH, adenosine, and L-methionine could not (**Fig. 6e and Supplementary Fig. 41b**).”

Accordingly, we have revised the relevant “Figure legends” as follows:

For Phesor:

“(e) Time course of the fluorescence ratio changes of Phesor expressed in permeabilized *E. coli* BL21(DE3) in response to exogenous gradient concentrations of L-phenylalanine, 500 μ M L-tyrosine, or 500 μ M L-tryptophan.”

For Valsor:

“(e) Time course of the fluorescence ratio changes of Valsor expressed in permeabilized *E. coli* BL21(DE3) in response to exogenous gradient concentrations of L-valine, 500 μ M L-leucine, or 500 μ M L-isoleucine.”

For Ilesor:

“(e) Time course of the fluorescence ratio changes of Ilesor expressed in permeabilized *E. coli* BL21(DE3) in response to exogenous gradient concentrations of L-isoleucine, 500 μ M L-valine, or 500 μ M L-leucine.”

For SAMsor:

“(e) Time course of the fluorescence ratio changes of SAMsor expressed in permeabilized *E. coli* BL21(DE3) in response to exogenous gradient concentrations of SAM, 100 μ M MTA, 10 μ M SAH, 100 μ M adenosine, or 500 μ M L-methionine.”

3. Line 663: Related to the comment 2, the authors describe as “Live-cell imaging of HEK293FT cells expressing either...”. However, in this protocol, HEK293FT cells are dead cells due to permeabilization with digitonin. The authors should revise the main text and Methods accordingly.

Response: Thanks for your good suggestion. We have clarified related description in the “Methods” section of the revised manuscript as follows:

“Characterization of Phesor, Valsor, Ilesor, and SAMsor in permeabilized human

cells

Imaging of HEK293FT cells expressing either Phesor, Valsor, Ilesor, or SAMsor was performed 24 h after transfection using a Zeiss 900 confocal microscope (Zeiss, Germany) equipped with a 405 nm diode laser, a 488 nm argon laser, a 20×/0.8 objective, and a full-spectrum fluorescence detector. To determine the dose-response curves and specificity of these biosensors in HEK293FT cells, the transfected cells were first washed twice with 37 °C pre-warmed HHBSS buffer (1× Hank's balanced salt solution supplemented with 20 mM 4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid [HEPES], ThermoFisher, USA). Then, 80 μM digitonin was added into the imaging medium to permeabilize cell membranes and remove basal intracellular ligand levels. Finally, gradient concentrations of target ligands or different analogs were added separately and changes in fluorescence were imaged by the following procedure: excitation at 405 and 488 nm, emission at 503–617 nm, 1024 × 1024 frame size, 8-bit depth, and time intervals of 30 s. For each set of experiments, images were acquired using the same confocal settings. The ratiometric image analysis was carried out using ImageJ software (National Institutes of Health, USA). cpSFYFP was used in parallel experiments to exclude potential effects due to pH fluctuations.”

Accordingly, we have made relevant changes in the “Figure legends” section of the revised manuscript as follows:

For Phesor:

“(g) Ratiometric images and fluorescence ratios of permeabilized HEK293FT cells expressing Phesor before and after the addition of 5 mM L-phenylalanine, L-tyrosine, or L-tryptophan. Scale bar, 10 μm. (h) Gradient responses of Phesor expressed in permeabilized HEK293FT cells to increasing concentrations of L-phenylalanine addition.”

For Valsor:

“(g) Ratiometric images and fluorescence ratios of permeabilized HEK293FT cells expressing Valsor before and after the addition of 15 mM L-valine, L-leucine, or L-isoleucine. Scale bar, 10 μm. (h) Gradient responses of Valsor expressed in permeabilized HEK293FT cells to increasing concentrations of L-valine addition.”

For Ilesor:

“(g) Ratiometric images and fluorescence ratios of permeabilized HEK293FT cells

expressing Ilesor before and after the addition of 5 mM L-valine, L-leucine, or L-isoleucine. Scale bar, 10 μ m. **(h)** Gradient responses of Ilesor expressed in permeabilized HEK293FT cells to increasing concentrations of L-isoleucine addition.”

For SAMsor:

“(g, h) Ratiometric images (g) and fluorescence ratios (h) of permeabilized HEK293FT cells expressing SAMsor or cpSFYFP before and after sequential addition of SAH (50 μ M), MTA (100 μ M), L-methionine (1 mM), adenosine (100 μ M), and SAM (20 μ M and 50 μ M). **(i)** Gradient responses of SAMsor expressed in permeabilized HEK293FT cells to increasing concentrations of SAM addition.”

4. Line 652: Details regarding the species for which codon optimization was performed should be clarified.

Response: Thanks for your reminding. The codon optimization for Phesor, Valsor, Ilesor, and SAMsor were performed for expression in human cells.

Related description has been clarified in the “Methods” section of the revised manuscript as follows:

“The DNA sequences of Phesor, Valsor, Ilesor, and SAMsor were human codon-optimized, synthesized, and cloned into the mammalian expression plasmid pcDNA3.1⁽⁺⁾ behind a Kozak sequence by General Biology Co., Ltd (China).”

5. Line 701: “((Schrödinger, USA)” should be “(Schrödinger, USA)”.

Response: Thanks for your reminding. The related description has been corrected in the revised manuscript.

To Reviewer #3:

This study presents a novel method for developing genetically encoded fluorescent biosensors (GEFBs) using the regulatory domains of allosteric enzymes as biorecognition elements. The authors demonstrate the versatility of this approach by constructing biosensors for four specific metabolites, L-phenylalanine, L-valine, L-isoleucine, and SAM. The study highlights the potential of allosteric regulatory domains as versatile tools for biosensor design, providing a way of biosensor development and addressing gaps in metabolite detection.

Response: We sincerely appreciate your valuable and constructive comments, which have greatly helped us to improve our manuscript. In response to your suggestions, we have conducted additional subcellular experiments and further demonstrated the clinical applicability of the biosensors using authentic human body fluid samples. The revised manuscript also provides a more comprehensive description of the advantages offered by these biosensors, particularly their capability for real-time and *in situ* monitoring of target metabolites in living cells, as well as their potential for clinical diagnostics. We believe that the quality of the revised manuscript has been significantly enhanced and hope it meets your satisfaction.

From the authors' description and the experimental results of this work, I do not particularly see the urgency of developing these metabolite biosensors and have not understood what specific situations would require the use of the biosensors developed in this work. About half of the results in this work are from bacteria, what are the advantages of using these biosensors compared to traditional methods such as HPLC in these common systems? The authors claim that these biosensors can monitor their targeted metabolites with high spatiotemporal resolution, but there are not results regarding the spatial resolution. The imaging results obtained in cultured HEK293FT cells do not distinguish subcellular localization, which did not showcase the advantage of fluorescence biosensors in providing spatial information under microscopic imaging.

Response: Thanks for your constructive comments. We appreciate the opportunity to clarify the unique advantages and critical applications of our biosensors, as well as to

demonstrate their utility for spatially resolved imaging. Genetically encoded fluorescent biosensor (GEFB) composed of biorecognition element and fluorescent reporter element is an emerging tool for metabolite assays. Traditional biorecognition elements for GEFBs include G protein-coupled receptors (GPCRs), bacterial periplasmic binding proteins (PBPs), and bacterial transcriptional regulators. A versatile method for developing neuropeptide GEFBs based on GPCRs was recently published in *Science* (*Science*, 2023, 382:eabq8173). GPCR-based biosensors specialize in detecting signal transduction but not intracellular metabolite concentrations due to their insolubility, while PBP-based biosensors typically require extensive affinity screening due to their poor selectivity (*Cell*, 2020, 183:1986–2002). Our group has previously focused on identifying novel transcriptional regulators from bacterial metabolic pathways and developing corresponding metabolite GEFBs (*Nat. Commun.*, 2025, 16:6913; *Nat. Commun.*, 2021, 12:3619; *Nat. Commun.*, 2021, 12:7108). However, we found that most transcriptional regulator-based GEFBs prefer sensing exogenous metabolites or metabolites at non-physiological levels.

Allosteric regulation of proteins is regarded as the “second secret of life”. The primary objective of this study is to demonstrate the feasibility of constructing GEFBs utilizing the regulatory domains of allosteric enzymes, thereby providing new insights for GEFB development. As demonstrated, the designed allosteric regulatory domain-based GEFBs exhibited soluble expression and good specificity, making them suitable for intracellular applications. Importantly, more than 30,000 chemicals, including many key endogenous metabolites for which GEFBs have yet to be developed, have been identified as allosteric ligands of allosteric enzymes (*Nucleic Acids Res.*, 2024, 52:D376–D383). In addition to the biosensors for L-phenylalanine, L-valine, L-isoleucine, and SAM presented in this study, similar strategies can be employed to develop biosensors targeting various endogenous metabolites. To emphasize this methodological advance, we have revised the title of the manuscript to: “**A versatile method for designing biosensors via regulatory domains of allosteric enzymes**”.

Additionally, we would like to note that the rationale for the selection of these four metabolites lies in their critical physiological and pathological roles, as well as the current lack of highly specific and spatiotemporally resolved detection methods. For example,

previously reported transcriptional regulator-based biosensors could not distinguish between the three aromatic amino acids (*Appl. Microbiol. Biotechnol.*, 2016, 100:6739–6753) or the three branched-chain amino acids (*Metab. Eng.*, 2012, 14:449–457). Numerous studies have reported the development of GEFBs targeting various cofactors, such as ATP (*Nat. Commun.*, 2024, 15:5299; *Proc. Natl. Acad. Sci. USA*, 2024, 121:e2314604121; *Neuron*, 2022, 110:770–782; *Nat. Commun.*, 2019, 10:711; *Nat. Protoc.*, 2017, 12:1542–1562), NADPH/NADP⁺ (*Nat. Commun.*, 2024, 15:10704; *Nat. Commun.*, 2023, 14:3277), and coenzyme A (*Nat. Chem. Biol.*, 2023, 19:346–355). SAM is considered the second most commonly used enzyme cofactor after ATP. However, the only reported intracellular SAM biosensor based on an RNA aptamer also responded to the SAM demethylated product S-adenosyl-L-homocysteine (SAH) and adenosine (*J. Am. Chem. Soc.*, 2020, 142:14117–14124). By utilizing the regulatory domains from PheA, IlvH, CimA, and PH1780, we successfully constructed four biosensors for the previously unavailable specific and highly spatiotemporally resolved detection of L-phenylalanine, L-valine, L-isoleucine, and SAM both *in vitro* and in living cells (**as demonstrated by the spatial resolution data provided below**).

The developed biosensors provide powerful tools for investigating the metabolism, physiology, and pathology of their target ligands. For example, the traditional screening of inhibitors for LAT1, a cancer therapeutic target involved in L-phenylalanine transport, relies on cumbersome radioisotope technology (*Sci. Rep.*, 2024, 14:4651). The *in situ* assay of intracellular L-phenylalanine through Phesor can support the high-throughput screening of LAT1 inhibitors. L-Valine and L-isoleucine have distinct effects on human metabolism and health (*Cell Metab.*, 2021, 33:905–922). The combined application of Valsor and Ilesor will help elucidate the unique metabolic roles of these two structurally similar branched-chain amino acids. In addition, SAM-dependent methyltransferases play crucial roles in biocatalysis and biosynthesis, and serve as valuable therapeutic targets for various diseases (*Chembiochem*, 2012, 13:2642–2655; *Nature*, 2021, 593:597–601). SAMsor will promote the high-throughput screening of SAM-dependent methyltransferases and facilitate the development of efficient inhibitors targeting disease-associated methyltransferases.

We acknowledge that substantial bacterial data were presented in our previous

manuscript. This emphasis arises from the significant industrial importance of these metabolites as high-value compounds, which are primarily produced through microbial fermentation. Using the developed biosensors, we elucidated the metabolic pathways and transport mechanisms of these metabolites in bacteria. For example, we discovered that both LivJ and LivK are involved in L-valine uptake, whereas only LivJ participates in L-isoleucine uptake. These findings provide a theoretical foundation for production improvement through transport engineering.

Nevertheless, we fully agree with your opinion that these metabolite biosensors require rigorous validation of their spatial resolution in living cells. Consequently, we have supplemented the manuscript with comprehensive subcellular experiments. Taking the L-phenylalanine biosensor Phesor as an example, the sequences for nuclear exclusion, mitochondrial localization, and nuclear localization were fused to the N-, N-, and C-terminus of Phesor to realize its subcellular positioning in HEK293FT cells. As shown in **Fig. 56a**, the fluorescence of Phesor was successfully restricted to the cytosol, mitochondria, and nucleus by introducing corresponding localization signals. Next, 750 μ M L-phenylalanine was added into the imaging medium of digitonin-pre-permeabilized HEK293FT cells. As shown in **Fig. 56**, Phesor expressed in different subcellular compartments exhibited identical responses to exogenous L-phenylalanine addition, whereas the fluorescence ratio of cpSFYFP remained unaffected. Then, the subcellular distribution of L-phenylalanine was investigated by comparing the fluorescence ratios of the differentially localized Phesor in resting HEK293FT cells (**Fig. 57**). As shown in **Fig. 57c**, the cpSFYFP-corrected fluorescence ratio of Phesor was nearly identical in the cytosol and the nucleus, but was lowest in the mitochondria. This result indicates a relatively lower abundance of L-phenylalanine in the mitochondria, which was consistent with previous results obtained using complicated mass spectrometry technology (*Cell*, 2016, 166:1324–1337). In addition, different subcellular localized Phesor could also respond to endogenous L-phenylalanine fluctuations induced by L-phenylalanine addition and LAT1 overexpression (**Fig. 57c**). These results demonstrate the applicability of Phesor for *in situ* monitoring of L-phenylalanine fluctuations in living cells with high spatial resolution.

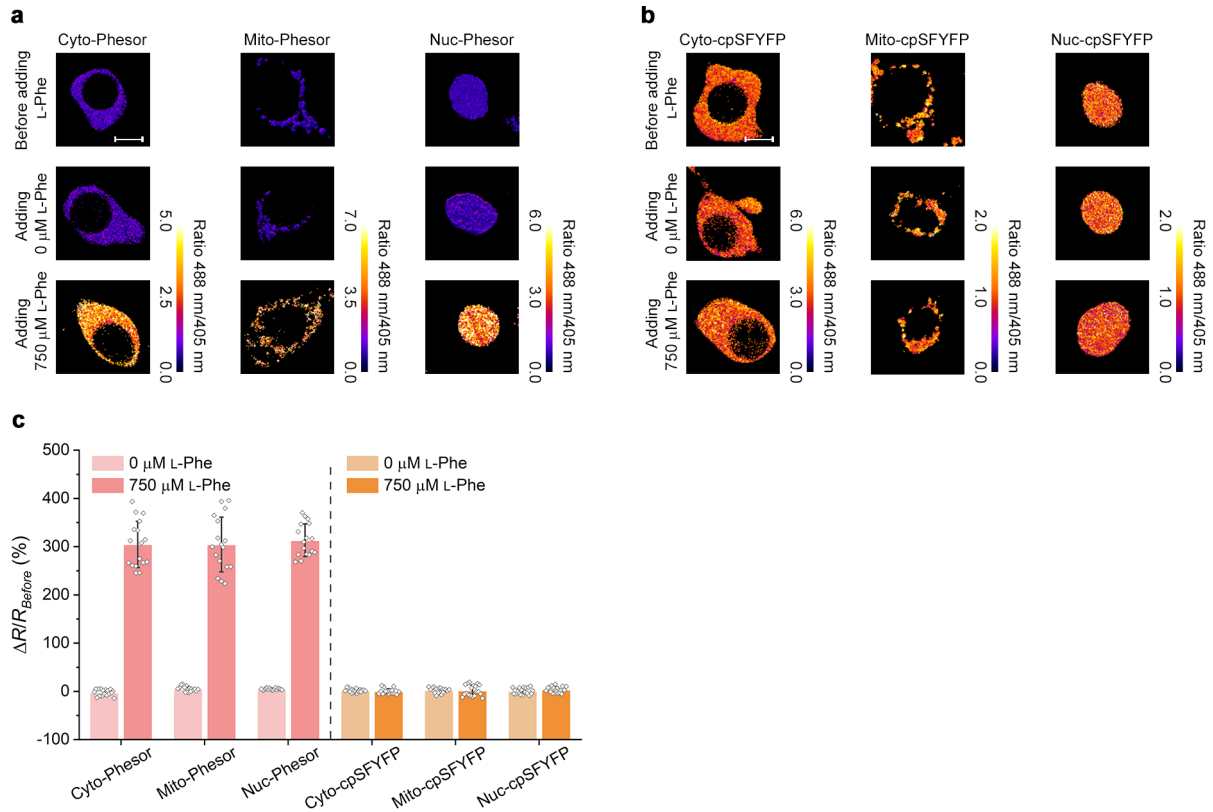


Figure 56 Characterization of different subcellular localized Phesor in HEK293FT cells. **(a, b)** Ratiometric images of single HEK293FT cell expressing Cyto-Phesor, Mito-Phesor, Nuc-Phesor **(a)**, or Cyto-cpSFYFP, Mito-cpSFYFP, Nuc-spSFYFP **(b)** before and after addition of 0 or 750 μM L-phenylalanine. Scale bar, 10 μm . **(c)** Fluorescence response of different subcellular localized Phesor or cpSFYFP to exogenous 750 μM L-phenylalanine addition. The HEK293FT cells were permeabilized with 80 μM digitonin. Data shown in panel **c** are means $\pm$ s.d. ($n = 17$ cells for different subcellular localized Phesor; $n = 19$ cells for different subcellular localized cpSFYFP). (**Supplementary Fig. 11 in the revised manuscript**)

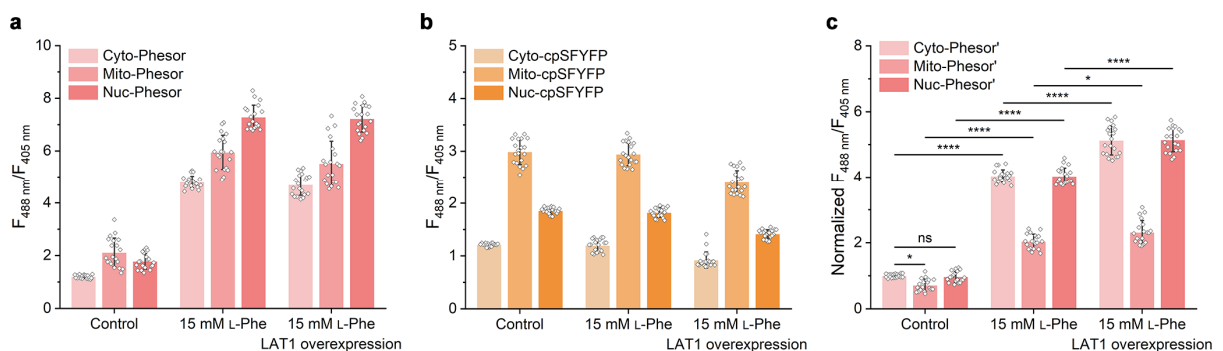


Figure 57 Analysis of subcellular distribution of L-phenylalanine using different subcellular localized Phesor. **(a, b)** Fluorescence ratios of HEK293FT cells expressing different subcellular localized Phesor **(a)** or cpSFYFP **(b)** in response to 15 mM L-phenylalanine addition and LAT1 overexpression. 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 subcellular localized Phesor required correction using cpSFYFP. **(c)** Corrected fluorescence ratios of HEK293FT cells expressing different subcellular localized Phesor by cpSFYFP. 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 test with Tukey's Multiple Comparison Test. **(Supplementary Fig. 12 in the revised manuscript)**

Similarly, all three other biosensors (Valsor, Ilesor, and SAMsor) could be correctly expressed in the cytosol, mitochondria, and nucleus of HEK293FT cells. These differentially localized biosensors exhibited identical fluorescence ratio changes in response to exogenous addition of target ligands (**Fig. 58, Fig. 60, and Fig. 62**). Notably, they also enabled *in situ* analysis of compartment-specific fluctuations in endogenous target ligands induced by treatment with various inhibitors (**Fig. 59, Fig. 61, and Fig. 63**). For example, in mammalian cells, L-valine and L-isoleucine are converted into branched-chain α -keto acids through transamination mediated by cytosolic branched-chain amino acid transaminase 1 (BCAT1) and mitochondrial branched-chain amino acid transaminase 2 (BCAT2). Treatment with the selective BCAT1 inhibitor gabapentin resulted in significant accumulation of L-valine and L-isoleucine in the cytosol and nucleus. The slightly elevated fluorescence ratios in mitochondria may be attributed to the transport of L-valine and L-isoleucine across the mitochondrial membrane (**Fig. 59c and Fig. 61c**). In addition, relatively lower abundances of L-valine, L-isoleucine, and SAM in the mitochondria were also observed, which were consistent with previously reported results (*Cell*, 2016, 166:1324–1337). Collectively, these results systematically demonstrate the robust performance of the four biosensors for compartmental monitoring of target ligands. These biosensors would pave the way for spatially resolved analysis of metabolic mechanisms and physiological functions at an unprecedented level.

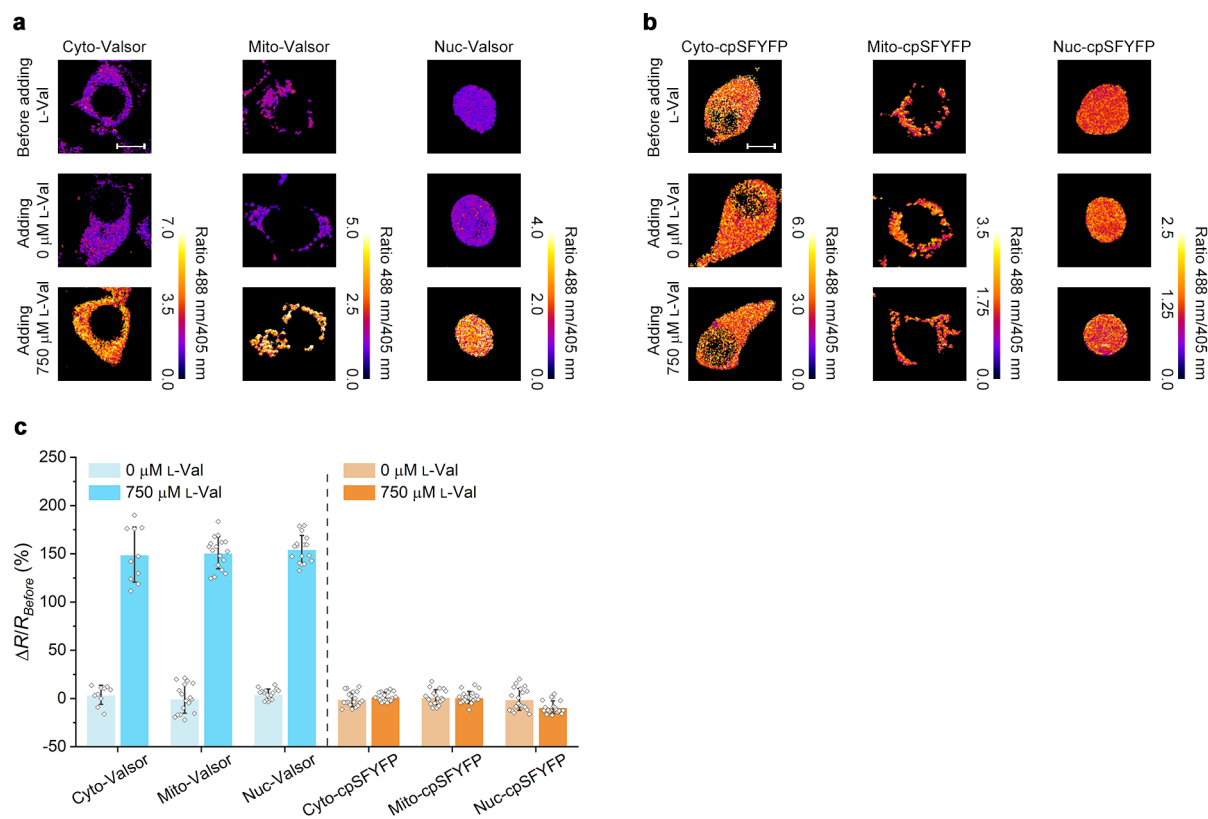


Figure 58 Characterization of different subcellular localized Valsor in HEK293FT cells. **(a, b)** Ratiometric images of single HEK293FT cell expressing Cyto-Valsor, Mito-Valsor, Nuc-Valsor **(a)**, or Cyto-cpSFYFP, Mito-cpSFYFP, Nuc-spSFYFP **(b)** before and after addition of 0 or 750 μM L-valine. Scale bar, 10 μm. **(c)** Fluorescence response of different subcellular localized Valsor or cpSFYFP to exogenous 750 μM L-valine addition. The HEK293FT cells were permeabilized with 80 μM digitonin. Data shown in panel **c** are means $\pm$ s.d. (n = 10, 10, 17, 17, 17, 17, 19, 19, 19, 19, 19 cells from left to right). **(Supplementary Fig. 24 in the revised manuscript)**

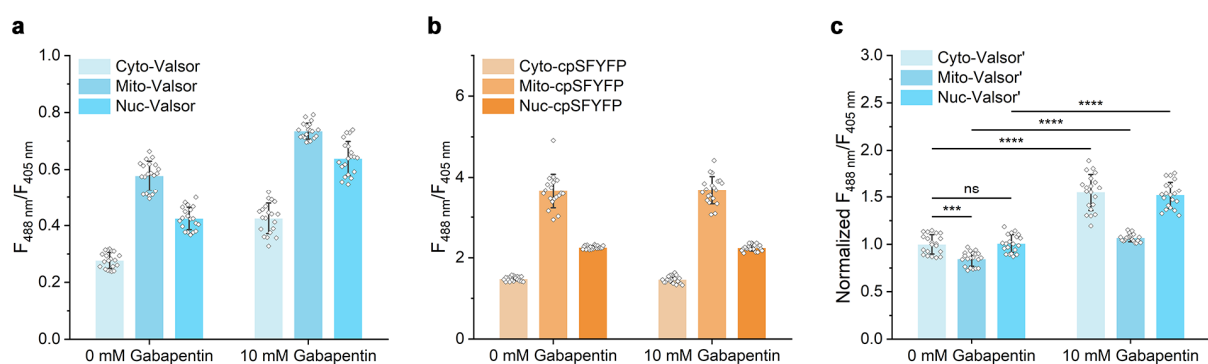


Figure 59 Analysis of subcellular distribution of L-valine using different subcellular localized Valsor. **(a, b)** Fluorescence ratios of HEK293FT cells expressing different subcellular localized Valsor **(a)** or cpSFYFP **(b)** in response to 10 mM gabapentin addition. Cells were treated with 0 or 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

subcellular localized Valsor required correction using cpSFYFP. **(c)** Corrected fluorescence ratios of HEK293FT cells expressing different subcellular localized Valsor by cpSFYFP. All data shown are means $\pm$ s.d. ($n = 20$ cells). ***, $P < 0.001$; ****, $P < 0.0001$; ns, no significant difference ($P \geq 0.05$); one-way ANOVA test with Tukey's Multiple Comparison Test. **(Supplementary Fig. 25 in the revised manuscript)**

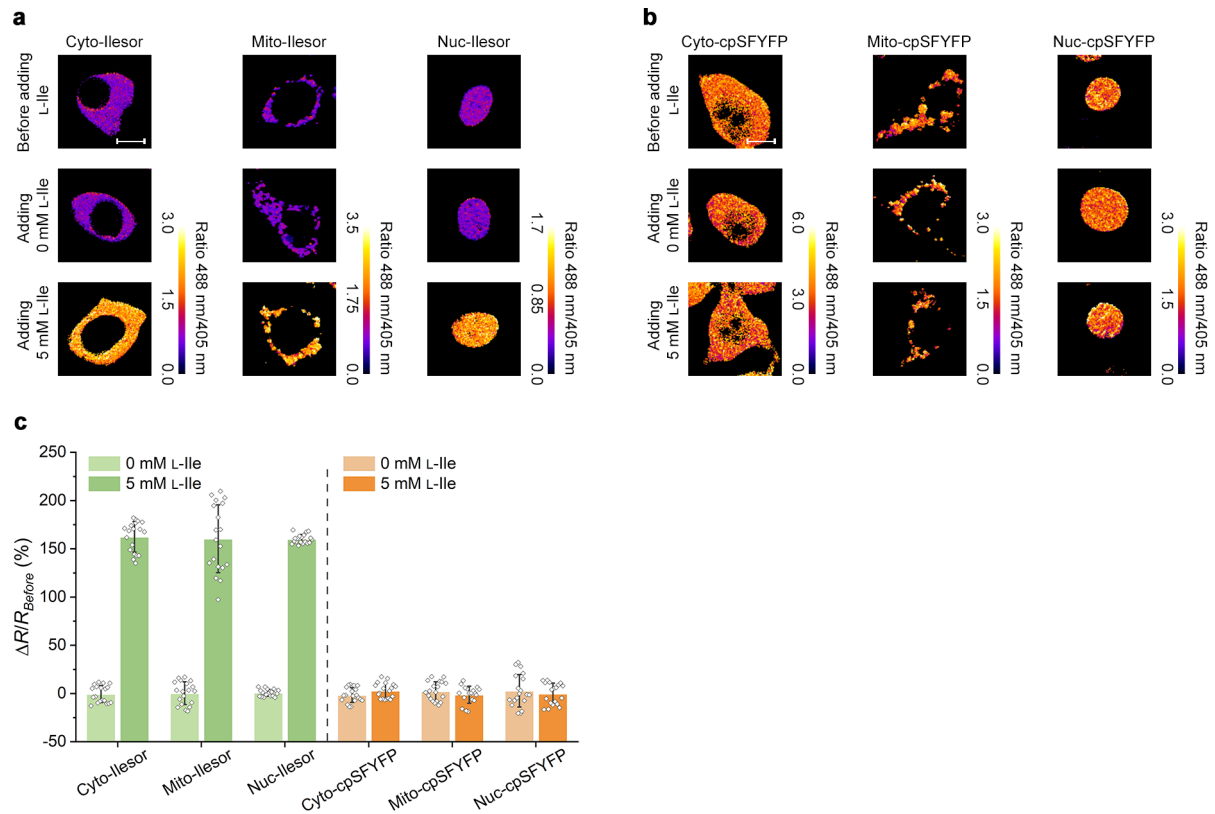


Figure 60 Characterization of different subcellular localized Ilesor in HEK293FT cells. **(a, b)** Ratiometric images of single HEK293FT cell expressing Cyto-Ilesor, Mito-Ilesor, Nuc-Ilesor **(a)**, or Cyto-cpSFYFP, Mito-cpSFYFP, Nuc-spSFYFP **(b)** before and after addition of 0 or 5 mM L-isoleucine. Scale bar, 10 μ m. **(c)** Fluorescence response of different subcellular 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). **(Supplementary Fig. 34 in the revised manuscript)**

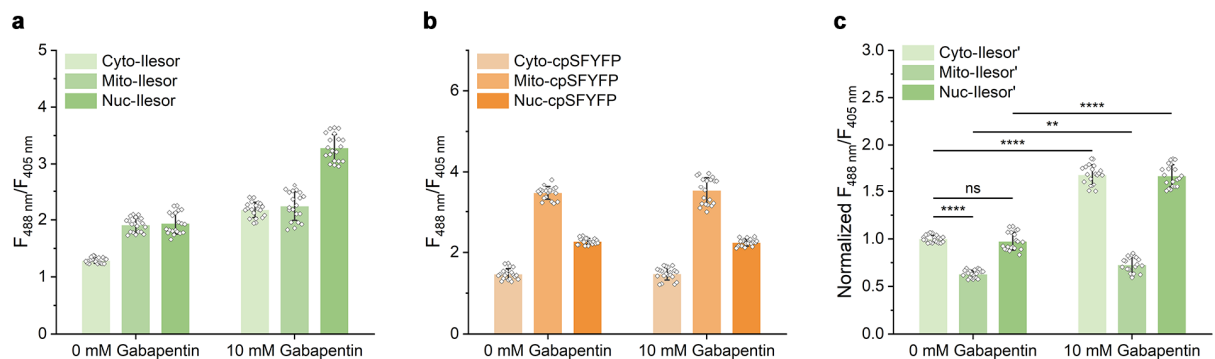


Figure 61 Analysis of subcellular distribution of L-isoleucine using different subcellular localized Ilesor.

(a, b) Fluorescence ratios of HEK293FT cells expressing different subcellular 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 subcellular localized Ilesor required correction using cpSFYFP. **(c)** Corrected fluorescence ratios of HEK293FT cells expressing different subcellular localized Ilesor by cpSFYFP. 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 test with Tukey's Multiple Comparison Test. **(Supplementary Fig. 35 in the revised manuscript)**

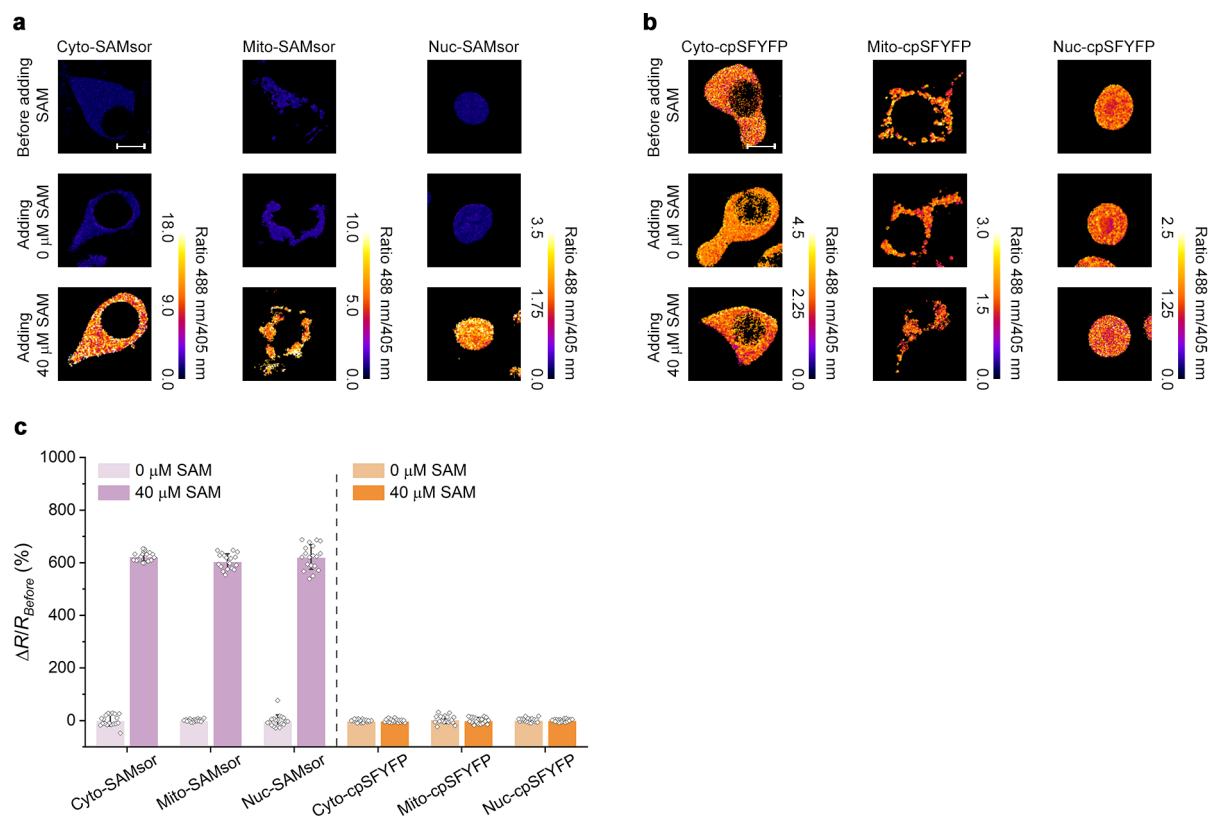


Figure 62 Characterization of different subcellular localized SAMsor in HEK293FT cells. **(a, b)** Ratiometric images of single HEK293FT cell expressing Cyto-SAMsor, Mito-SAMsor, Nuc-SAMsor **(a)**, or Cyto-cpSFYFP, Mito-cpSFYFP, Nuc-spSFYFP **(b)** before and after addition of 0 or 40 μM SAM. Scale bar, 10 μm . **(c)** Fluorescence response of different subcellular localized SAMsor or cpSFYFP to exogenous 40 μM SAM addition. The HEK293FT cells were permeabilized with 80 μM digitonin. Data shown in panel **c** are means $\pm$ s.d. ($n = 19$ cells). **(Supplementary Fig. 46 in the revised manuscript)**

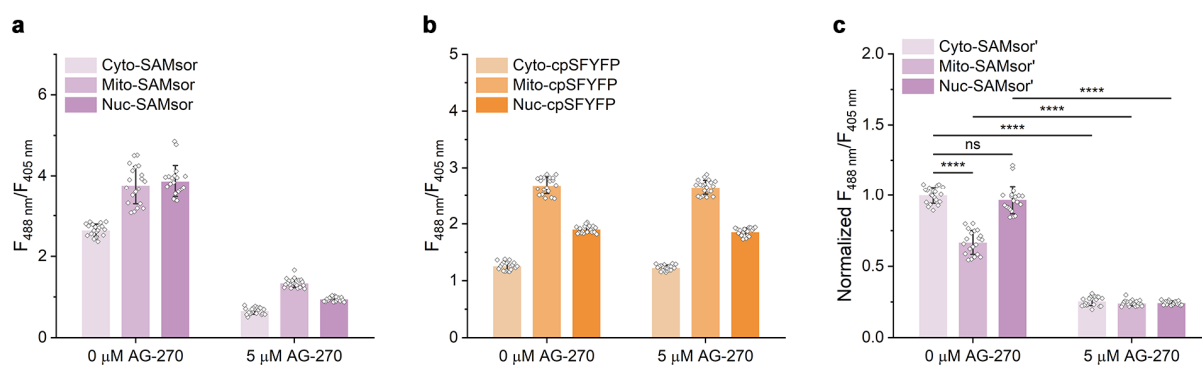


Figure 63 Analysis of subcellular distribution of SAM using different subcellular localized SAMsor. **(a, b)** Fluorescence ratios of HEK293FT cells expressing different subcellular localized SAMsor **(a)** or cpSFYFP **(b)** in response to 5 μ M AG-270 addition. Cells were treated with 0 or 5 μ M AG-270, 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 subcellular localized SAMsor required correction using cpSFYFP. **(c)** Corrected fluorescence ratios of HEK293FT cells expressing different subcellular localized SAMsor by cpSFYFP. 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 test with Tukey's Multiple Comparison Test. **(Supplementary Fig. 47 in the revised manuscript)**

In addition, according to your advice, we have included a new table to systematically compare the detection properties of HPLC and the present biosensors in the revised manuscript (**Table 4**). L-Phenylalanine and SAM are key biomarkers for the diagnosis and therapeutic monitoring of various human diseases (*Nat. Rev. Dis. Primers*, 2021, 7:36; *BMC Res. Notes*, 2016, 9(1):498). Besides the advantages in live-cell assays, we have also demonstrated the applicability of the Phesor and SAMsor_L (a newly developed, highly sensitive SAM biosensor designed for SAM detection in human body fluids) in clinical diagnostics using authentic human body fluid samples from healthy individuals and patients with phenylketonuria or lung cancer. Compared to traditional HPLC- or mass spectrometry-based methods, Phesor and SAMsor_L are easy to handle, cost-effective, rapid, and compatible with high-throughput assays (**Table 4**), making them ideal tools for point-of-care testing (POCT). In addition, branched-chain amino acids are valuable compounds widely used in the food, cosmetic, and pharmaceutical industries, with a steadily increasing global market (e.g., the average annual increase rate of L-valine exceeding 5%). Microbial fermentation serves as the primary source of branched-chain amino acids worldwide (*Bioresour. Technol.*, 2024, 397:130502). We also demonstrated the feasibility of Valsor and Ilesor for continuous

bioprocess monitoring using authentic bacterial fermentation samples. By combining with high-throughput analysis technologies (e.g., fluorescence-activated droplet sorting [FADS]; *Biochem. Eng. J.*, 2022, 186:108586), these biosensors could accelerate the engineering of the L-valine and L-isoleucine producing strains. **Detailed data regarding the clinical diagnostics and bioprocess monitoring are provided in our response to the Next Comment.**

Table 4 Comparison of detection performance between HPLC and the biosensors developed in this study. (**Supplementary Table 5 in the revised manuscript**)

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	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	> 100 µL	10 µL	10 µL	10 µL	/	10 µL
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.

Related data were presented in **Supplementary Fig. 11, Supplementary Fig. 12, Supplementary Fig. 24, Supplementary Fig. 25, Supplementary Fig. 34, Supplementary Fig. 35, Supplementary Fig. 46, Supplementary Fig. 47, and Supplementary Table 5** in the revised manuscript. Related descriptions regarding the spatial resolution results were added in the “Results” section of the revised manuscript as follows:

For Phesor:

“Phesor could also be expressed in different subcellular compartments and responded to endogenous L-phenylalanine fluctuations (**Supplementary Fig. 11 and Supplementary Fig. 12**). We further investigated the subcellular distribution of L-phenylalanine in HEK293FT cells and found a relatively lower abundance of L-phenylalanine in the mitochondria than in the cytosol and nucleus (**Supplementary Fig. 12c**), which was consistent with previous results obtained using complicated mass spectrometry technology¹⁹. These results demonstrate that Phesor can monitor L-phenylalanine fluctuations in human cells with high spatiotemporal resolution.”

For Valsor:

“Valsor could be correctly expressed in the cytosol, mitochondria, and nucleus, and responded to compartment-specific L-valine fluctuations induced by L-valine addition or gabapentin treatment (**Supplementary Fig. 24 and Supplementary Fig. 25**). Furthermore, we analyzed the subcellular distribution of L-valine and demonstrated its lower abundance in the mitochondria (**Supplementary Fig. 25c**).”

For Ilesor:

“Ilesor also allowed *in situ* detection of L-isoleucine in different subcellular compartments, and a lower abundance of mitochondrial L-isoleucine was identified (**Supplementary Fig. 34 and Supplementary Fig. 35**).”

For SAMsor:

“SAMsor expressed in different subcellular organelles also enabled compartment-specific SAM detection, with a lower mitochondrial SAM distribution observed (**Supplementary Fig. 46 and Supplementary Fig. 47**).”

Related descriptions regarding the advantages, significance, and application prospects of the biosensors were added in the “Discussion” section of the revised manuscript as follows:

“In this study, we successfully used the allosteric regulatory domain of PheA, the key enzyme involved in L-phenylalanine biosynthesis, to construct a specific biosensor Phesor. Phesor allowed for previously unavailable high spatiotemporal resolution detection of L-phenylalanine in living cells. Traditional screening of inhibitors of LAT1, a cancer therapeutic target involved in L-phenylalanine transport, was achieved using cumbersome radioisotope technology¹⁸. The *in situ* assay of intracellular L-phenylalanine through Phesor may also support high-throughput screening of LAT1 inhibitors. In addition, L-phenylalanine is a critical biomarker for phenylketonuria diagnosis and therapeutic monitoring²⁰. Compared to traditional HPLC- or mass spectrometry-based methods, Phesor is easy to handle, cost-effective, rapid, and compatible with high-throughput assays (**Supplementary Table 5**), making it an ideal tool for point-of-care diagnostics of L-phenylalanine-related diseases. We also used the concept of Phesor construction to obtain Valsor and Ilesor for specific detection of L-valine and L-isoleucine. L-Valine and L-isoleucine have distinct effects on human metabolism and health⁴¹. Deeper insights into their physiological and pathological roles may be achieved through Valsor- and Ilesor-supported spatiotemporal monitoring of L-valine and L-isoleucine. In addition, both biosensors enabled convenient and precise bioprocess monitoring for fermentative production of L-valine or L-isoleucine. Such biosensor-based high-throughput screening may accelerate the engineering of the L-valine and L-isoleucine producing strains^{40,42}.”

“SAM is an important metabolic intermediate and a universal cofactor in various cellular processes^{12,32}. The RNA aptamer-based SAM biosensor also responded to the SAM demethylated product SAH and adenosine⁴³. The SAMsor-enabled specific, real-time, and *in situ* assays of SAM may provide deeper insights into the functions of enzymes in SAM metabolism, support high-throughput screening of SAM-dependent methyltransferases, and facilitate the development of efficient inhibitors targeting methyltransferases related to various diseases^{44,45}. In addition, SAM level in body fluids is an important indicator of disease status^{36–38}. A more sensitive SAM biosensor, SAMsor_L, was also developed in this study with PH1780 as biorecognition element. Besides binding-pocket engineering, the screening of cpSFYFP insertion sites proved to be an effective strategy for affinity optimization. Importantly, SAMsor_L enables point-of-care quantification of SAM in human

body fluids, offering a practical tool for the clinical diagnostics and therapeutic monitoring of SAM-related diseases.”

Experimental methods related to subcellular localization and characterization were added in the “Supplementary Methods” section of the revised manuscript as follows:

“Subcellular localization of Phesor, Valsor, Ilesor, and SAMsor

The pcDNA3.1⁽⁺⁾ plasmid was used for the subcellular expression of Phesor, Valsor, Ilesor, SAMsor, or cpSFYFP in HEK293FT cells. For cytosolic localization, two repeats of the MAPKK nuclear export signal (MALQKKLEELDELDEQQRKRLEDL)₂ were fused to the N-terminus of these biosensors. For mitochondrial localization, a mitochondrial localization signal (MLSLRQSIRFFKPATRTLCSRYLL) was fused to the N-terminus of these biosensors. For nuclear localization, three repeats of the SV40 large T antigen nuclear localization signal (DPKKKRKV)₃ were fused to the C-terminus of these biosensors. The expression of subcellular localized Phesor, Valsor, Ilesor, SAMsor, or cpSFYFP in HEK293FT cells followed the same procedure as that used for the non-targeted biosensors. Functional characterization of these subcellular localized biosensors in HEK293FT cells was performed 24 h after transfection. The cells were washed twice with 37 °C pre-warmed HHBSS buffer, permeabilized with 80 µM digitonin, and then treated with 750 µM L-phenylalanine, 750 µM L-valine, 5 mM L-isoleucine, or 40 µM SAM. Fluorescence ratios were measured before and after the addition of different target ligands using a Zeiss 900 confocal microscope. Different subcellular localized cpSFYFP were used in parallel experiments to exclude potential effects due to pH fluctuations.

Application of Phesor, Valsor, Ilesor, and SAMsor for subcellular detection of target ligands in human cells

For detection of endogenous L-phenylalanine fluctuations at subcellular resolution using Phesor, 0 or 15 mM L-phenylalanine was added into the medium of HEK293FT cells transfected with different subcellular localized Phesor or co-transfected with different subcellular localized Phesor and LAT1. Changes in the fluorescence ratio of different subcellular localized Phesor were imaged 20 h after L-phenylalanine addition. For detection of endogenous L-valine or L-isoleucine fluctuations at subcellular resolution using Valsor or Ilesor, 0 or 10 mM gabapentin was added into the medium of HEK293FT cells transfected

with different subcellular localized Valsor or Ilesor. Changes in the fluorescence ratio of different subcellular localized Valsor or Ilesor were imaged 20 h after gabapentin treatment. For detection of endogenous SAM fluctuations at subcellular resolution using SAMsor, 0 or 5 μ M AG-270 was added into the medium of HEK293FT cells transfected with different subcellular localized SAMsor. Changes in the fluorescence ratio of different subcellular localized SAMsor were imaged 20 h after gabapentin treatment. Different subcellular localized cpSFYFP were used in parallel experiments to exclude potential effects due to pH fluctuations.”

We sincerely hope these modifications satisfactorily address your concerns. If additional clarifications or experimental data are desired, we would be pleased to continue refining our manuscript.

In general, only experiments conducted in living organisms are referred to as “in vivo.” It may not be quite appropriate to refer to the results obtained in cultured cells as “in vivo” and the entire field might be more interested in seeing the application of these powerful sensors for metabolite imaging and detection in higher systems. If these biosensors are only applied in bacteria and cultured cells, the significance of this work will be greatly limited. If there are unique advantages of the biosensors in in vitro diagnostics, this work lacks related results in this regard to demonstrate that these biosensors can indeed play an important role in the actual testing of patient samples.

Response: Thanks for your constructive comments. We agree that referring to results from cultured cells as “in vivo” was imprecise. Therefore, to improve the accuracy of our descriptions, we have systematically replaced the term “in vivo” with “in living cells” throughout the revised manuscript. For example:

“The applicability of the as-designed biosensors for L-phenylalanine, L-valine, L-isoleucine, and SAM detection is comprehensively characterized in living cells, fermentation systems, and clinical human body fluids.”

“These results suggest that Valsor, developed based on the regulatory subunit IlvH in allosteric enzyme 2-acetolactate synthase, can serve as a reliable tool for precise L-valine detection both in living cells and *in vitro*.”

“These results demonstrate that Ilesor, developed based on the regulatory domain Cim_{ALA} in allosteric enzyme CimA, is applicable for sensitive and precise detection of L-isoleucine both in living cells and *in vitro*.”

Furthermore, according to your suggestions, we have now systematically demonstrated the applicability of the designed biosensors in clinical diagnostics. Taking Phesor as an example, we collected 14 authentic serum samples and nine urine samples from healthy individuals. Then, we prepared 14 serum and 16 urine samples from simulated patients with hyperphenylalaninemia or phenylketonuria by spiking various concentrations of L-phenylalanine (30–550 μM). As shown in **Fig. 64a-c** and **Fig. 65**, quantification of L-phenylalanine in these samples using Phesor demonstrated good correlation and consistency with HPLC measurements, and the results were unaffected by the presence of different L-phenylalanine analogs. The measured L-phenylalanine concentrations in serum and urine from healthy individuals were 72.55–102.17 μM and 68.30–87.06 μM , respectively, consistent with previously reported values (35–120 μM in serum and ~ 62.89 μM in urine; *Nat. Rev. Dis. Primers*, 2021, 7:36; *Anal. Chem.*, 2021, 93:5709–5717).

Dried blood spots are the primary sample type used in newborn screening for phenylketonuria. To further assess the diagnostic potential of Phesor, we collected authentic dried blood spot samples from 19 healthy children and 23 children with phenylketonuria. As shown in **Fig. 64d**, blood L-phenylalanine concentrations measured using Phesor ranged from 31.10 to 119.91 μM in healthy subjects, while those in phenylketonuria patients ranged from 393.45 to 1,821.13 μM , higher than the clinical diagnostic cut-off value of 360 μM (*Nat. Rev. Dis. Primers*, 2021, 7:36). These results indicate that Phesor is applicable for point-of-care testing of L-phenylalanine in human body fluids, demonstrating its potential as a reliable tool for the diagnosis and therapeutic monitoring of L-phenylalanine-related diseases.

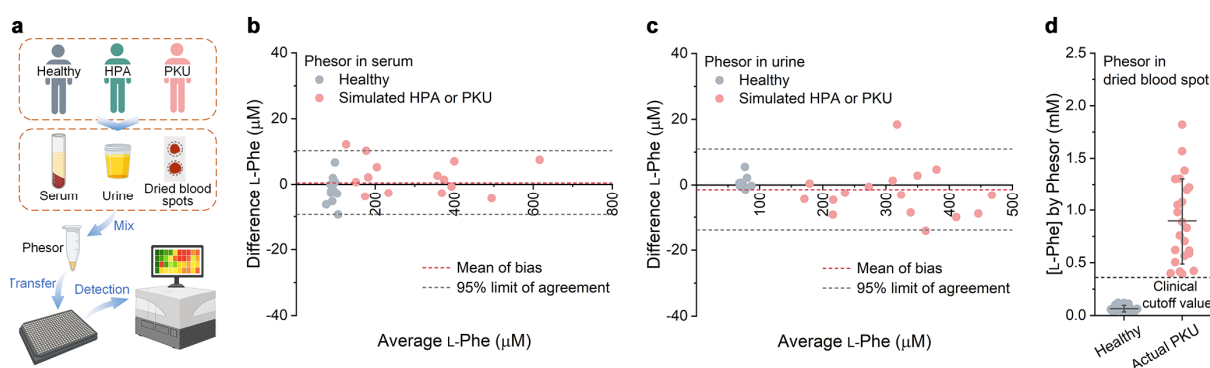


Figure 64 Application of Phesor for quantitative detection of L-phenylalanine in human body fluids. **(a)** Schematic representation of the workflow for quantitative detection of L-phenylalanine in human serum, urine, and dried blood spot samples using Phesor. **(b, c)** Bland-Altman analysis for L-phenylalanine in serum **(b)** and urine **(c)** samples from healthy adults and simulated patients with hyperphenylalaninemia (HPA) or phenylketonuria (PKU) measured by Phesor and HPLC. Bias (red dotted line) and 95% limits of agreement (gray dotted lines) were shown. **(d)** Quantitative detection of L-phenylalanine in authentic dried blood spot samples from healthy children and children with phenylketonuria (PKU) using Phesor. All data shown are means $\pm$ s.d. ($n = 3$ independent experiments). (**Fig. 2j-m in the revised manuscript**)

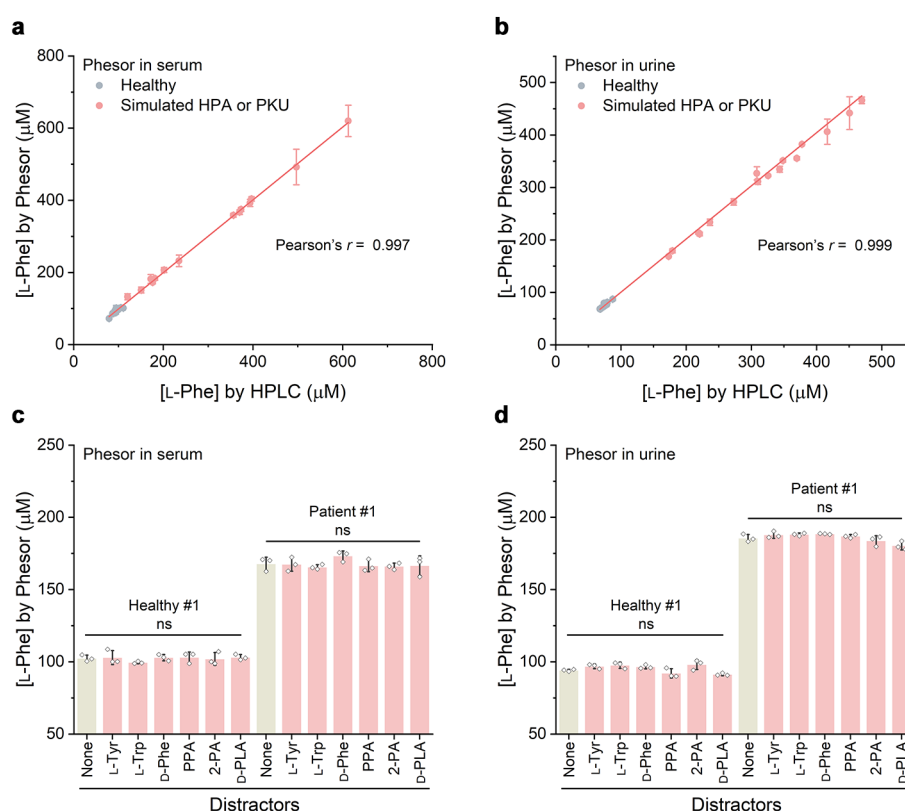


Figure 65 Analysis of the accuracy and anti-interference ability of Phesor for the quantitative detection of L-phenylalanine in serum and urine. **(a, b)** Correlation analysis between Phesor and HPLC for quantitative detection of L-phenylalanine in serum **(a)** and urine **(b)**. Quantitative results measured by Phesor were plotted against those obtained by HPLC. r , correlation coefficient. **(c, d)** Comparison of the quantitative results measured by Phesor and HPLC for L-phenylalanine in serum **(c)** and urine **(d)** in the presence of various distractors. ns, not significant.

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 = 3 independent experiments). ns, no significant difference ($P \geq 0.05$); one-way ANOVA test with Tukey's Multiple Comparison Test.

(Supplementary Fig. 13 in the revised manuscript)

In addition to L-phenylalanine, SAM is recognized as an important biomarker for evaluating human disease and health status. Elevated SAM levels in body fluids are highly associated with the occurrence of diseases including digestive cancer, lung cancer, and pneumonia. The limits of detection (LOD) of the SAM biosensor SAMsor was calculated to be 310 nM (Fig. 66), which exceeds the reported serum SAM concentrations in healthy individuals (~73.5–120 nM; *Metabolites*, 2022, 12:373).

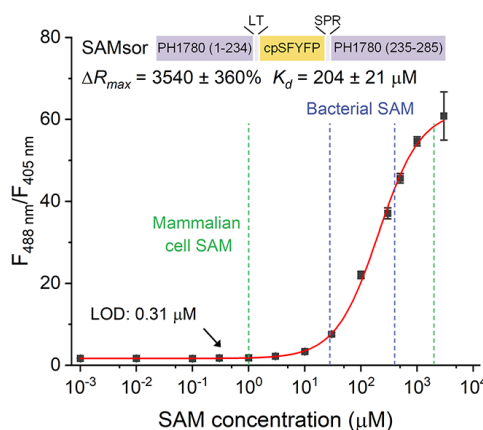


Figure 66 Dose-response curve of SAMsor for increasing concentrations of SAM. The structural composition of SAMsor and physiological concentrations of SAM in bacteria and mammalian cell were shown. All data shown are means $\pm$ s.d. (n = 3 independent experiments). **(Fig. 6c in the revised manuscript)**

In this study, the development of SAMsor involved screening for insertion sites of cpSFYFP within the biorecognition element PH1780 (yielding intermediate variant SAMsor-0.85, with cpSFYFP inserted between residues 234V/235I) (Fig. 67), followed by linker optimization. Fortunately, we identified that SAMsor-0.34, with cpSFYFP inserted between residues 111T/112V of PH1780, exhibited higher sensitivity to SAM (K_d value of ~0.9 μ M) compared to SAMsor-0.85 (K_d value of ~8.3 μ M) (Fig. 67c, d). Dose-response curve analysis indicated that SAMsor-0.34 achieved a ΔR_{max} of $80.0 \pm 4.4\%$, a low K_d of 900 ± 110 nM, and a low LOD of 144.10 nM for SAM (Fig. 67c and Fig. 68b). Subsequently, we conducted a

systematic optimization of the linker in SAMsor-0.34 and identified a more sensitive biosensor SAMsor_L with the N-terminal linker of “Leu-Glu” and the C-terminal linker of “Thr-Pro” from 2,715 mutants (Fig. 68c). SAMsor_L exhibited an increased ΔR_{max} of $128.5 \pm 1.1\%$, a lower K_d of 379 ± 41 nM, and a decreased LOD of 57.25 nM for SAM, making it suitable for SAM detection in human body fluids (Fig. 68d).

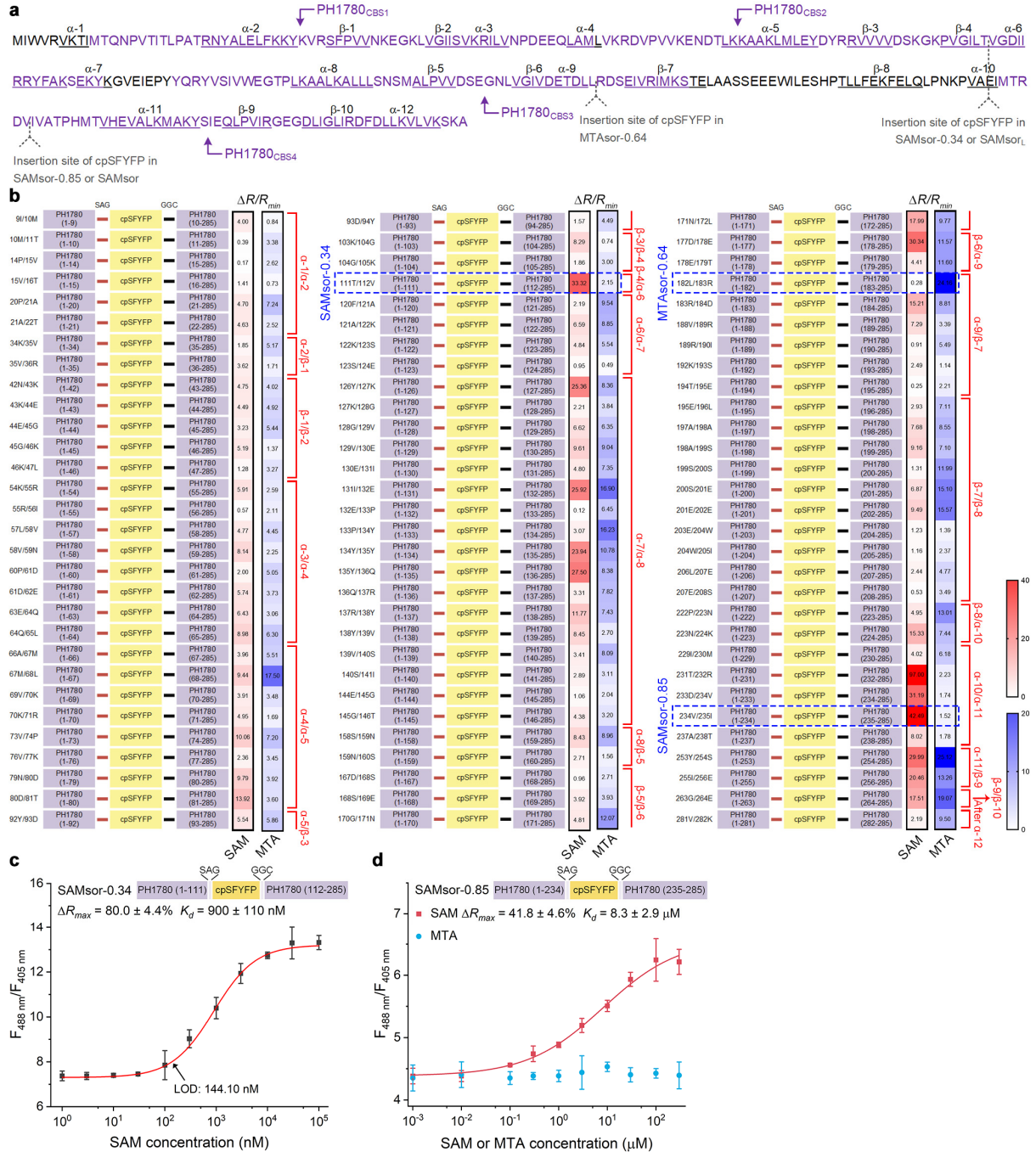


Figure 67 Construction of MTAsor-0.64, SAMsor-0.85 (precursor of SAMsor), and SAMsor-0.34 (precursor of SAMsor_L) based on insertion sites screening. **(a)** Schematic representations of the amino acid sequence and the predicted secondary structure of PH1780. The four CBS domains of PH1780 were shown

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 of the MTA biosensor MTAsor-0.64, SAM biosensor with high detection range SAMsor-0.85, and SAM biosensor with low detection range SAMsor-0.34 based on insertion sites screening. The response magnitudes ($\Delta R/R_{min}$) of different biosensor variants 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. **(c, d)** Dose-response curves of SAMsor-0.34 **(c)** and SAMsor-0.85 **(d)** for increasing concentrations of SAM. The low K_d value variant SAMsor-0.34 (precursor of SAMsor_L) together with the high K_d value variant SAMsor-0.85 (precursor of SAMsor) were submitted to further bioinformatics analysis. All data shown are means $\pm$ s.d. (n = 3 independent experiments). (**Fig. 5e, Fig. 7b, Supplementary Fig. 37a, and Supplementary Fig. 39 and in the revised manuscript**)

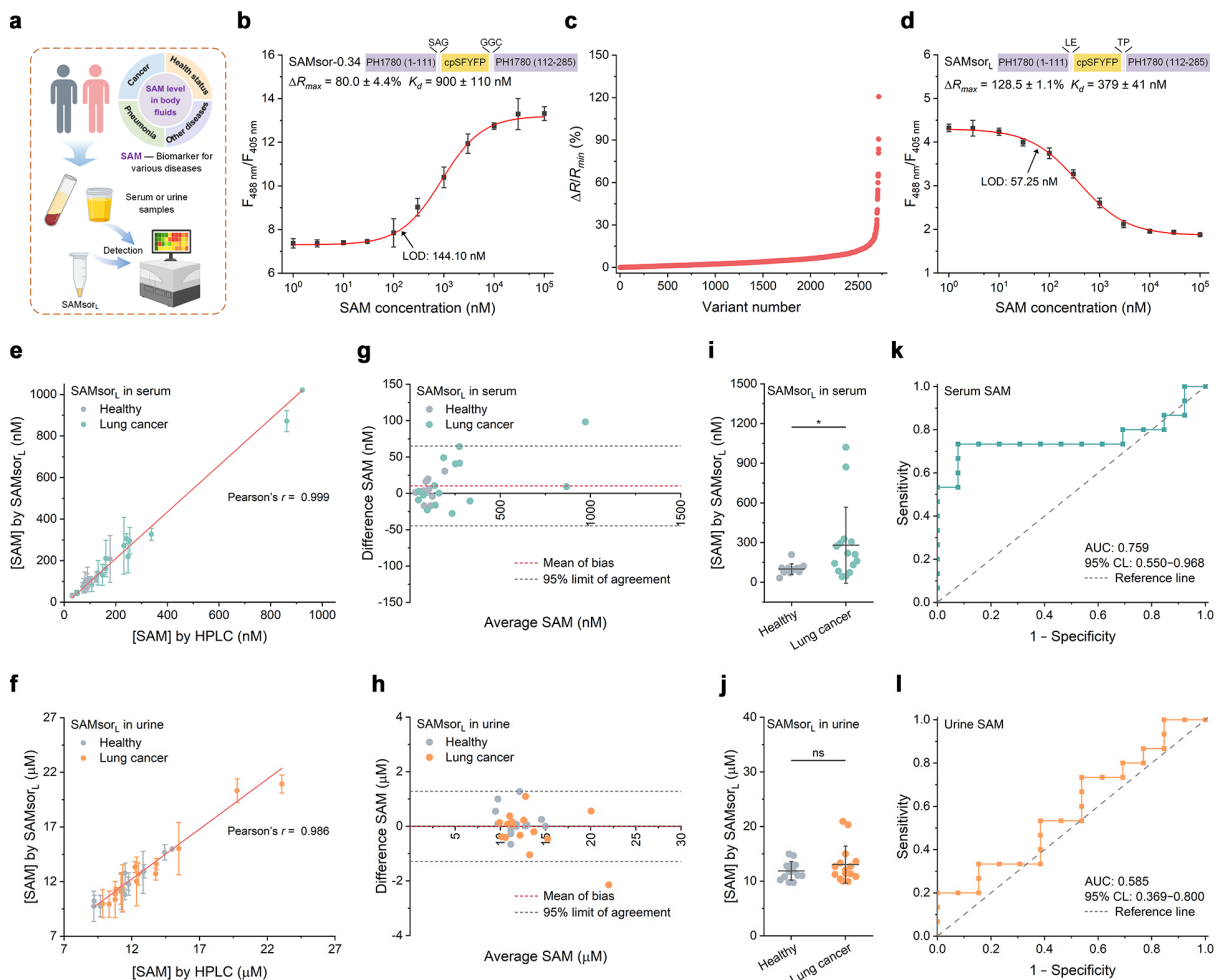


Figure 68 Optimization and application of the SAM biosensor SAMsor_L with low detection range. **(a)** Schematic representation of the workflow for quantitative detection of SAM in human serum and urine samples using SAMsor_L. **(b)** Dose-response curve of SAMsor-0.34 for increasing concentrations of SAM. The structural composition of SAMsor-0.34 was shown. **(c)** Optimization of SAMsor-0.34 by linker

random mutagenesis combined with high-throughput screening. The response magnitudes of crude extracts of 2,715 linker mutants were determined in the presence of 10 μM SAM. **(d)** Dose-response curve of SAMsor_L for increasing concentrations of SAM. The structural composition of SAMsor_L was shown. **(e, f)** Correlation analysis between SAMsor_L and HPLC for quantitative detection of SAM in serum **(e)** and urine **(f)** samples from healthy individuals and patients with lung cancer. Quantitative results measured by SAMsor_L were plotted against those obtained by HPLC. r , correlation coefficient. **(g, h)** Bland-Altman analysis for SAM in serum **(g)** and urine **(h)** samples from healthy individuals and patients with lung cancer measured by SAMsor_L and HPLC. Bias (red dotted line) and 95% limits of agreement (gray dotted lines) were shown. **(i, j)** Analysis of differences in serum **(i)** and urine **(j)** SAM levels between healthy individuals and patients with lung cancer measured by SAMsor_L. **(k, l)** ROC curves of patients with lung cancer compared with healthy individuals based on serum **(k)** or urine **(l)** SAM levels. The area under the curve (AUC) and 95% confidence level (CL) were shown. All data shown are means $\pm$ s.d. ($n = 3$ independent experiments). *, $P < 0.05$; ns, no significant difference ($P \geq 0.05$); two-tailed Student's t test. **(Fig. 7 in the revised manuscript)**

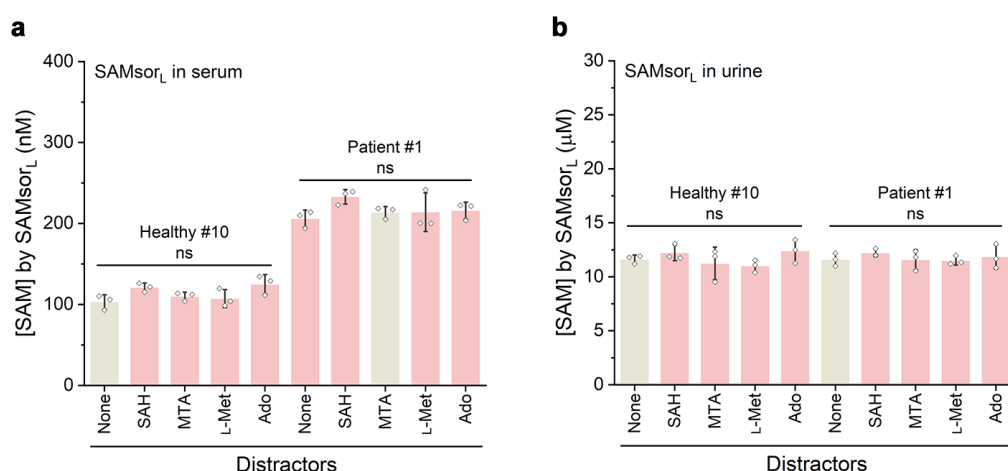


Figure 69 Analysis of the interference of different SAM analogs on the quantitative detection of SAM by SAMsor_L. **(a, b)** Comparison of the quantitative results of SAMsor_L for SAM in serum **(a)** and urine **(b)** from healthy individuals and patients with lung cancer in the absence or presence 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 $\pm$ s.d. ($n = 3$ independent experiments). ns, no significant difference ($P \geq 0.05$); one-way ANOVA test with Tukey's Multiple Comparison Test. **(Supplementary Fig. 48 in the revised manuscript)**

Next, we prepared authentic serum and urine samples from 13 healthy individuals and 15 patients with lung cancer. Quantification of SAM in these samples using SAMsor_L

exhibited good correlation and consistency with HPLC measurements, and the results were unaffected by the presence of different SAM analogs (**Fig. 68e-h and Fig. 69**). The measured SAM concentrations in serum and urine from healthy individuals were 100 ± 40 nM and 11.9 ± 1.7 μ M, respectively, consistent with previously reported values (~ 73.5 – 120 nM in serum and ~ 10.2 – 22.1 μ M in urine; *Metabolites*, 2022, 12:373; *Electrophoresis*, 2020, 41:209–214) (**Fig. 68i, j**). Notably, serum SAM levels were significantly elevated in lung cancer patients, approximately 2.81-fold higher than those in healthy individuals (**Fig. 68i**). However, no significant differences were observed in urine SAM levels between the two population groups (**Fig. 68j**). Receiver operating characteristic (ROC) analysis indicated that serum SAM could effectively differentiate patients with lung cancer from healthy subjects, with an area under the curve (AUC) of 0.759, while urine SAM could not (AUC = 0.585) (**Fig. 68k, l**). These results demonstrate that serum SAM has the potential to be a clinically valuable biomarker for the diagnosis and treatment of lung cancer. Furthermore, the SAM biosensor SAMsor_L is applicable for sensitive and precise detection of SAM in human body fluids.

Besides evaluating the performance of Phesor and SAMsor_L in clinical diagnostics, we have also assessed the applicability of Valsor and Ilesor for bioprocess monitoring in the revised manuscript. L-Valine and L-isoleucine are valuable compounds widely used in the food, cosmetic, and pharmaceutical industries, with a steadily increasing global market. Microbial fermentation serves as the primary source of branched-chain amino acids worldwide. We conducted fed-batch fermentation using the L-valine-producing strain *Klebsiella oxytoca* VKO-9 (*Bioresour. Technol.*, 2024, 395:130403) and the L-isoleucine-producing strains *Cupriavidus necator* CnIle6 and CnIle8 (*Bioresour. Technol.*, 2024, 398:130538), respectively. Authentic bacterial fermentation medium samples were collected at specific time points and measured through Valsor/Ilesor and HPLC. As shown in **Fig. 70a, b**, Valsor exhibited a high correlation and consistency with HPLC results in the quantitative detection of L-valine in fermentation medium samples. The presence of different distractors did not affect the quantitative results obtained using Valsor (**Fig. 70c**). Similarly, Ilesor also enabled precise quantification of L-isoleucine in these samples with no interference from different distractors (**Fig. 71**). These results indicate that Valsor and Ilesor are applicable for efficient and convenient bioprocess monitoring of L-valine and L-isoleucine fermentation.

Such biosensor-based high-throughput screening could accelerate the engineering of L-valine and L-isoleucine producing strains.

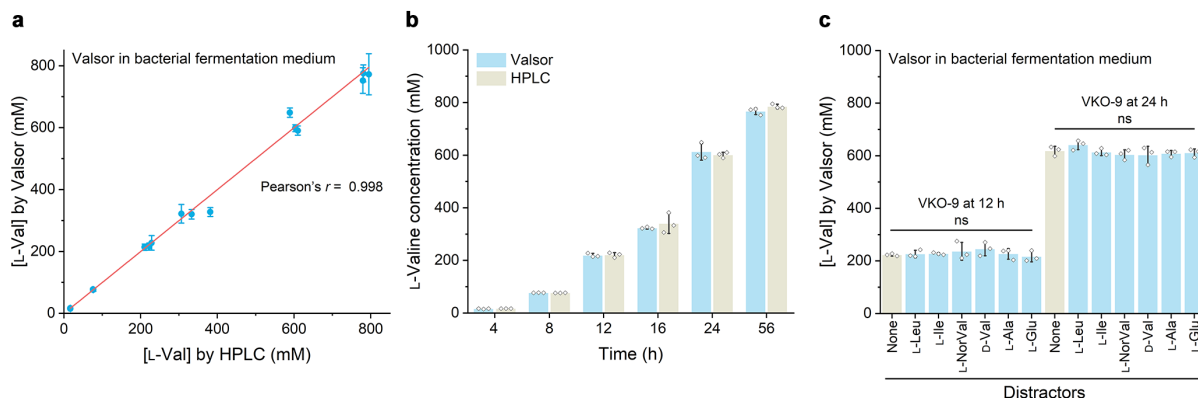


Figure 70 Application of Valsor for quantitative detection of L-valine in bacterial fermentation medium. **(a)** Correlation analysis **(a)** and consistency analysis **(b)** between Valsor and HPLC for quantitative detection of L-valine in bacterial fermentation medium. The samples were obtained from the fermentation medium of the VKO-9 strain. **(c)** Comparison of the quantitative results of Valsor for L-valine in bacterial fermentation medium in the absence or presence of 10 mM indicated L-valine analogs. All data shown are means $\pm$ s.d. ($n = 3$ independent experiments). ns, no significant difference ($P \geq 0.05$); one-way ANOVA test with Tukey's Multiple Comparison Test. (Fig. 3j-l in the revised manuscript)

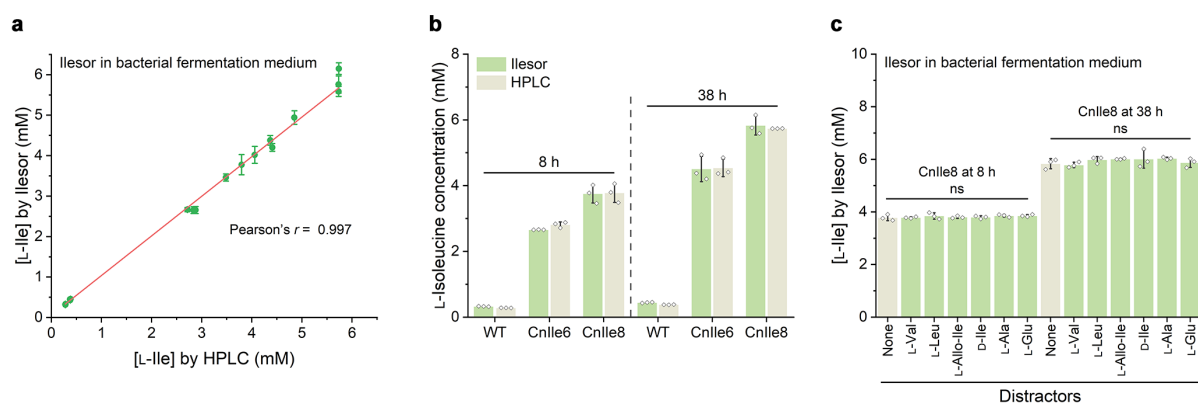


Figure 71 Application of Ilesor for quantitative detection of L-isoleucine in bacterial fermentation medium. **(a)** Correlation analysis **(a)** and consistency analysis **(b)** between Ilesor and HPLC for quantitative detection of L-isoleucine in bacterial fermentation medium. The samples were obtained from the fermentation medium of the *C. necator* H16 WT, CnIle6, and CnIle8 strains. **(c)** Comparison of the quantitative results of Ilesor for L-isoleucine in bacterial fermentation medium in the absence or presence of 10 mM indicated L-isoleucine analogs. All data shown are means $\pm$ s.d. ($n = 3$ independent experiments). ns, no significant difference ($P \geq 0.05$); one-way ANOVA test with Tukey's Multiple Comparison Test. (Fig. 4j-l in the revised manuscript)

Related data were presented in Fig. 2j-m, Fig. 3j-l, Fig. 4j-l, Fig. 5e, Fig. 6c, Fig. 7, Supplementary Fig. 13, Supplementary Fig. 37a, Supplementary Fig. 39,

Supplementary Fig. 48, and Supplementary Table 2 in the revised manuscript. Related descriptions were added in the “Results” section of the revised manuscript as follows:

For Phesor:

“L-Phenylalanine is a well-established biomarker for phenylketonuria²⁰. We collected 14 serum and 9 urine samples from healthy individuals, and prepared 14 serum and 16 urine samples from simulated patients with hyperphenylalaninemia or phenylketonuria by spiking various concentrations of L-phenylalanine (**Fig. 2j**). Quantification of L-phenylalanine in these samples using Phesor demonstrated good correlation and consistency with high-performance liquid chromatography (HPLC) measurements, and the results were unaffected by the presence of different L-phenylalanine analogs (**Fig. 2k, l and Supplementary Fig. 13**). The measured L-phenylalanine concentrations in serum and urine from healthy individuals were 72.55–102.17 μM and 68.30–87.06 μM , respectively, consistent with previously reported values (**Supplementary Table 2**). Furthermore, we collected authentic dried blood spot samples from 19 healthy children and 23 children with phenylketonuria. Blood L-phenylalanine concentrations measured using Phesor ranged from 31.10 to 119.91 μM in healthy subjects, while those in phenylketonuria patients ranged from 393.45 to 1,821.13 μM , higher than the clinical diagnostic cut-off value of 360 μM (**Fig. 2m**)²⁰. These results demonstrate that the GEFB Phesor based on the regulatory domain PheA_{ACT} in allosteric enzyme PheA is applicable for sensitive and precise detection of L-phenylalanine in human body fluids.”

For Valsor:

“L-Valine is a high-value bioactive compound, widely used in food, pharmaceutical, and feed industries²⁴. We conducted fed-batch fermentative production of L-valine using the *Klebsiella oxytoca* VKO-9 strain²⁴. As shown in **Fig. 3j, k**, Valsor exhibited a high correlation and consistency with HPLC results in the quantitative detection of L-valine in fermentation medium samples. The presence of different distractors did not affect the quantitative results obtained using Valsor (**Fig. 3l**). These results suggest that Valsor, developed based on the regulatory subunit IlvH in allosteric enzyme 2-acetolactate synthase, can serve as a reliable tool for precise L-valine detection both in living cells and *in vitro*.”

For Ilesor:

“In addition, we conducted fermentative production of L-isoleucine using *Cupriavidus necator* H16 and two L-isoleucine-producing strains, CnIle6 and CnIle8²⁷. Purified Ilesor enabled precise quantification of L-isoleucine in fermentation medium samples with no interference from different distractors (**Fig. 4j-l**). These results demonstrate that Ilesor, developed based on the regulatory domain CimA_{LA} in allosteric enzyme CimA, is applicable for sensitive and precise detection of L-isoleucine both in living cells and *in vitro*.”

For SAMsor_L:

“SAM is also recognized as an important biomarker for evaluating human disease and health status³⁶. Elevated SAM levels in body fluids are highly associated with the occurrence of diseases including digestive cancer, lung cancer, and pneumonia (**Fig. 7a**)^{37,38}. The limit of detection (LOD) of SAMsor for SAM was calculated to be 310 nM (**Fig. 6c**), which exceeds the reported serum SAM concentrations in healthy individuals (~73.5–120 nM) (**Supplementary Table 2**). Fortunately, we found that the 34th variant (denoted as SAMsor-0.34, with cpSFYFP inserted between residues 111T/112V of PH1780) exhibited higher sensitivity to SAM among the 90 insertion variants of the SAM biosensor (**Fig. 7b**, **Supplementary Fig. 37b**, and **Supplementary Fig. 39**). Dose-response curve analysis indicated that SAMsor-0.34 achieved a ΔR_{max} of $80.0 \pm 4.4\%$, a low K_d of 900 ± 110 nM, and a low LOD of 144.10 nM for SAM (**Fig. 7b**). Then, we conducted a systematic optimization of the linker in SAMsor-0.34 and identified a more sensitive biosensor SAMsor_L with the N-terminal linker of “Leu-Glu” and the C-terminal linker of “Thr-Pro” from 2,715 mutants (**Fig. 7c**). SAMsor_L exhibited an increased ΔR_{max} of $128.5 \pm 1.1\%$, a lower K_d of 379 ± 41 nM, and a decreased LOD of 57.25 nM for SAM (**Fig. 7d**).

We prepared authentic serum and urine samples from 13 healthy individuals and 15 patients with lung cancer. Quantification of SAM in these samples using SAMsor_L exhibited good correlation and consistency with HPLC measurements, and the results were unaffected by the presence of different SAM analogs (**Fig. 7e-h** and **Supplementary Fig. 48**). The measured SAM concentrations in serum and urine from healthy individuals were 100 ± 40 nM and 11.9 ± 1.7 μ M, respectively, consistent with previously reported values (**Fig. 7i, j** and **Supplementary Table 2**). Notably, serum SAM levels were significantly elevated in lung cancer patients, approximately 2.81-fold higher than those in healthy individuals (**Fig.**

7i). However, no significant differences were observed in urine SAM levels between the two population groups (**Fig. 7j**). Receiver operating characteristic (ROC) analysis indicated that serum SAM could effectively differentiate patients with lung cancer from healthy subjects, with an area under the curve (AUC) of 0.759, while urine SAM could not (AUC = 0.585) (**Fig. 7k, l**). These results demonstrate that GEFBs with adjustable affinities can be derived from the same allosteric regulatory domain. Furthermore, the SAM biosensor SAM_{sortL} is applicable for sensitive and precise detection of SAM in human body fluids.”

Related experimental methods were added in the “Methods” section and “Supplementary Methods” section of the revised manuscript as follows:

“Preparation of human body fluid samples and bacterial fermentation medium samples

Serum samples from healthy individuals and patients with lung cancer were obtained by collecting venous blood in clot activator tubes, clotting for 2 h at room temperature, and then centrifuging at $2,000 \times g$ for 30 min at 4 °C. The resulting supernatant was filtered through a 0.22 μm filter and stored at $-80\text{ }^{\circ}\text{C}$ until further analysis. Urine samples from healthy individuals and patients with lung cancer were collected in sterile centrifuge tubes, filtered through a 0.22 μm filter, and then stored at $-80\text{ }^{\circ}\text{C}$. Dried blood spot samples from healthy children and children with phenylketonuria were prepared by sorbing $\sim 25\text{--}75\text{ }\mu\text{L}$ of whole blood onto filter paper cards. To extract L-phenylalanine from the cards, the blood spot areas were intactly excised, divided into small fragments, and transferred into 500 μL Tris-HCl buffer (pH 7.4). The mixture was then sonicated for 20 min, vortexed for 2 min, and stirred overnight. The supernatant was separated by centrifugation at $12,000 \times g$ for 10 min, completely dried in a vacuum centrifuge (Concentrator Plus, Eppendorf, Germany) at 45°C , resuspended in an appropriate volume of Tris-HCl (pH 7.4), filtered through a 0.22 μm filter, and then stored at $-80\text{ }^{\circ}\text{C}$.

The L-valine-containing bacterial fermentation medium samples were generated by fed-batch fermentation of the *K. oxytoca* VKO-9 strain using glucose as the substrate²⁴. 1 mL samples were collected at specific time points, centrifuged at $4,000 \times g$ for 10 min at 4 °C, and then filtered through a 0.22 μm filter. The L-isoleucine-containing bacterial fermentation medium samples were generated by fed-batch fermentation of the *C. necator* H16 WT strain,

CnIle6 strain, and CnIle8 strain using glucose as the substrate²⁷. 1 mL samples were collected at specific time points, centrifuged at $4,000 \times g$ for 10 min at 4 °C, and then filtered through a 0.22 µm filter. The prepared bacterial fermentation medium samples were stored at -80 °C until further analysis.

Determination of L-phenylalanine, L-valine, L-isoleucine, and SAM in different biological samples by Phesor, Valsor, Ilesor, and SAMsor_L

For the determination of L-phenylalanine in human serum and urine using Phesor, various concentrations of L-phenylalanine were spiked into the prepared serum and urine samples from healthy individuals to simulate body fluids from patients with hyperphenylalaninaemia or phenylketonuria. Both authentic samples from healthy subjects and simulated samples were mixed with diluted Phesor in a black 384-well plate at a volume ratio of 1:1. After incubating for 10 min at room temperature, the fluorescence ratios of Phesor were determined. The absolute quantification of L-phenylalanine was achieved by substituting the measured Phesor fluorescence ratio into the formula of the dose-response curve established based on serum or urine standards with known concentrations of L-phenylalanine. Measurement and quantification of L-phenylalanine in authentic dried blood spot samples were performed using the same procedure.

For the determination of L-valine or L-isoleucine in bacterial fermentation medium samples, the prepared samples were diluted to an appropriate concentration using 50 mM Tris-HCl (pH 7.4). Then, 10 µL samples were mixed with 10 µL diluted Valsor or Ilesor in a black 384-well plate. After incubating for 20 min at room temperature, the fluorescence ratios of Valsor or Ilesor were determined. The absolute quantification of L-valine or L-isoleucine was achieved by substituting the measured fluorescence ratio of Valsor or Ilesor into the formula of the dose-response curves established based on fermentation medium standards with known concentrations of L-valine or L-isoleucine.

Measurement and quantification of SAM in authentic human serum and urine samples from healthy individuals and patients with lung cancer using SAMsor_L were performed using the same procedure as Phesor, with the urine samples required to be diluted to an appropriate concentration using 50 mM Tris-HCl (pH 7.4)."

"Determination of L-phenylalanine, L-valine, L-isoleucine, and SAM by HPLC

For the determination of L-phenylalanine in human body fluids using HPLC, the serum samples were mixed with methanol at a volume ratio of 1:1, vortexed for 2 min, centrifuged at $12,000 \times g$ for 15 min, and filtered through a $0.22 \mu\text{m}$ filter. The urine samples and dried blood spot samples after extraction were boiled at 105°C for 15 min, centrifuged at $12,000 \times g$ for 15 min, and filtered through a $0.22 \mu\text{m}$ filter. The prepared samples were then analyzed by an HPLC system (Agilent 1100 series, Agilent Technologies, Germany) equipped with a ZORBAX SB-C18 column ($5 \mu\text{m}$, $4.6 \text{ mm} \times 150 \text{ mm}$, Agilent Technologies, Germany) and a diode-array detector. The quantification was carried out using a $10 \mu\text{L}$ injection volume, 10% acetonitrile at a flow rate of 0.6 mL min^{-1} as mobile phase, a detection wavelength of 210 nm , and a total analysis time of 30 min.

For the determination of L-valine and L-isoleucine in bacterial fermentation medium using HPLC, the samples required pre-column derivatization by phenyl isothiocyanate (PITC)-acetonitrile and triethylamine (TEA)-acetonitrile. Briefly, $400 \mu\text{L}$ samples were mixed with $200 \mu\text{L}$ 0.1 M PITC-acetonitrile and $200 \mu\text{L}$ 1 M TEA-acetonitrile, and placed for 1 h at room temperature. Then, $800 \mu\text{L}$ *n*-hexane was added and the mixture was vortexed for 1 min. After being placed for 10 min at room temperature, the lower liquid layer was extracted and submitted to HPLC analysis after filtration through a $0.22 \mu\text{m}$ filter. The column and detector used for HPLC analysis were consistent with L-phenylalanine detection. The quantification was carried out using a gradient elution program consisting of mobile phase A (0.76% [wt/vol] NaAc and 7% [vol/vol] acetonitrile, pH 6.5) and mobile phase B (80% [vol/vol] acetonitrile) at a flow rate of 0.6 mL min^{-1} : 0–11 min, from 0% to 7% B; 11–13.9 min, from 7% to 12% B; 13.9–14 min, from 12% to 15% B; 14–29 min, from 15% to 34% B; 29–37 min, 100% B; 37–45 min, 0% B. The injection volume was $5 \mu\text{L}$, the column temperature was 40°C , and the detection wavelength was 254 nm .

For the determination of SAM in human body fluids using HPLC, the serum samples were mixed with methanol at a volume ratio of 1:1, vortexed for 2 min, centrifuged at $12,000 \times g$ for 15 min, and filtered through a $0.22 \mu\text{m}$ filter. The urine samples were boiled for 15 min at 105°C , centrifuged at $12,000 \times g$ for 15 min, and filtered through a $0.22 \mu\text{m}$ filter. The column and detector used for HPLC analysis were consistent with L-phenylalanine detection. The quantification was carried out using a gradient elution program consisting of mobile

phase A (8 mM octanesulfonic acid sodium salt and 50 mM NaH₂PO₄, adjusted to pH 3.0 with H₃PO₄) and mobile phase B (methanol) at a flow rate of 0.5 mL min⁻¹: 0–16 min, 20% B; 16–16.9 min, from 20% to 40% B; 16.9–42 min, 40% B; 42–42.9 min, from 40% to 20% B; 42.9–63 min, 20% B. The injection volume was 20 µL, the column temperature was 25 °C, and the detection wavelength was 254 nm.”

Other comments:

Fig. 1c, 2h, 3b, 4b, 5a, and 6h demonstrates the detailed metabolic pathways associated with these metabolites to be detected. However, these figures contain a large number of components that are irrelevant to the sensors, and their content is not the main findings of this work. I doubt whether it is necessary to retain these in the main figures.

Response: Thanks for your good suggestion. According to your advice, these metabolic pathway diagrams have been relocated to the “Supplementary Information” file and are now presented as **Supplementary Fig. 1, Supplementary Fig. 7, Supplementary Fig. 14, Supplementary Fig. 26, Supplementary Fig. 36, and Supplementary Fig. 42.**

The development and characterization are similar between different sensors, while some details are not clearly described such as the residues mutated in the evolution.

Response: Thanks for your good suggestion. We apologize for the lack of detailed descriptions regarding the specific mutations and development processes in the previous manuscript. In accordance with your advice, we have included four new supplementary figures in the revised manuscript to clearly illustrate the step-by-step development of Phesor, Valsor, Ilesor, SAMsor, and SAMsor_L. These figures explicitly depict the amino acid sequences of the final biosensors, their intermediate variants, and the original biorecognition elements, highlighting the key residues mutated during evolution (**Fig. 72, Fig. 73, Fig. 74, and Fig. 75**).

Taking Phesor as an example, we first inserted cpSFYFP between residues 365I/366T of the PheA_{ACT} domain with an N-terminal linker of “Ser-Ala-Gly” and a C-terminal linker of “Gly-Gly-Cys”, generating the intermediate variant Phesor-0.38. This construct was subsequently optimized through random linker mutagenesis, yielding the final biosensor

Phesor, which features an N-terminal linker of “Ala-Arg-Leu” and a C-terminal linker of “Gln-His-Leu” (Fig. 72).

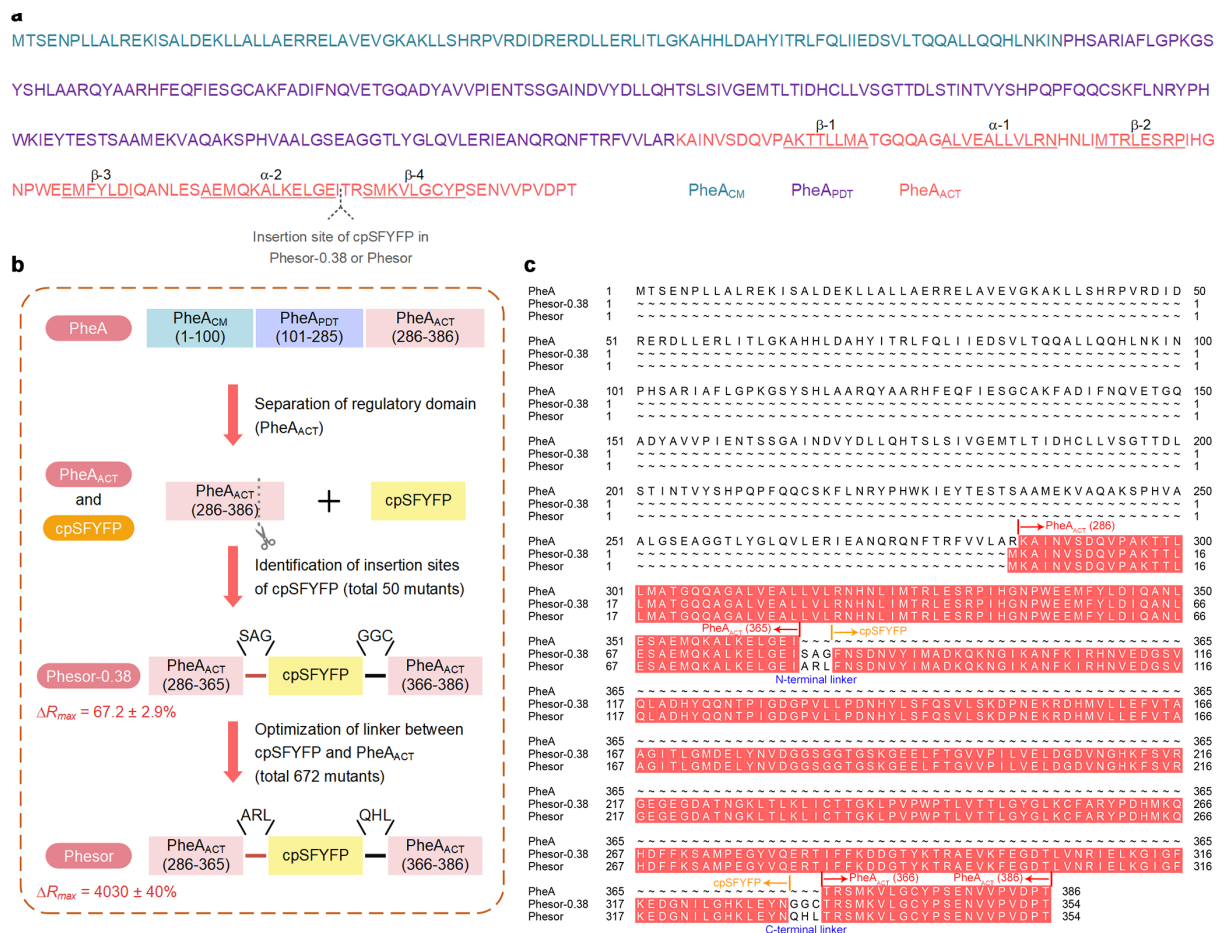


Figure 72 Workflow for the development of L-phenylalanine biosensor Phesor. **(a)** Schematic representations of the amino acid sequence and the predicted secondary structure of PheA. The chorismate mutase (PheA_{CM}) domain, prephenate dehydratase (PheA_{PDT}) domain, and ACT (PheA_{ACT}) domain of 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 residues 286K-365I/366T-386T of the PheA_{ACT} with an N-terminal linker of “Ala-Arg-Leu” and a C-terminal linker of “Gln-His-Leu”. **(c)** Sequence alignment between PheA, Phesor-0.38, and Phesor. The PheA_{ACT} region, the cpSFYFP region, and the N- and C-terminal linkers were indicated. (Supplementary Fig. 2 in the revised manuscript)

Similar strategies were applied to demonstrate the development processes for Valsor, Ilesor, SAMsor, and SAMsor_L (Fig. 73, Fig. 74, and Fig. 75).

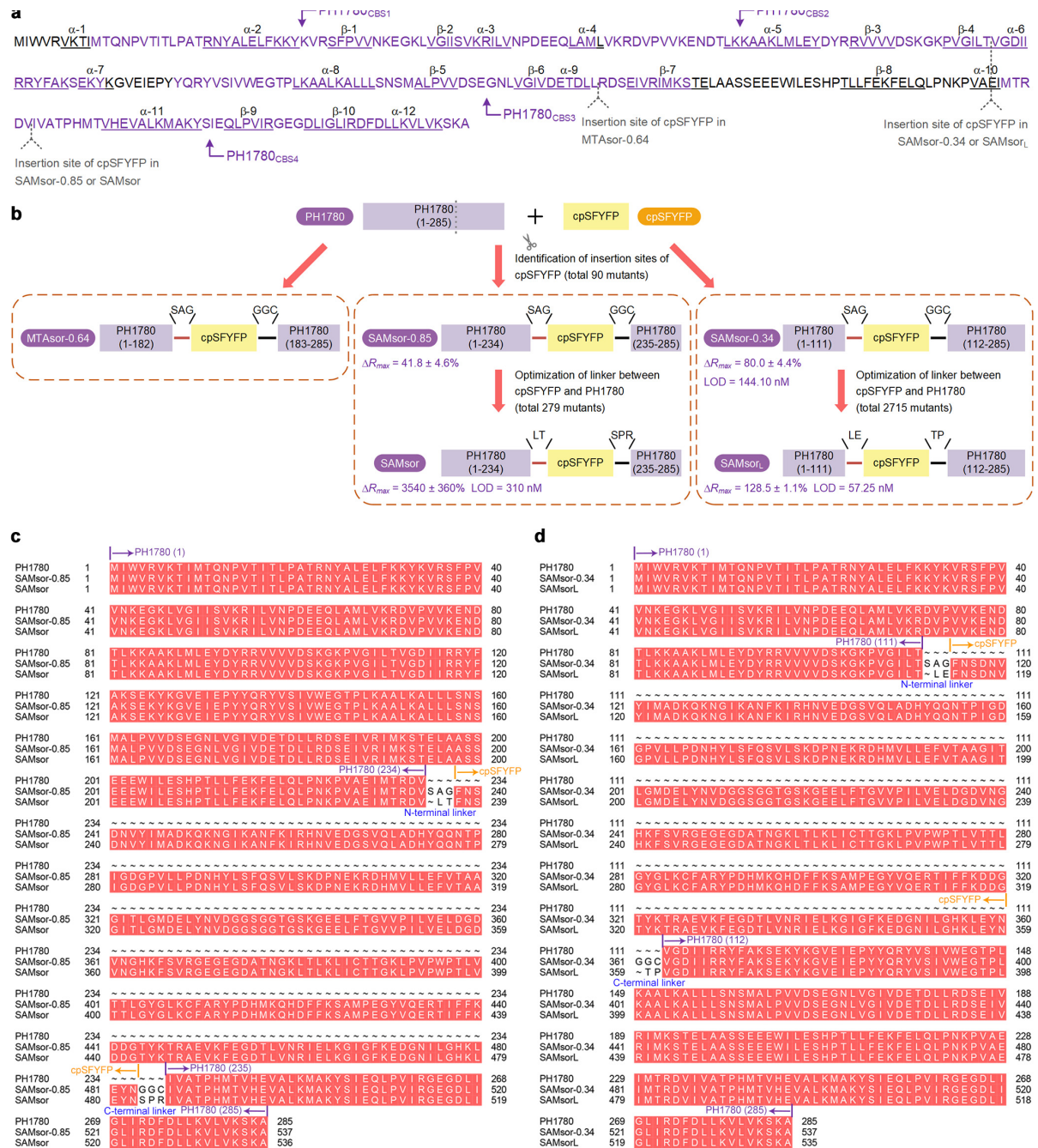


Figure 75 Workflow for the development of SAM biosensors SAMsor and SAMsor_L. **(a)** Schematic representations of the amino acid sequence and the predicted secondary structure of PH1780. The four CBS domains of PH1780 were shown in purple. The predicted α -helices and β -sheets of PH1780 were indicated 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 residues 1M-182L/183R-285A of the 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 residues 1M-234V/235I-285A of the 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 residues 1M-111T/112V-285A of the PH1780 with an N-terminal linker of “Leu-Glu” and a C-terminal linker of “Thr-Pro”. **(c)** Sequence alignment between PH1780, SAMsor-0.85, and SAMsor. The PH1780 region, the cpSFYFP region, and the N- and C-terminal linkers were indicated. **(d)** Sequence alignment between PH1780, SAMsor-0.34, and SAMsor_L. The PH1780 region, the cpSFYFP region, and the N- and C-terminal linkers were indicated. **(Supplementary Fig. 37 in the revised manuscript)**

Related data were presented in **Supplementary Fig. 2, Supplementary Fig. 15, Supplementary Fig. 27, and Supplementary Fig. 37** in the revised manuscript. In addition, detailed information regarding the key residue mutations and the optimization process for each biosensor has been elaborated in the “Results” section of the revised manuscript as follows:

For Phesor:

“Firstly, we constructed 50 biosensor variants by inserting cpSFYFP between amino acid residues located in the loop region of the PheA_{ACT} domain with an N-terminal linker of “Ser-Ala-Gly” and a C-terminal linker of “Gly-Gly-Cys” (**Supplementary Fig. 2 and Supplementary Fig. 4**). The 38th variant (denoted as Phesor-0.38, with cpSFYFP inserted between residues 365I/366T of the PheA_{ACT} domain) exhibited a higher maximum fluorescence ratio change (ΔR_{max}) of $67.2 \pm 2.9\%$ and an appropriate apparent dissociation constant (K_d) of $440 \pm 80 \mu\text{M}$, making it suitable for L-phenylalanine detection both in living cells and in human body fluids (**Fig. 2a and Supplementary Table 2**). Subsequently, we conducted random mutagenesis and high-throughput screening for linkers connecting the cpSFYFP and the PheA_{ACT} domain. Among a total of 672 mutants, the biosensor with the N-terminal linker of “Ala-Arg-Leu” and the C-terminal linker of “Gln-His-Leu” possessed the highest response magnitude to L-phenylalanine (**Fig. 2b and Supplementary Fig. 2b, c**). This biosensor was denoted as Phesor. Its amino acid sequence is provided in **Supplementary Table 3.**”

For Valsor:

“We constructed seven biosensor variants by inserting cpSFYFP into the following locations: the N-terminus or C-terminus of the intact IlvH, the middle of the two ACT domains of IlvH, the N-terminus or C-terminus of the separated IlvH_{ACT1} domain, or the N-

terminus or C-terminus of the separated IlvH_{ACT2} domain (**Supplementary Fig. 16**). The variant Valsor-0.03, created by inserting cpSFYFP into the middle of the two ACT domains of intact IlvH, possessed a higher ΔR_{max} of $109.2 \pm 2.1\%$ and an appropriate K_d of 414 ± 43 μ M for L-valine (**Fig. 3b**). Subsequently, we systematically optimized the linker in Valsor-0.03 and identified a high-response magnitude biosensor, Valsor, with the N-terminal linker of “Tyr-Thr” and the C-terminal linker of “Ala-Gly-Leu”, from 173 mutants (**Fig. 3c and Supplementary Fig. 15b, c**).”

For Ilesor:

“We constructed 36 biosensor variants by inserting cpSFYFP between amino acid residues located in the loop region of the Cim_{ALA} (**Supplementary Fig. 27a and Supplementary Fig. 28**). The 23rd variant (denoted as Ilesor-0.23, with cpSFYFP inserted between residues 463K/464T of the Cim_{ALA}) exhibited a higher ΔR_{max} of $97.0 \pm 2.4\%$ and an appropriate K_d of $1,700 \pm 660$ μ M for L-isoleucine (**Fig. 4b**). A high-response magnitude mutant with the N-terminal linker of “Phe-Arg-Thr” and the C-terminal linker of “Pro-His-Leu”, denoted as Ilesor, was then screened from 375 mutants through linker random mutagenesis (**Fig. 4c and Supplementary Fig. 27b, c**).”

For SAMsor:

“The 85th variant (denoted as SAMsor-0.85, with cpSFYFP inserted between residues 234V/235I of PH1780) showed high selectivity for SAM (**Fig. 5d**). Subsequently, we performed a systematic optimization of the SAM biosensor SAMsor-0.85 (**Supplementary Fig. 37b**). A high-response magnitude mutant with the N-terminal linker of “Leu-Thr” and the C-terminal linker of “Ser-Pro-Arg”, denoted as SAMsor, was screened from 279 mutants through linker random mutagenesis (**Fig. 6b and Supplementary Fig. 37b, c**).”

For SAMsor_L:

“Fortunately, we found that the 34th variant (denoted as SAMsor-0.34, with cpSFYFP inserted between residues 111T/112V of PH1780) exhibited higher sensitivity to SAM among the 90 insertion variants of the SAM biosensor (**Fig. 7b, Supplementary Fig. 37b, and Supplementary Fig. 39**). Dose-response curve analysis indicated that SAMsor-0.34 achieved a ΔR_{max} of $80.0 \pm 4.4\%$, a low K_d of 900 ± 110 nM, and a low LOD of 144.10 nM for SAM (**Fig. 7b**). Then, we conducted a systematic optimization of the linker in SAMsor-0.34 and

identified a more sensitive biosensor SAMsor_L with the N-terminal linker of “Leu-Glu” and the C-terminal linker of “Thr-Pro” from 2,715 mutants (**Fig. 7c**).”

We have also supplemented a new table in the revised manuscript to systematically summarize the detection properties (e.g., maximum fluorescence ratio change [ΔR_{max}], affinity [K_d], limit of detection [LOD], and oligomeric state) of the final biosensors (**Table 5**). We believe these revisions significantly improve the clarity and transparency of our biosensor development process and hope they meet your satisfaction.

Table 5 Detection properties of Phesor, Valsor, Ilesor, SAMsor, SAMsor_L, and cpSFYFP. (Supplementary Table 4 in the revised manuscript)

Properties	Phesor	Valsor	Ilesor	SAMsor	SAMsor _L	cpSFYFP
ΔR_{max}	4,030 $\pm$ 40%	875 $\pm$ 52%	1,325.3 $\pm$ 7.1%	3,540 $\pm$ 360%	128.5 $\pm$ 1.1%	/
K_d	1,760 $\pm$ 40 μ M	2,170 $\pm$ 110 μ M	3,620 $\pm$ 400 μ M	204 $\pm$ 21 μ M	0.379 $\pm$ 0.041 μ M	/
Hill slope	1.13 $\pm$ 0.03	1.03 $\pm$ 0.04	0.98 $\pm$ 0.05	1.15 $\pm$ 0.04	0.91 $\pm$ 0.07	/
LOD (<i>in vitro</i>) ^a	48.52 μ M	48.39 μ M	26.41 μ M	0.31 μ M	0.057 μ M	/
LOD (in human cells) ^a	67.39 μ M	75.17 μ M	47.93 μ M	0.45 μ M	/	/
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)	9.03	8.68	9.08	8.87	/	8.54
pK _a (with ligand) ^b	8.23	8.50	8.72	7.72	/	8.49 (L-phenylalanine) 8.49 (L-valine) 8.69 (L-isoleucine) 8.60 (SAM)

^aThe limit of detection (LOD) is calculated by interpolating the average background counts + (or -) 3 $\times$ standard deviation value (*Nat. Commun.*, 2021, 12:3619).

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

Related data were presented in **Supplementary Table 4** in the revised manuscript. In addition, descriptions related to the characterization of properties of the biosensors have also been more detailed in the revised manuscript as follows:

For Phesor:

“Purified Phesor existed as a dimer both in the absence and presence of L-phenylalanine (Supplementary Fig. 5a, b). It had two excitation peaks around 416 and 490 nm, with a single emission peak around 508 nm (405 nm excitation) or 516 nm (488 nm excitation) (Supplementary Fig. 5c, d). Upon binding with 1 mM L-phenylalanine, Phesor exhibited a 5.30-fold increase and 2.30-fold decrease in fluorescence when excited at 488 and 405 nm, respectively (Supplementary Fig. 5c, d). Dose-response curve analysis indicated that the detection range of Phesor encompasses the physiological concentrations of L-phenylalanine both in bacteria and in mammalian cells (Fig. 2c). Furthermore, it achieved a high ΔR_{max} ($4,030 \pm 40\%$), an appropriate K_d ($1,760 \pm 40 \mu\text{M}$; close to the value of $2.69 \pm 0.13 \text{ mM}$ determined by thermal shift assay [TSA]), and a Hill slope close to 1 for L-phenylalanine (Fig. 2c, Supplementary Fig. 5e, and Supplementary Table 4). As a ratiometric biosensor, Phesor displayed a robust response independent of its own concentration (Supplementary Fig. 5f). In addition, Phesor showed high specificity for L-phenylalanine, with no significant fluorescence changes in response to the other 19 amino acids, as well as D-phenylalanine, phenylpyruvate, 2-phenylacetamide, *N*-acetyl-L-phenylalanine, and D-phenyllactate (Supplementary Fig. 3 and Supplementary Fig. 5g). It also responded rapidly to variations in L-phenylalanine concentration within the assay system (Supplementary Fig. 5h, i). The increased fluorescence ratios induced by L-phenylalanine addition could return to basal levels upon the removal of L-phenylalanine from the system (Fig. 2d and Supplementary Fig. 5i). Consistent with other cpFP-based biosensors^{6,7}, the ratiometric response of Phesor was pH-dependent, with the pK_a varying from 9.03 to 8.23 upon L-phenylalanine binding (Supplementary Fig. 5j and Supplementary Table 4). Thus, an L-phenylalanine-unresponsive control biosensor with similar pH-sensitive properties, cpSFYFP, was introduced for parallel experiments (Supplementary Fig. 6a and Supplementary Table 4)⁷. As shown in Supplementary Fig. 5k, the fluorescence ratio of Phesor corrected by cpSFYFP remained stable between pH 5.0 and 8.2.”

For Valsor:

“Valsor existed as a dimer and had two excitation peaks around 426 and 504 nm, with a single emission peak around 509 nm (405 nm excitation) or 518 nm (488 nm excitation) (Supplementary Fig. 18a-d). Dose-response curve analysis indicated that Valsor achieved a wide detection range, with an increased ΔR_{max} ($875 \pm 52\%$), an appropriate K_d ($2,170 \pm 110 \mu\text{M}$; close to the value of $4.46 \pm 0.50 \text{ mM}$ determined by TSA), and a Hill slope close to 1 for L-valine (Fig. 3d, Supplementary Fig. 18e, and Supplementary Table 4). The pK_a value of Valsor was determined as 8.68, and decreased to 8.50 upon L-valine binding (Supplementary Table 4). In addition, Valsor demonstrated a robust response independent of its own concentration, high specificity, good reversibility, and correctable pH-stability from pH 5.0 to 10.0 for L-valine detection (Supplementary Fig. 17d-f, Supplementary Fig. 18f-j, and Supplementary Fig. 19a).”

For Ilesor:

“Consistent with the quaternary structure of CimA_{LA}²⁵, Ilesor exists as a mixture of dimers and tetramers both in the absence and presence of L-isoleucine (Supplementary Fig. 30a, b). It had two excitation peaks around 420 and 504 nm, with a single emission peak around 507 nm (405 nm excitation) or 517 nm (488 nm excitation) (Supplementary Fig. 30c, d). Dose-response curve analysis indicated that Ilesor achieved a suitable detection range, an increased ΔR_{max} ($1,325.3 \pm 7.1\%$), an appropriate K_d ($3,620 \pm 400 \mu\text{M}$; close to the value of $2.22 \pm 0.30 \text{ mM}$ determined by TSA), and a Hill slope close to 1 for L-isoleucine (Fig. 4d, Supplementary Fig. 30e, and Supplementary Table 4). The pK_a value of Ilesor was determined as 9.08, and decreased to 8.72 upon L-isoleucine binding (Supplementary Table 4). In addition, Ilesor demonstrated a robust response independent of its own concentration, high specificity, good reversibility, and correctable pH-stability from pH 5.0 to 8.2 for L-isoleucine detection (Supplementary Fig. 19a, Supplementary Fig. 29d-f, and Supplementary Fig. 30f-j).”

For SAMsor:

“SAMsor existed as a dimer and had two excitation peaks around 420 and 504 nm, with a single emission peak around 514 nm (405 nm excitation) or 518 nm (488 nm excitation) (Supplementary Fig. 40a-d). Dose-response curve analysis indicated that SAMsor achieved

an increased ΔR_{max} ($3,540 \pm 360\%$), an appropriate K_d ($204 \pm 21 \mu\text{M}$; close to the value of $0.295 \pm 0.061 \text{ mM}$ determined by TSA), a Hill slope close to 1, and a wide detection range suitable for detecting physiological concentrations of SAM in mammalian cells and bacteria (Fig. 6c, Supplementary Fig. 40e, and Supplementary Table 2). The pK_a value of SAMsor was determined as 8.87, and decreased to 7.72 upon SAM binding (Supplementary Table 4). SAMsor displayed a robust response independent of its own concentration (Supplementary Fig. 40f). None of the 20 amino acids, along with physiologic concentrations of MTA, SAH, or adenosine could induce fluorescence ratio changes of SAMsor (Fig. 6d and Supplementary Fig. 40g-j). In addition, SAMsor demonstrated good reversibility and correctable pH-stability from pH 5.0 to 10.0 for SAM detection (Supplementary Fig. 40k-m and Supplementary Fig. 41a).”

The on-kinetics of Phesor are better. Slow kinetics of Valsor and Ilesor

In Fig. 2g, 3h, 4h, and 6g, it seems that the on-kinetics of Phesor and SAMsor are good while those of Valsor and Ilesor are relatively slow. Moreover, the apparent K_d of Valsor and Ilesor are also higher (over 2 mM). Is it possible to discuss the reasons for such differences based on the inherent properties of the allosteric domain used for sensor development, thus provide theoretical bases for improving the kinetics and affinity of these sensors?

Response: Thanks for your good suggestion. As you correctly noted, in our previous manuscript, Valsor and Ilesor exhibited slower on-kinetics to exogenous gradient concentrations of L-valine and L-isoleucine when tested in toluene- and EDTA-permeabilized *E. coli* BL21(DE3). However, we observed significantly faster response kinetics when these biosensors were expressed in digitonin-permeabilized HEK293FT cells (Fig. 76). To investigate whether incomplete bacterial permeabilization caused this kinetic discrepancy, we extended the toluene/EDTA co-treatment duration from 12 h to 24 h. As shown in Fig. 77, Valsor responded rapidly to L-valine addition under the optimized conditions, while Ilesor still exhibited significant response retardation.

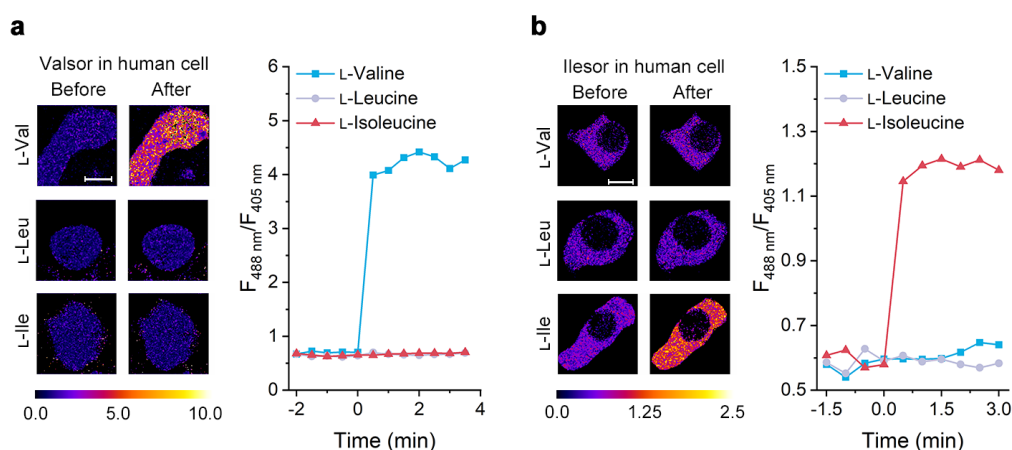


Figure 76 Analysis of response kinetics of Valsor and Ilesor in HEK293FT cells. **(a)** Ratiometric images and fluorescence ratios of permeabilized HEK293FT cells expressing Valsor before and after the addition of 15 mM L-valine, L-leucine, or L-isoleucine. Scale bar, 10 μm . **(b)** Ratiometric images and fluorescence ratios of permeabilized HEK293FT cells expressing Ilesor before and after the addition of 5 mM L-valine, L-leucine, or L-isoleucine. Scale bar, 10 μm . (Fig. 3g and Fig. 4g in the revised manuscript)

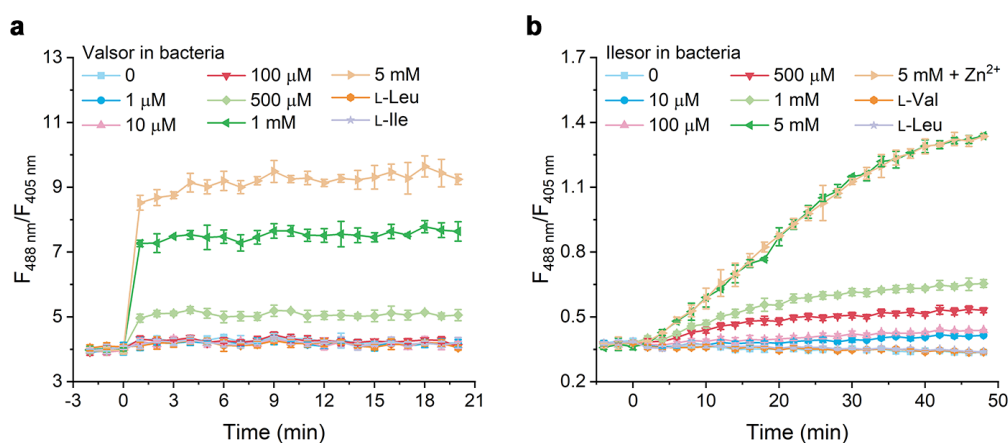


Figure 77 Analysis of response kinetics of Valsor and Ilesor in *E. coli* BL21(DE3). **(a)** Time course of the fluorescence ratio changes of Valsor expressed in permeabilized *E. coli* BL21(DE3) in response to exogenous gradient concentrations of L-valine, 500 μM L-leucine, or 500 μM L-isoleucine. **(b)** Time course of the fluorescence ratio changes of Ilesor expressed in permeabilized *E. coli* BL21(DE3) in response to exogenous gradient concentrations of L-isoleucine, 500 μM L-valine, or 500 μM L-leucine. 10 mM Zn^{2+} was used to facilitate the assembly of Ilesor tetramers. All data shown are means $\pm$ s.d. ($n \geq 3$ independent experiments). (Fig. 3e and Fig. 4e in the revised manuscript)

According to the suggestions of Reviewer #2 and Reviewer #4, we analyzed the oligomeric states of Phesor, Valsor, Ilesor, and SAMsor purified from *E. coli* BL21(DE3). As shown in Fig. 78, Phesor, Valsor, and SAMsor existed as dimers both in the absence and presence of their target ligands, consistent with the reported native quaternary structures of their biorecognition elements (*J. Biol. Chem.*, 2006, 281:33825–33829; *J. Biol. Chem.*, 1998,

273:6248–6253; *J. Mol. Biol.*, 2006, 357:951–963; *Arch. Biochem. Biophys.*, 2013, 540:70–81). In contrast, the Cim_{ALA} domain, the biorecognition element of Ilesor, was previously reported to initially assemble into a dimer, which can further oligomerize into a tetramer through Zn²⁺-mediated bridging (*Biochem. J.*, 2009, 421:133–143). As shown in **Fig. 78c**, purified Ilesor was found to exist as a mixture of dimers and tetramers rather than predominantly as a tetramer. The chelation of Zn²⁺ with 10 mM EDTA caused Ilesor to shift to a dimeric state. To investigate whether the slow on-kinetics of Ilesor in bacteria resulted from incomplete tetramer formation, we supplemented excess Zn²⁺ ions to the toluene and EDTA-permeabilized Ilesor-expressing *E. coli* BL21(DE3). As shown in **Fig. 77b**, Zn²⁺ addition failed to accelerate the response of Ilesor to exogenous L-isoleucine, indicating that the kinetic performance of Ilesor is independent of its quaternary structure.

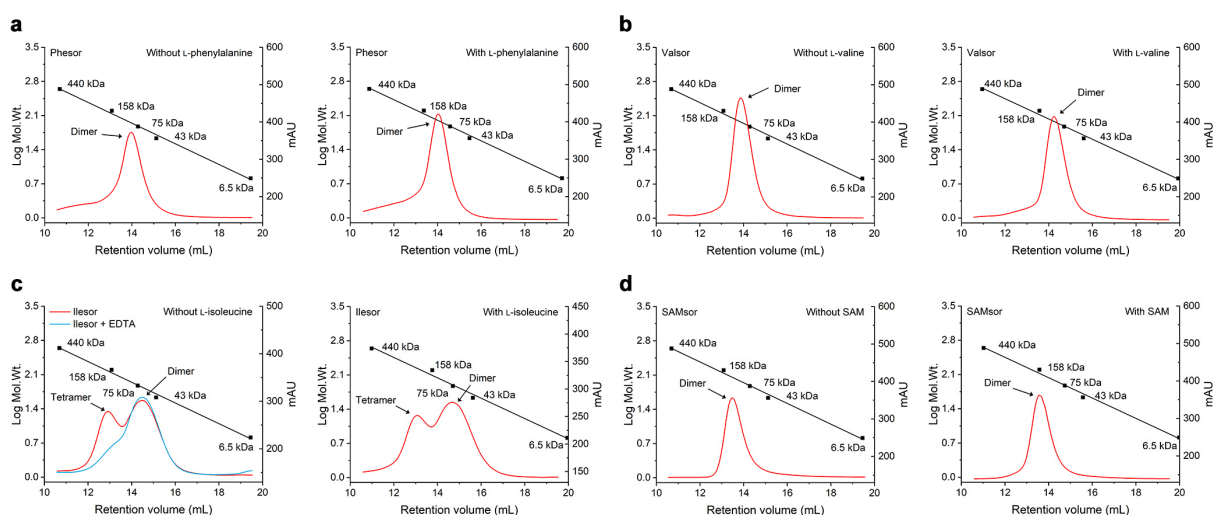


Figure 78 Characterization of Phesor, Valsor, Ilesor, and SAMsor *in vitro*. **(a–d)** Analysis of oligomeric state of Phesor **(a)**, Valsor **(b)**, Ilesor **(c)**, and SAMsor **(d)** in the absence or presence of 1 mM L-phenylalanine, 1 mM L-valine, 1 mM L-isoleucine, and 100 μ M SAM by gel filtration chromatography. 10 mM EDTA was added for chelating Zn²⁺ ions at the side-by-side dimer interface of the Ilesor tetramer. Red curve, chromatogram of purified Phesor; black line, standard curve for protein molecular mass standards. (Supplementary Fig. 5a, b, Supplementary Fig. 18a, b, Supplementary Fig. 30a, b, and Supplementary Fig. 40a, b in the revised manuscript)

Biosensors expressed in different biological environments may exhibit distinct properties (*Methods Mol. Biol.*, 2014, 1071:29–47; *Chem. Soc. Rev.*, 2023, 52:1189–1214; *Chembiochem*, 2025, 26:e202401055), such as response magnitude and binding affinity, which can be partially attributed to variations in protein folding and maturation across

different expression systems (*J. Cell Biochem. Suppl.*, 2001, 36:117–28; *J. Mol. Biol.*, 2005, 353:397–409; *Biochemistry*, 2007, 46:3775–3783). For example, GFP and its variants have been demonstrated to follow distinct folding trajectories in bacteria and mammalian cells, resulting in differences in structural stability (*J. Cell Biochem. Suppl.*, 2001). Furthermore, previous studies have demonstrated that the folding and stability of biosensors are closely associated with their fluorescence intensity and response kinetics. For example, the inefficient folding of the voltage biosensor VSFP-cpmKate(180) directly impaired the signal transduction between its biorecognition element and fluorescent reporter element, leading to reduced response magnitude and slow kinetics (*Front. Neuroeng.*, 2009, 2:14; *Biotechnol. J.*, 2025, 20:e70071). We speculate that the slow on-kinetics of Ilesor in bacterial environments may result from impaired folding and stability of its monomer. The application of Ilesor for real-time L-isoleucine monitoring in bacterial systems may require specific optimization strategies, such as the introduction of additional superfolder mutations (*Nat. Methods*, 2018, 15:936–939; *Nat. Methods*, 2022, 19:1612–1621) or rational binding site design (*Nat. Methods*, 2019, 16:649–657; *Chem. Rev.*, 2024, 124:12573–12660). Ilesor exhibits rapid response kinetics in mammalian cells. Addressing its bacterial-specific folding limitations would require extensive engineering efforts, which are difficult for us at this stage and may be beyond the scope of this methodological study. Consequently, we have acknowledged this limitation and added a brief discussion in the “Results” section of the revised manuscript as follows:

“Ilesor expressed in permeabilized *E. coli* BL21 (DE3) specifically responded to the exogenous L-isoleucine addition in a dose-dependent manner (**Fig. 4e and Supplementary Fig. 19b**). Notably, Ilesor exhibited slow on-kinetics that were independent of its oligomeric state, potentially due to impaired folding or maturation in bacterial environments. Additional optimizations, such as introducing superfolder mutations or engineering binding sites⁴, may be required to improve the temporal resolution of Ilesor in bacteria. Based on the Ilesor-supported bacterial endogenous L-isoleucine assay, we confirmed that LivJ, but not LivK, functions in L-isoleucine uptake of *E. coli* BL21(DE3) (**Fig. 4f and Supplementary Fig. 19d**).”

Related data were presented in **Fig. 3e, g, Fig. 4e, g, Supplementary Fig. 5a, b,**

Supplementary Fig. 18a, b, Supplementary Fig. 30a, b, and Supplementary Fig. 40a, b in the revised manuscript.

In addition, we would like to note that the K_d value of allosteric regulatory domain-based GEFBs can be strategically modulated by selecting the insertion site of cpSFYFP within the biorecognition element. All of the K_d values of Phesor (~1.66 mM), Valsor (~2.45 mM), Ilesor (~3.62 mM), and SAMsor (~204 μ M) were rationally designed to match the physiological concentrations of their target ligands in mammalian cells (~0.84 mM for L-phenylalanine, ~1.51 mM for L-valine, ~1.76 mM for L-isoleucine, and ~354 μ M for SAM; *Nat. Chem. Biol.*, 2016, 12:482–489; *Commun. Biol.*, 2022, 5:313). Taking Ilesor as an example, its development involved screening insertion sites of cpSFYFP within CimA_{LA}, which yielded intermediate variant Ilesor-0.23, followed by linker optimization (**Fig. 74**). Notably, multiple biosensor variants with a wide range of affinities (K_d from ~59 to ~17,000 μ M) were identified during the insertion site screening (**Fig. 79**). For example, Ilesor-0.17, with cpSFYFP inserted between residues 457R/458I of CimA_{LA}, exhibited the lowest K_d value (~59 μ M) (**Fig. 79e**). In contrast, Ilesor-0.23, with cpSFYFP inserted between residues 463K/464T, exhibited a relatively higher K_d value (~1,700 μ M) (**Fig. 79i**). Due to its suitable affinity for detecting physiological levels of L-isoleucine, Ilesor-0.23 was then selected for further linker optimization.

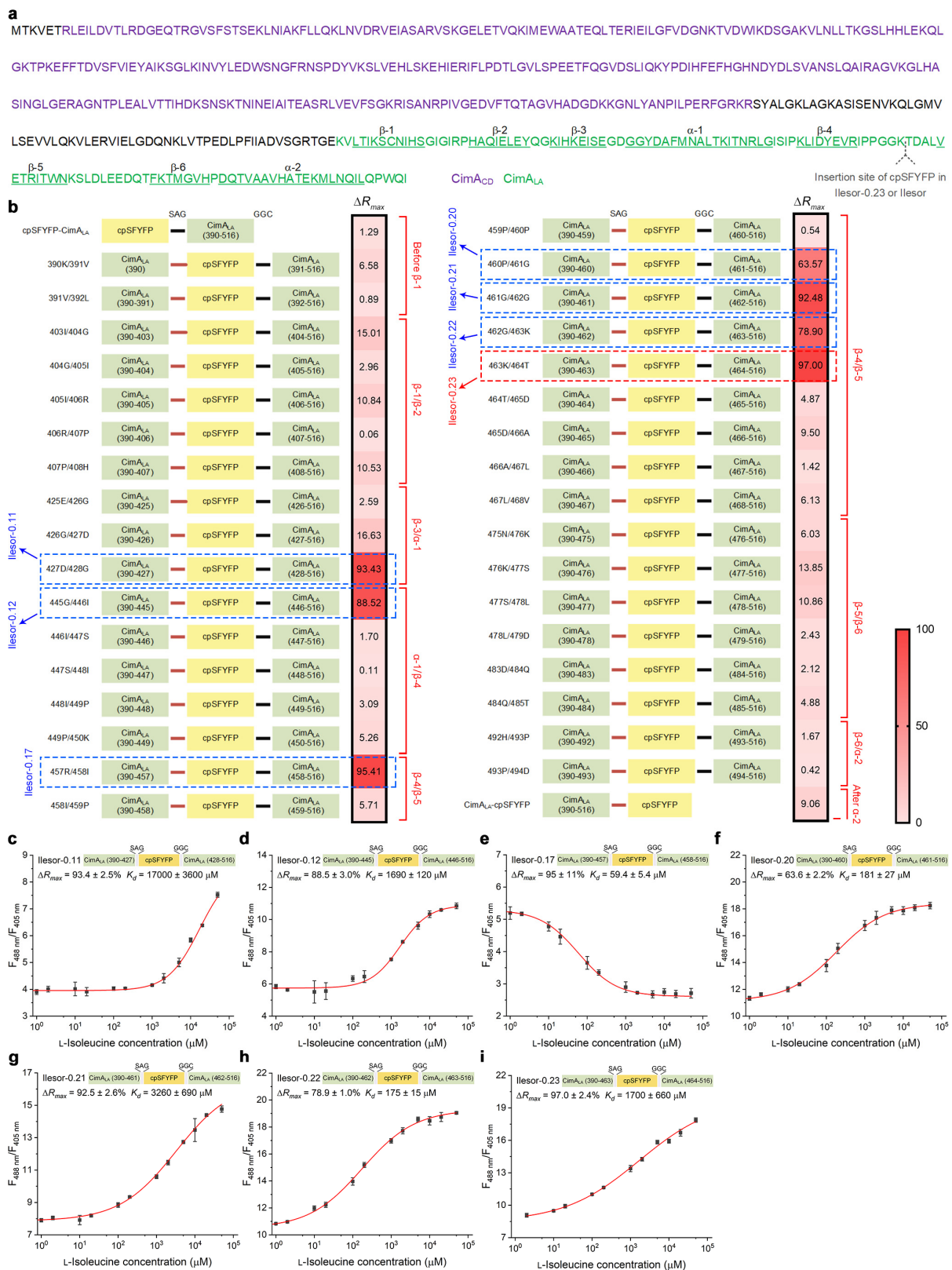


Figure 79 Construction of Ilesor-0.23 (precursor of Ilesor) by insertion sites screening. **(a)** Schematic representations of the amino acid sequence and the predicted secondary structure of CimA. The catalytic (CimA_{CD}) domain and LeuA regulatory (CimA_{LA}) domain of CimA were shown in purple and 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 of the L-isoleucine biosensor Ilesor-0.23 based on insertion sites screening. The ΔR_{max} of different biosensor variants to L-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. **(c-i)** Dose-response curves of Ilesor-0.11 **(c)**, Ilesor-0.12 **(d)**, Ilesor-0.17 **(e)**, Ilesor-0.20 **(f)**, Ilesor-0.21 **(g)**, Ilesor-0.22 **(h)**, and Ilesor-0.23 **(i)** for increasing concentrations of L-isoleucine. The low K_d value variant Ilesor-0.17 together with the high K_d value variant Ilesor-0.23 (precursor of Ilesor) were submitted to further bioinformatics analysis. All data shown are means $\pm$ s.d. ($n = 3$ independent experiments) **(Fig. 4b, Supplementary Fig. 27a, and Supplementary Fig. 28 in the revised manuscript)**

Consistent with the observations in Ilesor, multiple insertion variants spanning a range of K_d values were also obtained during the development of Phesor (K_d from ~ 57 to $\sim 3,830$ μM), Valsor (K_d from ~ 35 to ~ 414 μM), and SAMsor (K_d from ~ 0.9 to ~ 8.3 μM) **(Fig. 67, Fig. 80, and Fig. 81)**. From these variants, Phesor-0.38 (K_d of ~ 440 μM), Valsor-0.03 (K_d of ~ 414 μM), and SAMsor-0.85 (K_d of ~ 8.3 μM) with moderate K_d values were selected for further linker optimization **(Fig. 67d, Fig. 80e, and Fig. 81e)**. Notably, as detailed in our response to the previous Comment, we developed another SAM biosensor with a low detection range, SAMsor_L, by optimizing the linker of the low K_d -value variant SAMsor-0.34. Similarly, other biosensors with tailored affinities can also be strategically designed by adjusting cpSFYFP insertion sites.

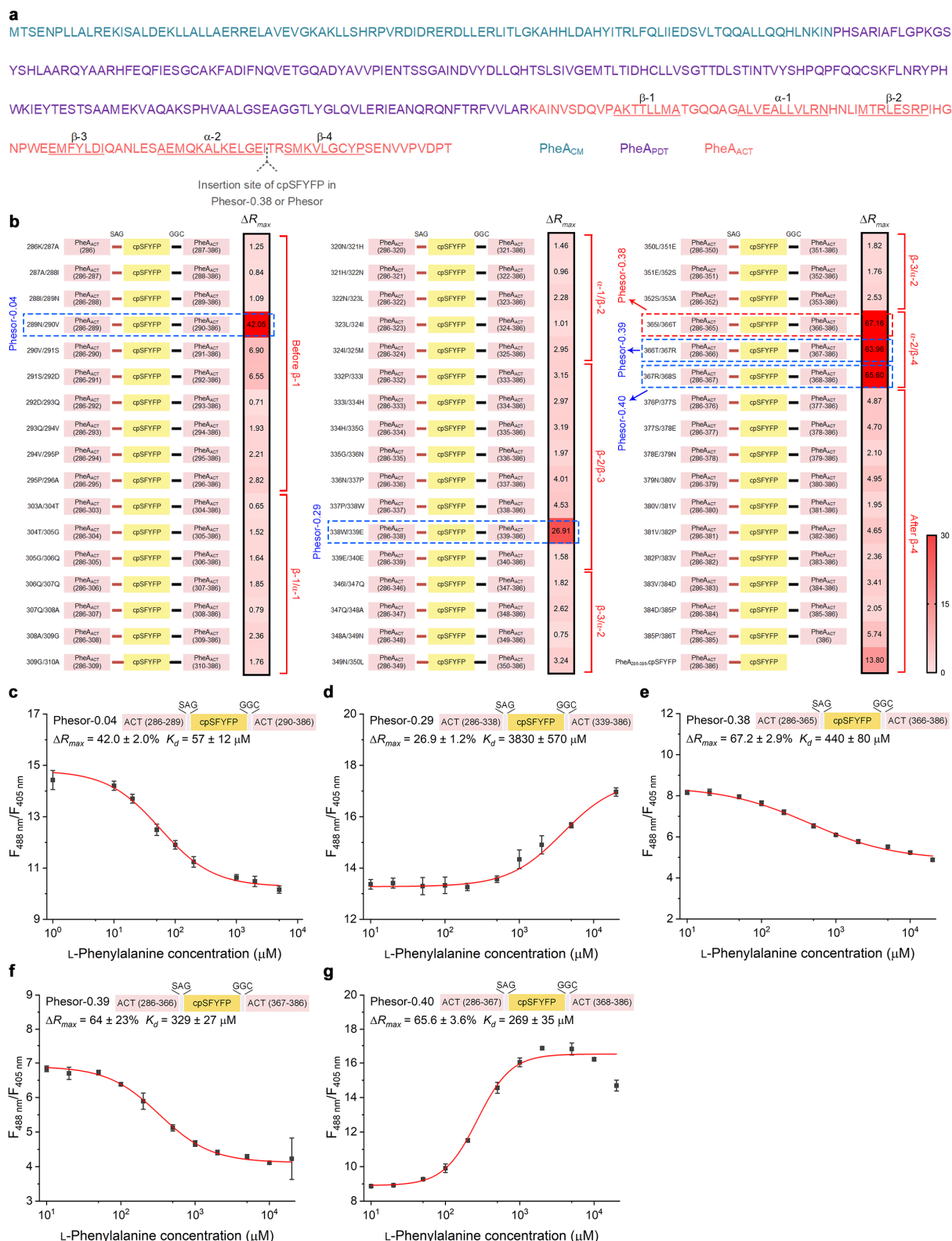


Figure 80 Construction of Phesor-0.38 (precursor of Phesor) by insertion sites screening. **(a)** Schematic representations of the amino acid sequence and the predicted secondary structure of PheA. The chorismate mutase (PheA_{CM}) domain, prephenate dehydratase (PheA_{PD}) domain, and ACT (PheA_{ACT}) 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 of the L-phenylalanine biosensor Phesor-0.38 based on insertion sites screening. The ΔR_{max} of different biosensor variants 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. **(c-g)** Dose-response curves of Phesor-0.04 **(c)**, Phesor-0.29 **(d)**, Phesor-0.38 **(e)**, Phesor-0.39 **(f)**, and Phesor-0.40 **(g)** for increasing concentrations of L-phenylalanine. The low K_d value variant Phesor-0.04 together with the high K_d value variant Phesor-0.38 (precursor of Phesor) were submitted to further bioinformatics analysis. All data shown are means $\pm$ s.d. ($n \geq 3$ independent experiments). **(Fig. 2a, Supplementary Fig. 2a, and Supplementary Fig. 4 in the revised manuscript)**

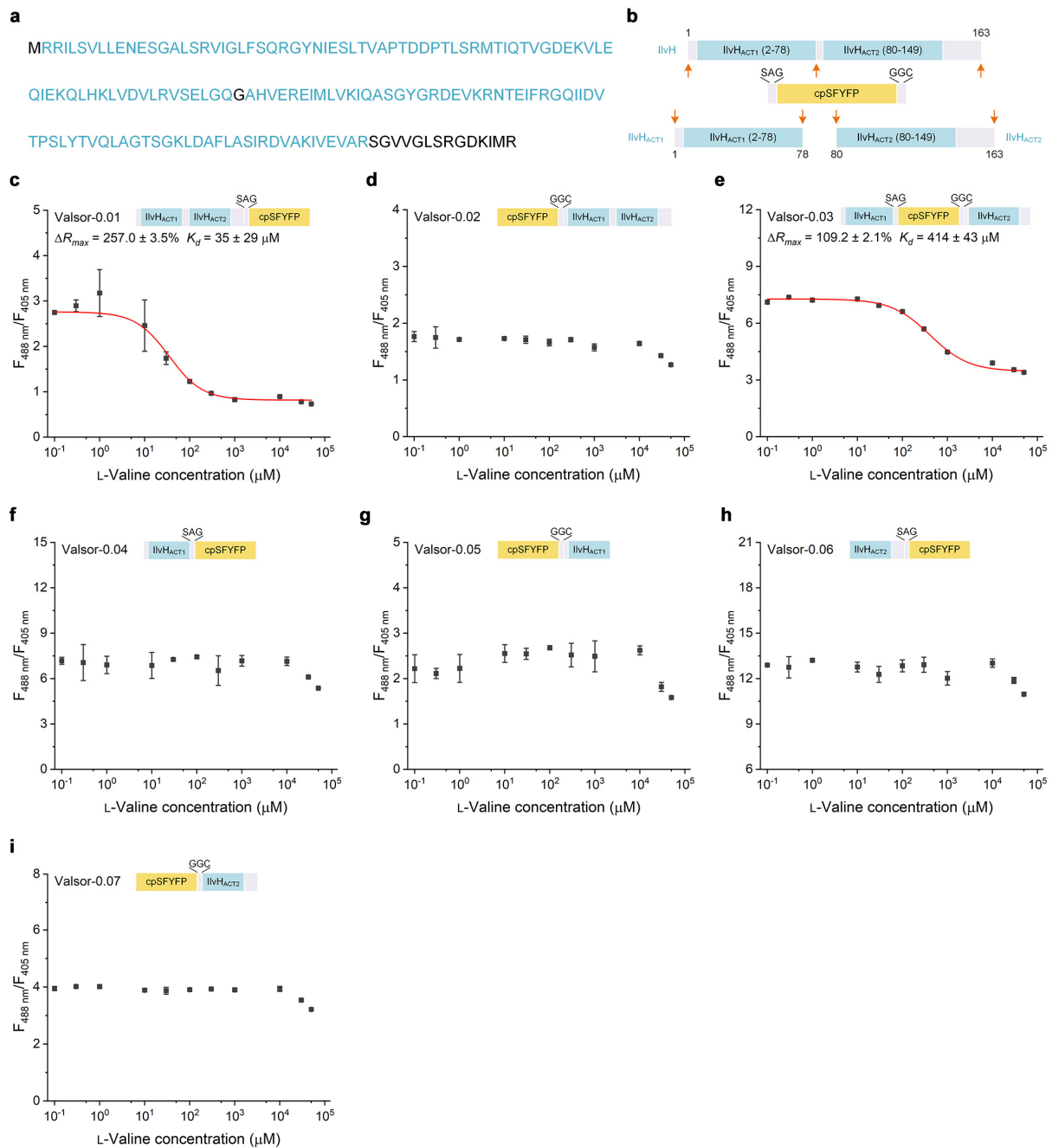


Figure 81 Construction of Valsor-0.03 (precursor of Valsor) by insertion sites screening. **(a)** Schematic representation of the amino acid sequence of IlvH. The two ACT domains of IlvH were shown in cyan. **(b)** Schematic representation of the construction of the initial seven L-valine biosensor insertion variants. **(c-i)** Dose-response curves of Valsor-0.01 **(c)**, Valsor-0.02 **(d)**, Valsor-0.03 **(e)**, Valsor-0.04 **(f)**, Valsor-0.05 **(g)**, Valsor-0.06 **(h)**, and Valsor-0.07 **(i)** for increasing concentrations of L-valine. The low K_d value variant Valsor-0.01 together with the high K_d value variant Valsor-0.03 (precursor of Valsor) were submitted to further bioinformatics analysis. All data shown are means $\pm$ s.d. ($n = 3$ independent experiments). **(Fig. 3b and Supplementary Fig. 16 in the revised manuscript)**

In addition, we investigated the structural basis for the affinity differences among Ilesor variants. The reported native dissociation constant of CimA_{LA} for L-isoleucine is $\sim 19 \mu\text{M}$ (*Biochem. J.*, 2009, 421:133–143), which is close to the K_d value of Ilesor-0.17 ($\sim 59.4 \mu\text{M}$) but significantly lower than that of Ilesor-0.23 ($\sim 1,700 \mu\text{M}$) **(Fig. 82)**. We speculated that the distinct cpSFYFP insertion sites caused varying structural perturbations in the biorecognition elements, which may account for the observed affinity differences.

Then, we performed homology modeling using AlphaFold 3 for CimA, Ilesor-0.17, and Ilesor-0.23, and compared the structural similarity of their CimA_{LA} domains. As shown in **Fig. 82**, a higher structural similarity, indicated by a lower root mean square deviation (RMSD) of **0.150 Å**, was observed between the CimA_{LA} domains of Ilesor-0.17 and CimA compared to that between Ilesor-0.23 and CimA (RMSD of **0.179 Å**). This result suggests that the structure of CimA_{LA} in Ilesor-0.17 more closely resembles its native conformation, providing a structural explanation for its higher affinity.

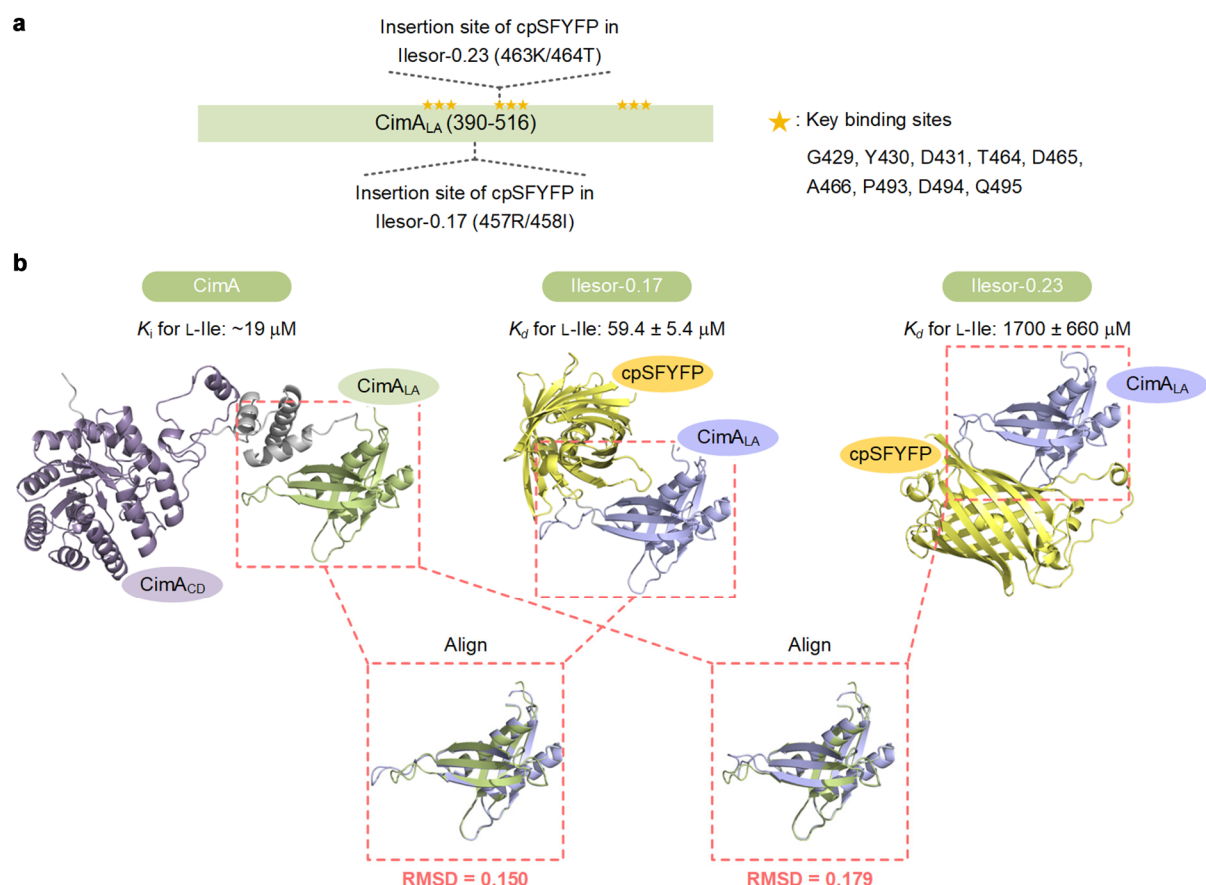


Figure 82 Analysis of the effect of cpSFYFP insertion sites on the affinity of the resulting biosensor variants. **(a)** Schematic representation of the cpSFYFP insertion sites in the CimA_{LA} domain of Ilesor-0.17 (a low- K_d L-isoleucine biosensor variant) and Ilesor-0.23 (a high- K_d variant). Key L-isoleucine binding sites were shown. **(b)** Structure 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. (**Supplementary Fig. 49e, f in the revised manuscript**)

Next, we performed similar analyses for the other three biosensors. As shown in **Fig. 83**, **Fig. 84**, and **Fig. 85**, in low- K_d variants, the insertion site of cpSFYFP was consistently positioned distal to the ligand-binding sites. The allosteric regulatory domains in these variants retained higher structural similarity (lower RMSD) to their native conformations.

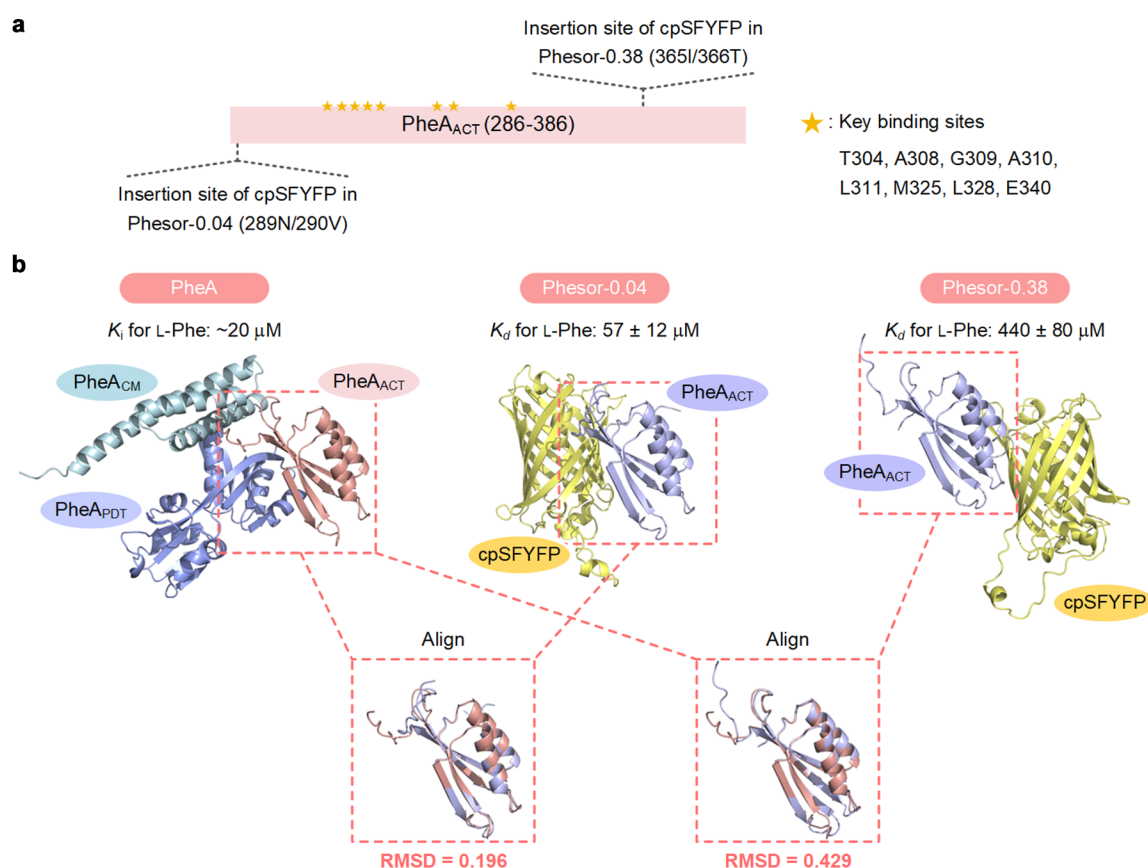


Figure 83 Analysis of the effect of cpSFYFP insertion sites on the affinity of the resulting biosensor variants. **(a)** Schematic representation of the cpSFYFP insertion sites in the PheA_{ACT} domain of Phesor-0.04 (a low- K_d L-phenylalanine biosensor variant) and Phesor-0.38 (a high- K_d variant). Key L-phenylalanine binding sites were shown. **(b)** Structure 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. (Supplementary Fig. 49a, b in the revised manuscript)

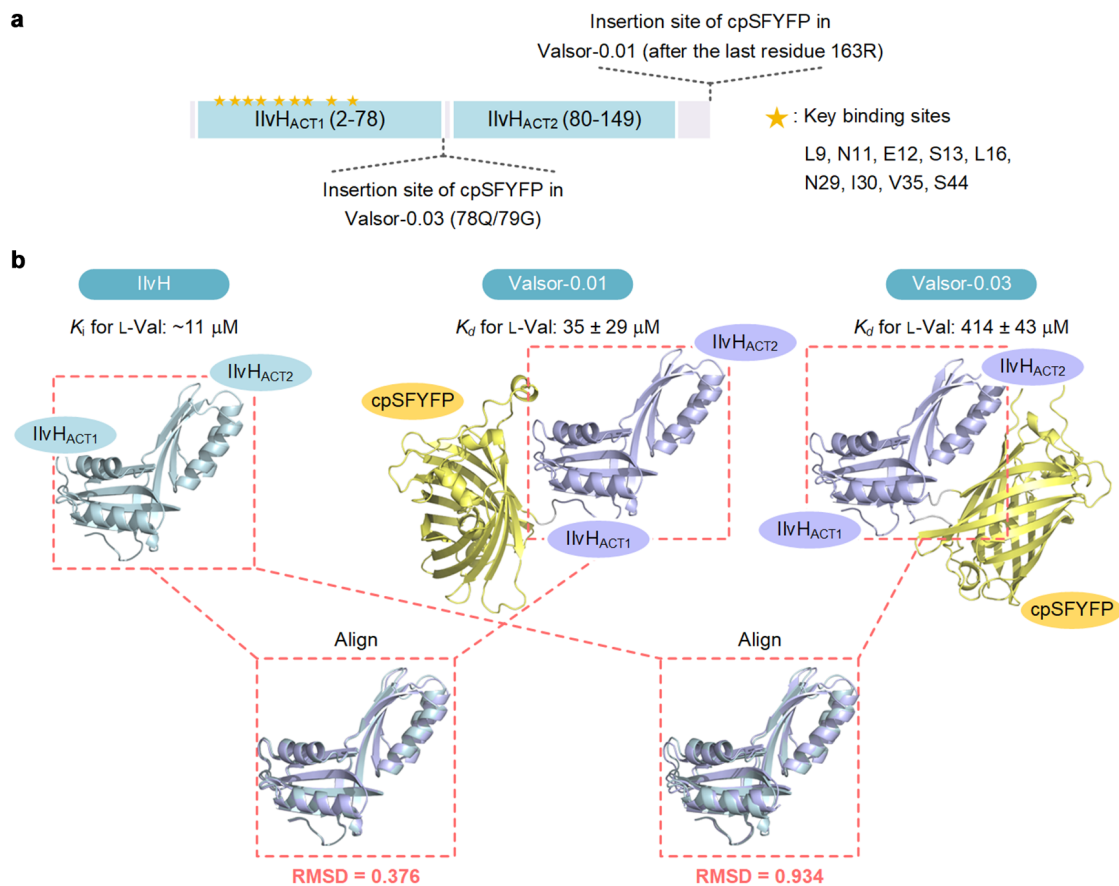


Figure 84 Analysis of the effect of cpSFYFP insertion sites on the affinity of the resulting biosensor variants. **(a)** Schematic representation of the cpSFYFP insertion sites in the IlvH domain of Valsor-0.01 (a low- K_d L-valine biosensor variant) and Valsor-0.03 (a high- K_d variant). Key L-valine binding sites were shown. **(b)** Structure 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. (Supplementary Fig. 49c, d in the revised manuscript)

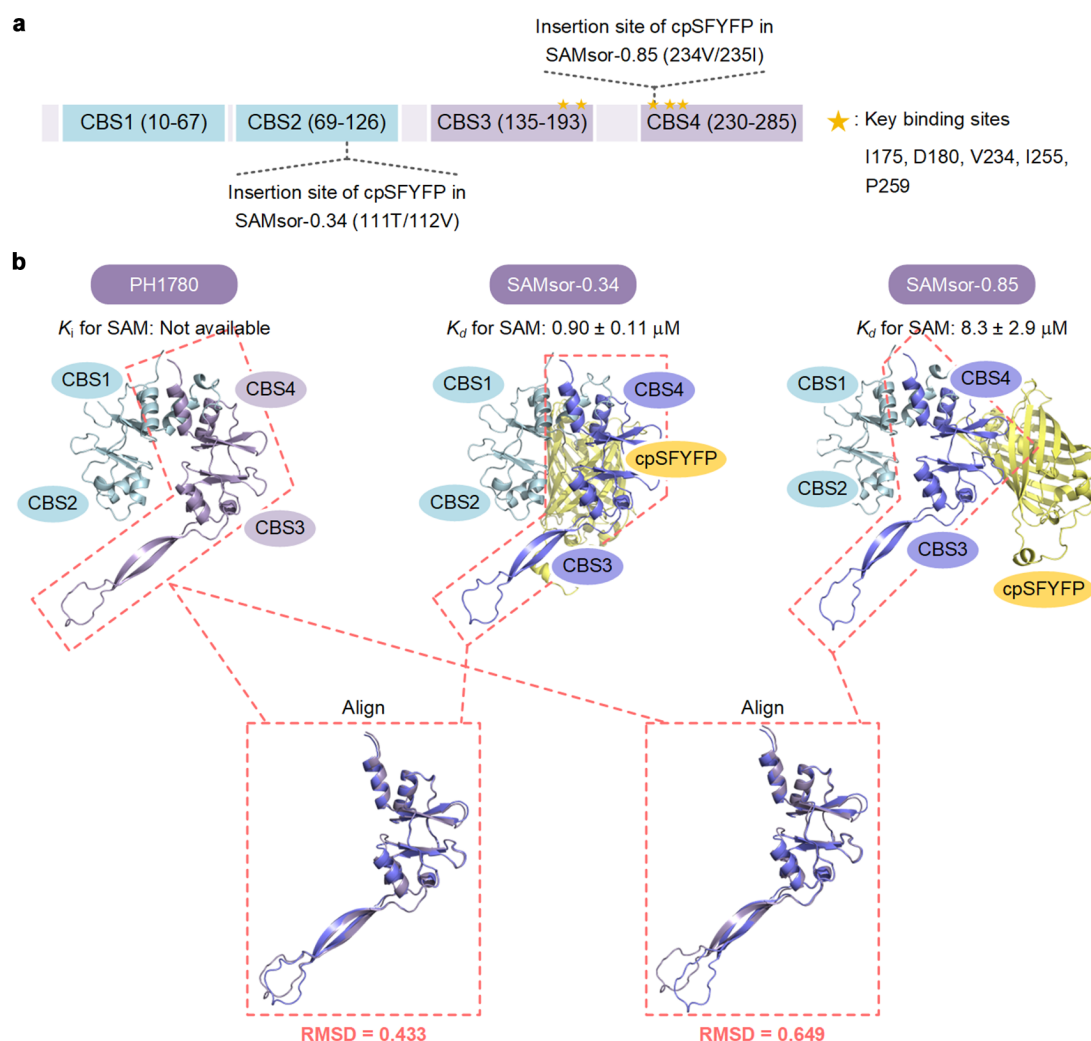


Figure 85 Analysis of the effect of cpSFYFP insertion sites on the affinity of the resulting biosensor variants. **(a)** Schematic representation of the cpSFYFP insertion sites in the PH1780 domain of SAMsor-0.34 (a low- K_d SAM biosensor variant) and SAMsor-0.85 (a high- K_d variant). Key SAM binding sites were shown. **(b)** Structure 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. (**Supplementary Fig. 49g, h in the revised manuscript**)

In conclusion, the relatively high K_d values of Valsor and Ilesor reflect a rational design based on the selection of the cpSFYFP insertion site. By positioning cpSFYFP near to or distal from the ligand-binding pocket, the affinities of allosteric regulation domain-based GEFBs can be predictably modulated. This structure-function relationship provides a rational foundation for future engineering of GEFBs with predictable affinity, thereby expanding the toolbox for metabolic imaging and *in vitro* diagnostics across varying concentration scales.

Related data were presented in **Fig. 2a, Fig. 3b, Fig. 4b, Supplementary Fig. 2a, Supplementary Fig. 4, Supplementary Fig. 16, Supplementary Fig. 27a, Supplementary Fig. 28, and Supplementary Fig. 49** in the revised manuscript. Related descriptions were added in the “Discussion” section of the revised manuscript as follows:

“In this study, we demonstrated the feasibility of utilizing regulatory domains of allosteric enzymes as biorecognition elements for GEFBs development. By coupling the ACT domain from homomultimeric enzyme (PheA_{ACT}), ACT-containing regulatory subunit from heteromultimeric enzyme (IlvH), or LA domain from homomultimeric enzyme (CimA_{LA}) with cpSFYFP, we developed three biosensors for detection of L-phenylalanine, L-valine, and L-isoleucine, respectively. We also confirmed that the multi-ligand-binding PH1780 can serve as a biorecognition element to derive diverse specific biosensors, and then constructed two SAM-specific biosensors with complementary detection ranges based on PH1780 and cpSFYFP. These biosensors assembled into proper quaternary structures consistent with those of the native PheA_{ACT} domain, IlvH, CimA_{LA} domain, and CBS domain^{13,15,21,25,29}. Each ligand molecule bound independently to individual monomers within the multimeric biosensors (hill slope close to 1). These results demonstrate that the developed biosensors preserved the intrinsic ligand-binding characteristics of their native allosteric regulatory domains. The methodology for developing GEFBs based on allosteric regulatory domains could be summarized in four steps: (i) selection of specific biorecognition elements, (ii) identification of optimal cpSFYFP insertion sites within loop regions to obtain a variant with obvious responsiveness and suitable affinity, (iii) optimization of linkers to maximize responsiveness, and (iv) functional testing (**Fig. 1a**). Notably, the affinities of allosteric regulatory domain-based GEFBs can be strategically modulated by shifting the insertion site of cpSFYFP. Besides SAMsor-0.34, multiple insertion variants with lower K_d values than Phesor, Valsor, and Ilesor were identified, such as Phesor-0.04 ($57 \pm 12 \mu\text{M}$), Valsor-0.01 ($35 \pm 29 \mu\text{M}$), and Ilesor-0.17 ($59.4 \pm 5.4 \mu\text{M}$) (**Supplementary Fig. 4, Supplementary Fig. 16, and Supplementary Fig. 28**). The higher affinities of these variants might be attributed to the insertion site of cpSFYFP being positioned distal to the binding pocket, which minimizes structural disruption and maintains higher architectural fidelity of the biorecognition element to its native conformation (**Supplementary Fig. 49**). This structure-function relationship

provides a rational foundation for future GEFB engineering, thereby expanding the toolbox for metabolic imaging and *in vitro* diagnostics across varying concentration scales.”

We sincerely hope these modifications satisfactorily address your concerns. If additional clarifications or experimental data are desired, we would be pleased to continue refining our manuscript.

To Reviewer #4:

This is a thoroughly interesting manuscript. The regulatory domains of allosteric enzymes are used as the binding domain in fluorescent probes by fusing to cpSFYFP. They have shown that they can elicit fluorescence response to the allosteric domain binding ligands. What is attractive about this work is the versatility they have shown. They have used ACT domains from both homo and heteromeric enzyme assemblies, used a LeuA domain and used a multiligand binding CBS domain. Not only have they shown that these engineered proteins show sensitivity in their fluorescent response to both in vitro and in vivo.

I am very supportive of the publication of these results and believe they are of wide interest to those interested in protein engineering and protein based sensors. Such approaches have a wide range of useful applications - many of which are referenced in this manuscript.

There are a number of aspects of this manuscript that warrant attention before publication. Many of these comments relate to the relatively scant experimental detail that is provided.

Response: We sincerely appreciate your recognition of the versatility and broad applicability of our proposed strategy. We are also particularly grateful for your insightful comments. In accordance with your suggestions, we have thoroughly revised the manuscript to provide comprehensive experimental descriptions and rigorous data presentation. We believe that these revisions have fully addressed your concerns and hope the revised version meets your satisfaction.

1. While the response to the ligands is clear, the dissociation constants measured through fluorescence are high and the levels of ligands used in these assays are generally high - in the mM range. I'm aware that many of these allosteric domains should have micro molar dissociation comments. There is not comment on the disruption to binding. While the mutagenesis results support the binding sites are indeed intact I would be more comfortable with some alternative assessment of binding and binding stoichiometry - also see comments below about quaternary structure.

Response: Thanks for your constructive comments, which are important for elucidating

the working mechanism of the allosteric regulatory domain-based biosensors. Here, we would like to provide a combined response to your Comment 1 and Comment 3 to comprehensively address your concerns regarding binding affinity, quaternary structure, and protein concentration effects.

In accordance with your advice, we first performed an alternative assessment of the binding properties between the four biosensors (Phesor, Valsor, Ilesor, and SAMsor) and their target ligands (L-phenylalanine, L-valine, L-isoleucine, and *S*-adenosyl-L-methionine [SAM]) using thermal shift assays (TSA). As shown in **Fig. 86**, ligand binding induced significant changes in the thermal stability of all biosensors. The apparent dissociation constants (K_d) determined by TSA were 2.69 ± 0.13 mM for Phesor, 4.46 ± 0.50 mM for Valsor, 2.22 ± 0.30 mM for Ilesor, and 0.295 ± 0.061 mM for SAMsor. These values align closely with those derived from the fluorescence-based dose-response curves (1.66 ± 0.11 mM for Phesor, 2.45 ± 0.20 mM for Valsor, 3.62 ± 0.40 mM for Ilesor, and 0.204 ± 0.021 mM for SAMsor) (**Fig. 86 and Fig. 87**). These consistent results confirm the reliability of the affinity values reported in our manuscript.

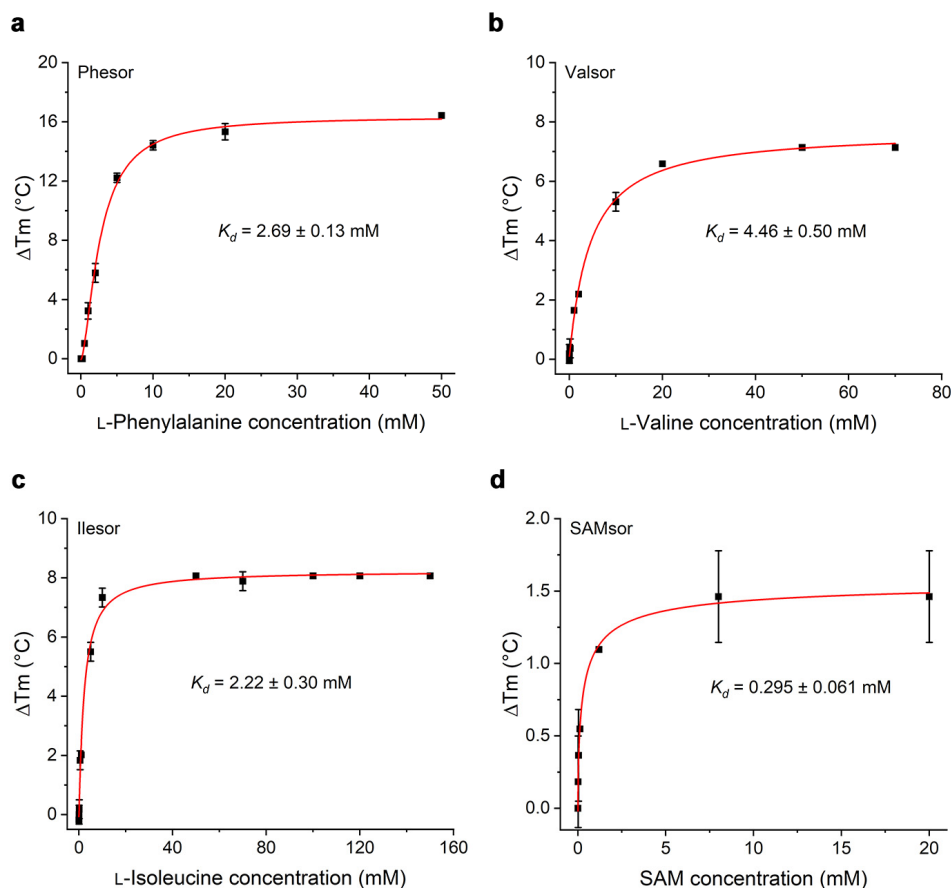


Figure 86 Analysis of the binding properties of Phesor, Valsor, Ilesor, and SAMsor to target ligands by thermal shift assays (TSA). **(a-d)** Binding properties between Phesor and L-phenylalanine **(a)**, Valsor and L-valine **(b)**, Ilesor and L-isoleucine **(c)**, or SAMsor and SAM **(d)**. The ΔT_m values indicated the changes in T_m values compared to control (with 0 mM target ligand). All data shown are means $\pm$ s.d. ($n = 3$ independent experiments). (Supplementary Fig. 5e, Supplementary Fig. 18e, Supplementary Fig. 30e, and Supplementary Fig. 40e in the revised manuscript)

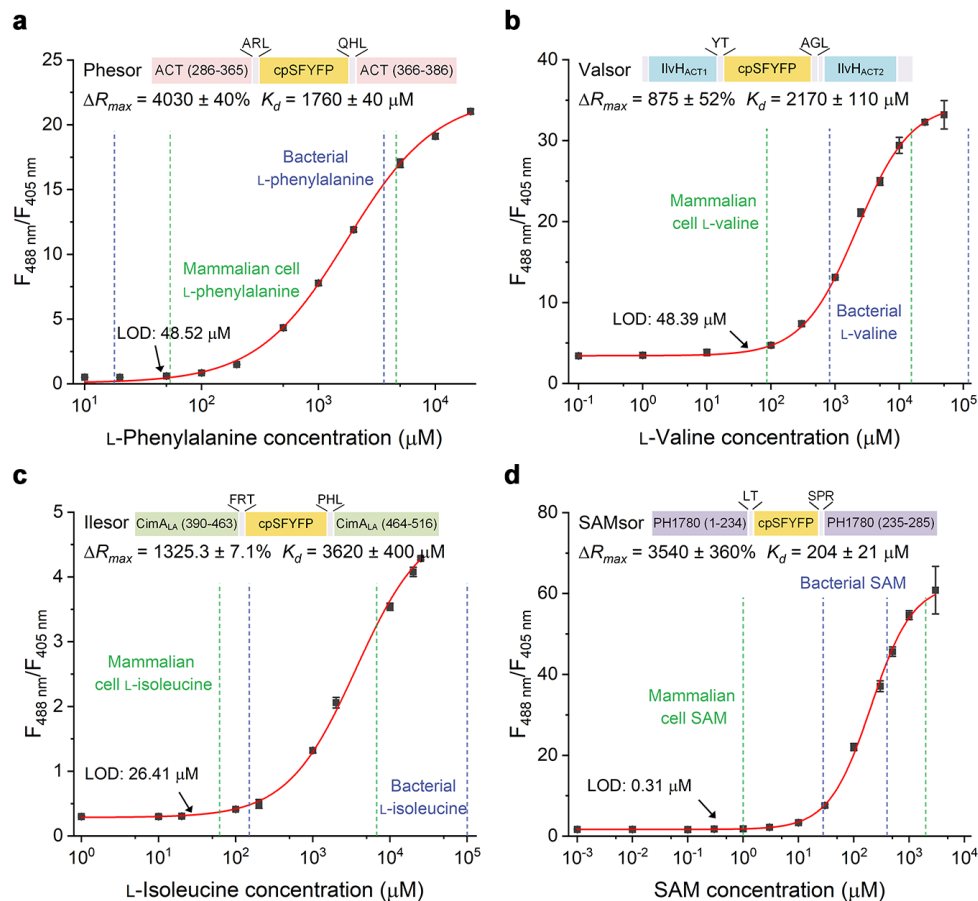


Figure 87 Characterization of Phesor, Valsor, Ilesor, and SAMsor *in vitro*. **(a-d)** Dose-response curve of Phesor **(a)**, Valsor **(b)**, Ilesor **(c)**, and SAMsor **(d)** for increasing concentrations of L-phenylalanine, L-valine, L-isoleucine, and SAM. The structural composition of the four biosensors and physiological concentrations of target ligands in bacteria and mammalian cell were shown. All data shown are means $\pm$ s.d. ($n = 3$ independent experiments). (Fig. 2c, Fig. 3d, Fig. 4d, and Fig. 6c in the revised manuscript)

Subsequently, we determined the oligomeric states of Phesor, Valsor, Ilesor, and SAMsor using gel filtration chromatography. As shown in Fig. 88, Phesor, Valsor, and SAMsor exist as dimers both in the absence and presence of target ligands, consistent with the reported native dimeric states of their corresponding biorecognition elements (*J. Biol. Chem.*, 2006, 281:33825–33829; *J. Biol. Chem.*, 1998, 273:6248–6253; *J. Mol. Biol.*, 2006, 357:951–963; *Arch. Biochem. Biophys.*, 2013, 540:70–81). In contrast, the CimA_{LA} domain (the

biorecognition element of Ilesor) was previously reported to initially assemble into a dimer, which can further oligomerize into a tetramer through Zn^{2+} -mediated bridging. Purified Cim_{ALA} domain was found to exist as a mixture of dimers and tetramers, and the addition of Zn^{2+} chelator EDTA shifts the equilibrium toward a predominantly dimeric state (*Biochem. J.*, 2009, 421:133–143). Consistent with these native characteristics, purified Ilesor eluted as a mixture of dimers and tetramers. Treatment with 10 mM EDTA converted it primarily to the dimeric form (**Fig. 88c**). Collectively, these results demonstrate that the allosteric enzyme-based biosensors preserve the native oligomerization characteristics of their original regulatory domains. They can assemble into the proper quaternary structures required for ligand binding.

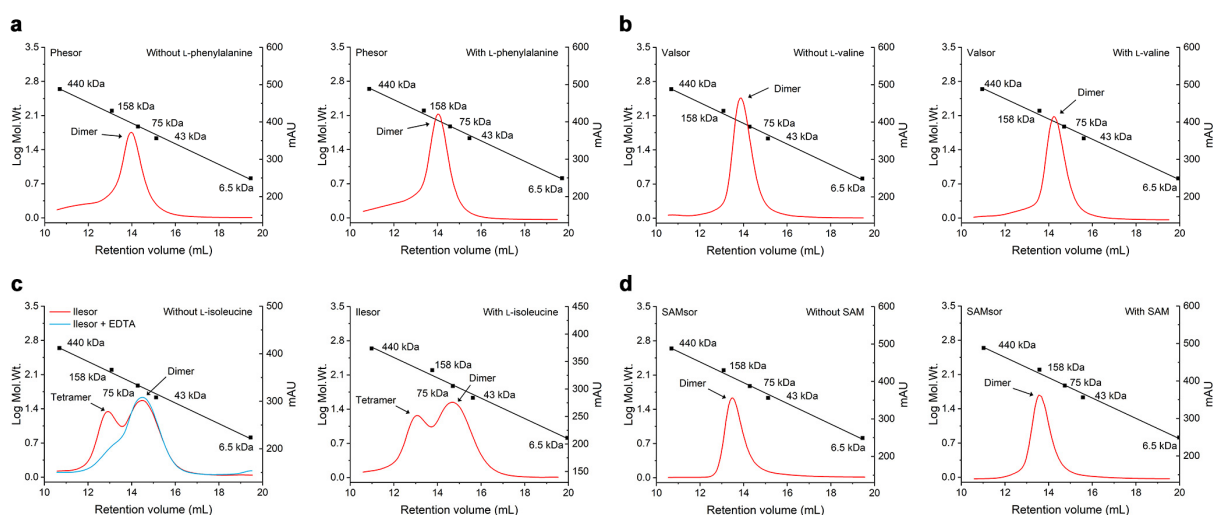


Figure 88 Characterization of Phesor, Valsor, Ilesor, and SAMsor *in vitro*. **(a-d)** Analysis of oligomeric state of Phesor **(a)**, Valsor **(b)**, Ilesor **(c)**, and SAMsor **(d)** in the absence or presence of 1 mM L-phenylalanine, 1 mM L-valine, 1 mM L-isoleucine, and 100 μM SAM by gel filtration chromatography. 10 mM EDTA was added for chelating Zn^{2+} ions at the side-by-side dimer interface of the Ilesor tetramer. Red curve, chromatogram of purified Phesor; black line, standard curve for protein molecular mass standards. (Supplementary Fig. 5a, b, Supplementary Fig. 18a, b, Supplementary Fig. 30a, b, and Supplementary Fig. 40a, b in the revised manuscript)

Next, to address your concerns regarding the potential influence of biosensor concentration on performance, we analyzed the dose-response curves of Phesor, Valsor, Ilesor, and SAMsor at varying biosensor concentrations (0.5, 1.0, and 3.0 μM). As shown in **Fig. 89**, the fluorescent ratios, response magnitudes, and affinities of all four biosensors remained unaffected across different protein concentrations. These results indicate that these

biosensors possess robust responses independent of their own concentration.

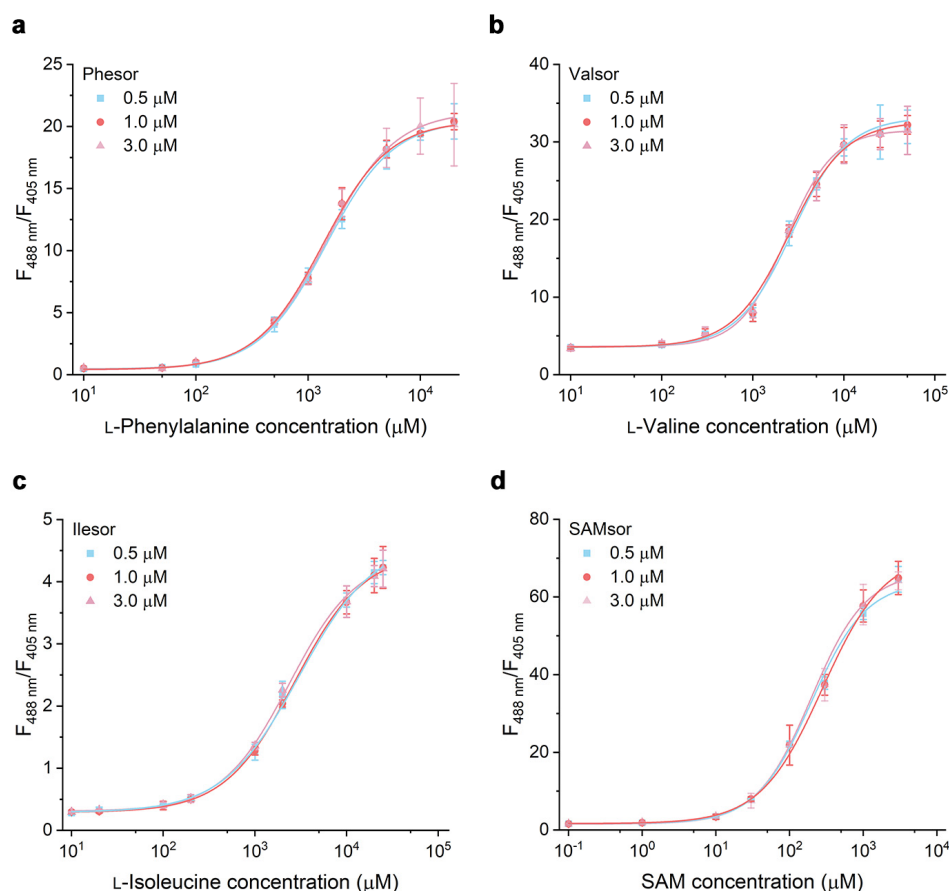


Figure 89 Analysis of the effect of biosensor concentration on the detection of target ligand. **(a-d)** Dose-response curves of Phesor **(a)**, Valsor **(b)**, Ilesor **(c)**, and SAMsor **(d)** at various concentrations (0.5, 1, or 3 μM) for L-phenylalanine, L-valine, L-isoleucine, and SAM. All data shown are means $\pm$ s.d. ($n = 3$ independent experiments). (Supplementary Fig. 5f, Supplementary Fig. 18f, Supplementary Fig. 30f, and Supplementary Fig. 40f in the revised manuscript)

As you correctly noted, the native allosteric regulatory domains utilized in this study exhibit affinities in the micromolar range: ~ 20 μM for PheA_{ACT} (used in Phesor), ~ 11 μM for IlvH (used in Valsor), and ~ 19 μM for CimA_{ALA} (used in Ilesor). PH1780, employed in SAMsor, was previously an uncharacterized protein. However, it is important to highlight that the K_d values of allosteric regulatory domain-based GEFBs can be strategically modulated by selecting the insertion site of cpSFYFP within the biorecognition element. The K_d values of Phesor (~ 1.66 mM), Valsor (~ 2.45 mM), Ilesor (~ 3.62 mM), and SAMsor (~ 204 μM) were all rationally tuned to match the physiological concentrations of their target ligands in mammalian cells (~ 0.84 mM for L-phenylalanine, ~ 1.51 mM for L-valine, ~ 1.76 mM for L-isoleucine, and ~ 354 μM for SAM; *Nat. Chem. Biol.*, 2016, 12:482–489; *Commun.*

Biol., 2022, 5:313) (**Fig. 87**).

Taking Phesor as an example, its development involved screening insertion sites of cpSFYFP within PheA_{ACT} (yielding intermediate variant Phesor-0.38), followed by linker optimization. Notably, multiple biosensor variants with a wide range of affinities (K_d from ~57 to ~3,830 μM) were identified during the insertion site screening (**Fig. 90**). Among them, Phesor-0.04, with cpSFYFP inserted near the N-terminus of PheA_{ACT}, exhibited the lowest K_d (~57 μM), close to the native dissociation constant of ~20 μM (**Fig. 90c**). In contrast, Phesor-0.38, with cpSFYFP inserted in the interior of PheA_{ACT}, exhibited a relatively higher K_d (~440 μM) (**Fig. 90e**). We speculated that the distinct cpSFYFP insertion sites caused varying structural perturbations in the biorecognition elements, which may account for the observed affinity differences.

Next, we performed homology modeling using AlphaFold 3 for PheA, Phesor-0.04, and Phesor-0.38, and compared the structural similarity of their PheA_{ACT} domains. As shown in **Fig. 91**, a higher structural similarity, indicated by a lower root mean square deviation (RMSD) of **0.196 Å**, was observed between the PheA_{ACT} domains of Phesor-0.04 and PheA compared to that between Phesor-0.38 and PheA (RMSD of **0.429 Å**). This result suggests that the PheA_{ACT} structure in Phesor-0.04 more closely resembles its native conformation, providing a structural basis for its higher affinity.

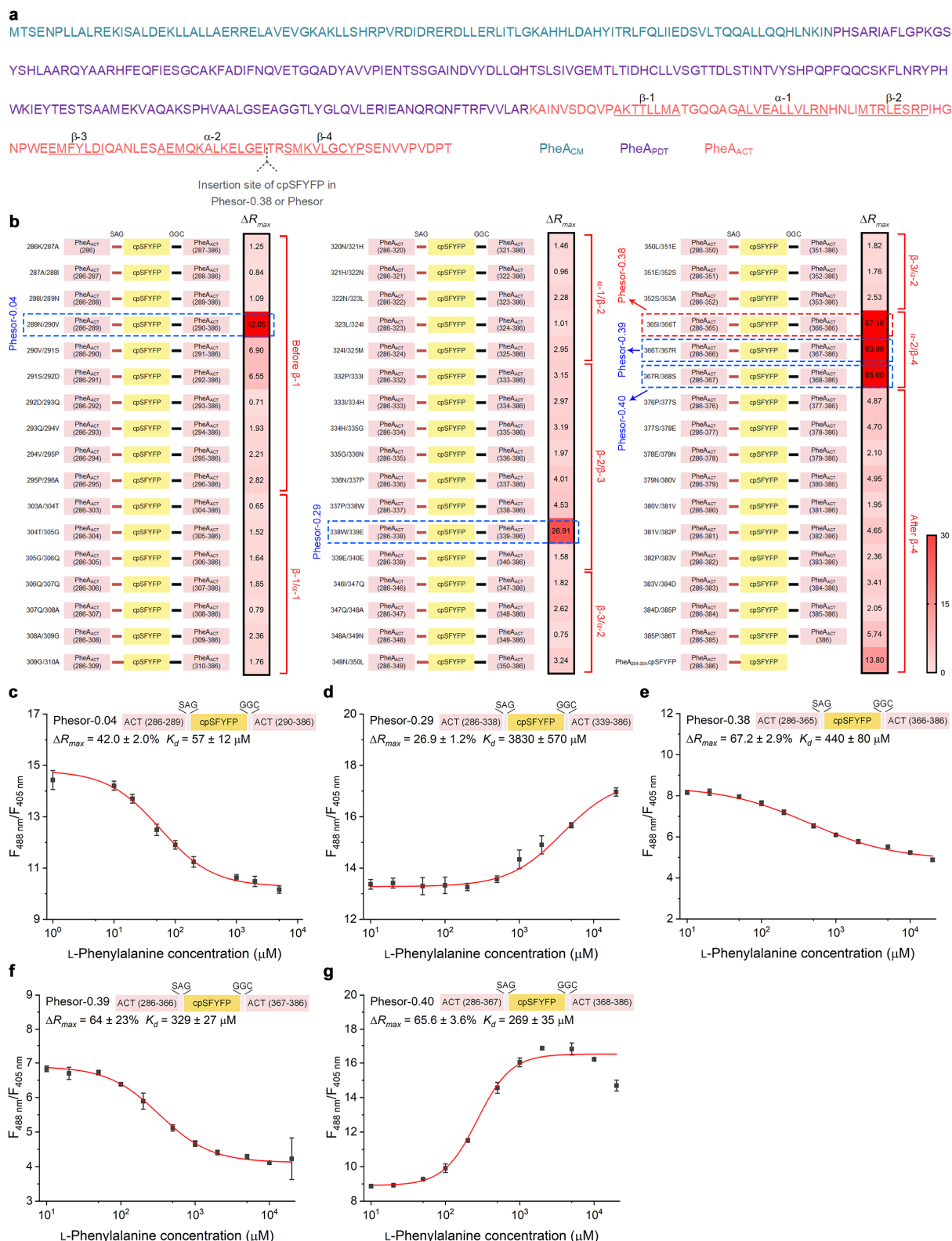


Figure 90 Construction of Phesor-0.38 (precursor of Phesor) by insertion sites screening. **(a)** Schematic representations of the amino acid sequence and the predicted secondary structure of PheA. The chorismate mutase (PheA_{CM}) domain, prephenate dehydratase (PheA_{PD}) domain, and ACT (PheA_{ACT}) 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 of the L-phenylalanine biosensor Phesor-0.38 based on insertion sites screening. The ΔR_{max} of different biosensor variants 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. **(c-g)** Dose-response curves of Phesor-0.04 **(c)**, Phesor-0.29 **(d)**, Phesor-0.38 **(e)**, Phesor-0.39 **(f)**, and Phesor-0.40 **(g)** for increasing concentrations of L-phenylalanine. The low K_d value variant Phesor-0.04 together with the high K_d value variant Phesor-0.38 (precursor of Phesor) were submitted to further bioinformatics analysis. All data shown are means $\pm$ s.d. ($n \geq 3$ independent experiments). **(Fig. 2a, Supplementary Fig. 2a, and Supplementary Fig. 4 in the revised manuscript)**

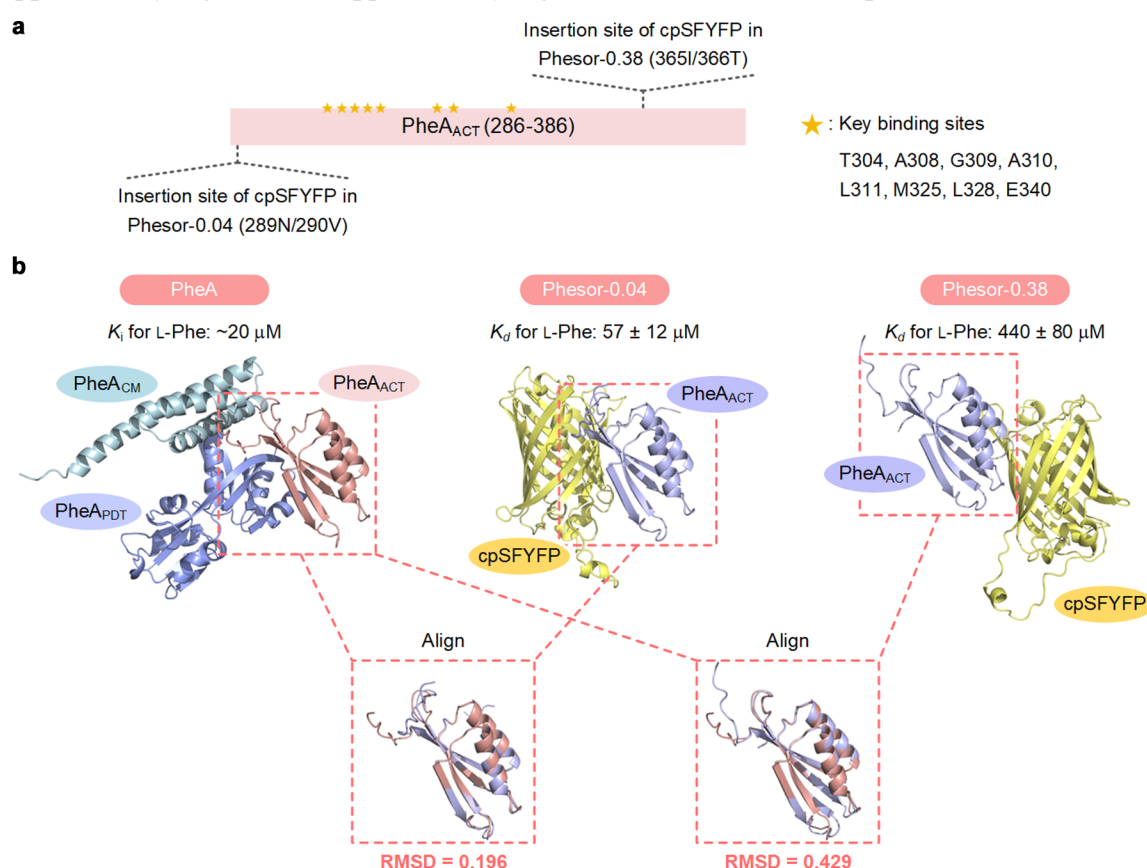


Figure 91 Analysis of the effect of cpSFYFP insertion sites on the affinity of the resulting biosensor variants. **(a)** Schematic representation of the cpSFYFP insertion sites in the PheA_{ACT} domain of Phesor-0.04 (a low- K_d L-phenylalanine biosensor variant) and Phesor-0.38 (a high- K_d variant). Key L-phenylalanine binding sites were shown. **(b)** Structure 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. **(Supplementary Fig. 49a, b in the revised manuscript)**

Consistent with the observations in Phesor, multiple insertion variants spanning a range

of K_d values were also obtained during the development of Valsor (K_d from ~ 35 to ~ 414 μM), Ilesor (K_d from ~ 59 to $\sim 17,000$ μM), and SAMsor (K_d from ~ 0.9 to ~ 8.3 μM) (Fig. 92, Fig. 94, and Fig. 96). From these variants, Valsor-0.03 (K_d of ~ 414 μM), Ilesor-0.23 (K_d of $\sim 1,700$ μM), and SAMsor-0.85 (K_d of ~ 8.3 μM) were selected for further linker optimization due to their moderate affinities (Fig. 92e, Fig. 94i, and Fig. 96d). Importantly, in low- K_d variants, the insertion site of cpSFYFP was consistently positioned distal to the ligand-binding sites. The allosteric regulatory domains in these variants retained higher structural similarity (lower RMSD) to the native conformations (Fig. 93, Fig. 95, and Fig. 97).

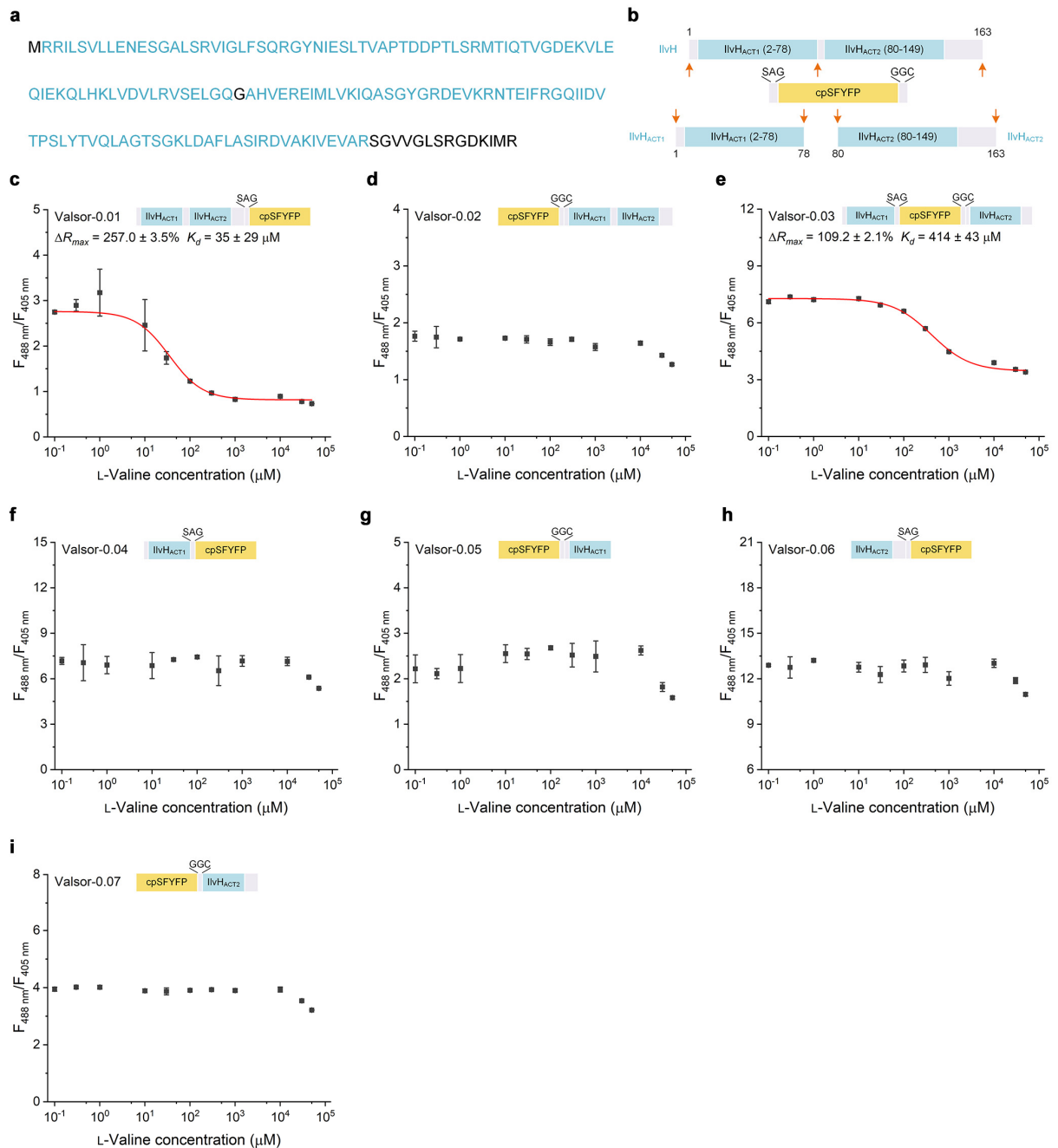


Figure 92 Construction of Valsor-0.03 (precursor of Valsor) by insertion sites screening. **(a)** Schematic representation of the amino acid sequence of IlvH. The two ACT domains of IlvH were shown in cyan. **(b)** Schematic representation of the construction of the initial seven L-valine biosensor insertion variants. **(c-i)** Dose-response curves of Valsor-0.01 **(c)**, Valsor-0.02 **(d)**, Valsor-0.03 **(e)**, Valsor-0.04 **(f)**, Valsor-0.05 **(g)**, Valsor-0.06 **(h)**, and Valsor-0.07 **(i)** for increasing concentrations of L-valine. The low K_d value variant Valsor-0.01 together with the high K_d value variant Valsor-0.03 (precursor of Valsor) were submitted to further bioinformatics analysis. All data shown are means $\pm$ s.d. ($n = 3$ independent experiments). **(Fig. 3b and Supplementary Fig. 16 in the revised manuscript)**

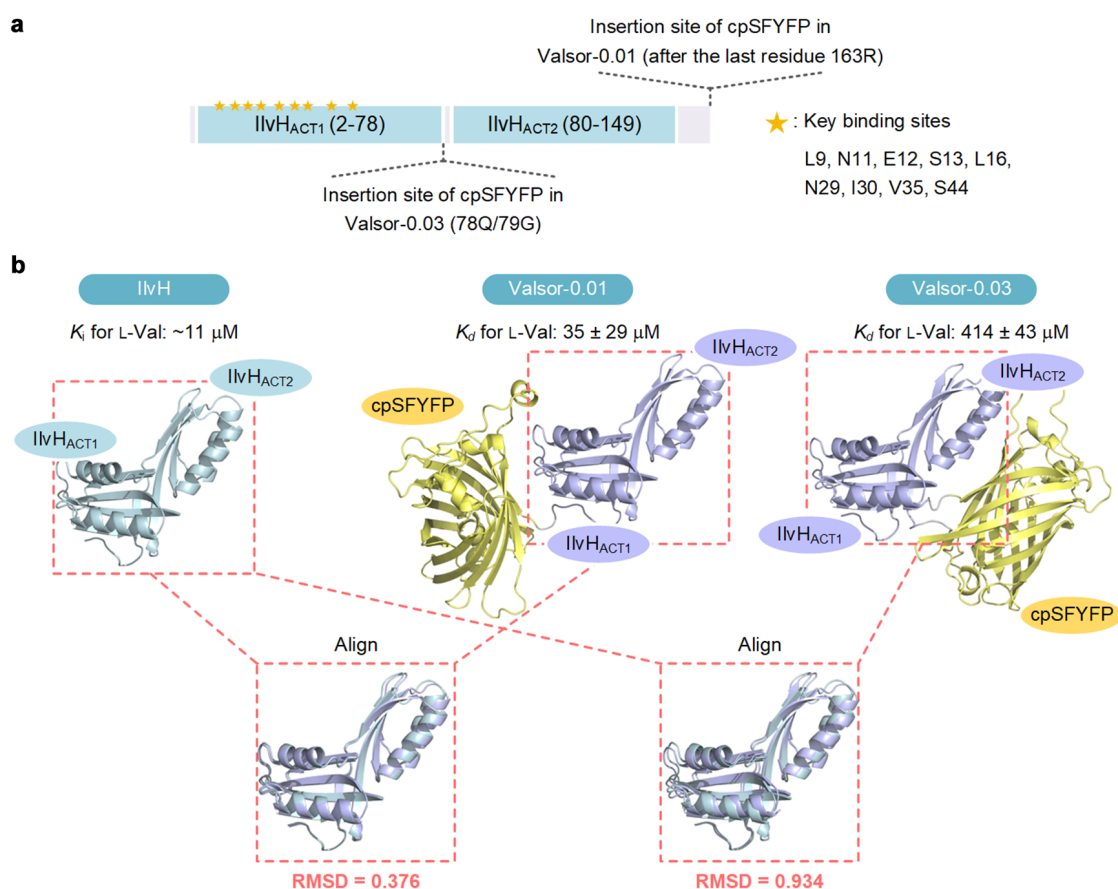


Figure 93 Analysis of the effect of cpSFYFP insertion sites on the affinity of the resulting biosensor variants. **(a)** Schematic representation of the cpSFYFP insertion sites in the IlvH domain of Valsor-0.01 (a low- K_d L-valine biosensor variant) and Valsor-0.03 (a high- K_d variant). Key L-valine binding sites were shown. **(b)** Structure 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. **(Supplementary Fig. 49c, d in the revised manuscript)**

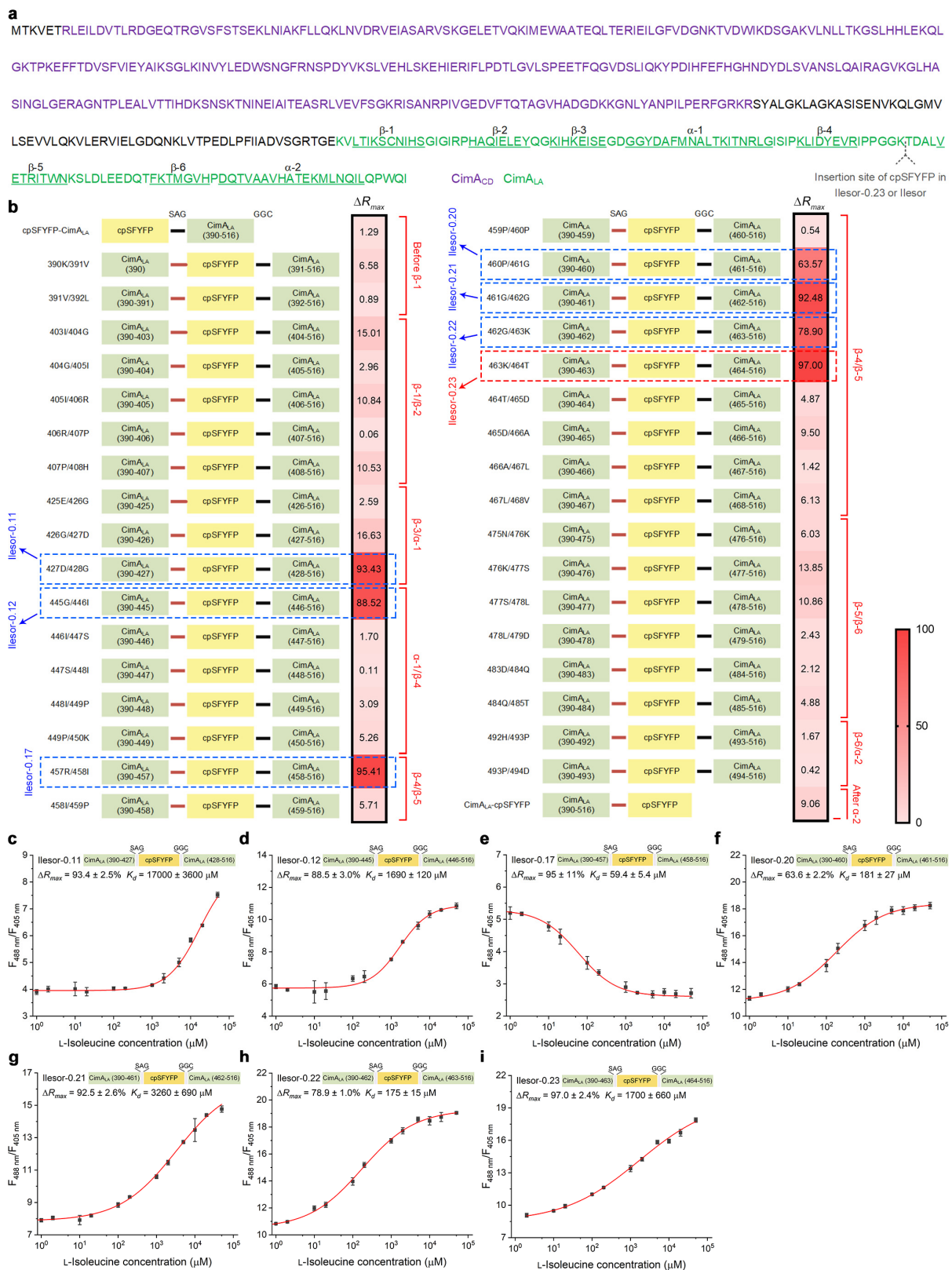


Figure 94 Construction of Ilesor-0.23 (precursor of Ilesor) by insertion sites screening. **(a)** Schematic representations of the amino acid sequence and the predicted secondary structure of CimA. The catalytic (CimA_{CD}) domain and LeuA regulatory (CimA_{LA}) domain of CimA were shown in purple and 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 of the L-isoleucine biosensor Ilesor-0.23 based on insertion sites screening. The ΔR_{max} of different biosensor variants to L-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. **(c-i)** Dose-response curves of Ilesor-0.11 **(c)**, Ilesor-0.12 **(d)**, Ilesor-0.17 **(e)**, Ilesor-0.20 **(f)**, Ilesor-0.21 **(g)**, Ilesor-0.22 **(h)**, and Ilesor-0.23 **(i)** for increasing concentrations of L-isoleucine. The low K_d value variant Ilesor-0.17 together with the high K_d value variant Ilesor-0.23 (precursor of Ilesor) were submitted to further bioinformatics analysis. All data shown are means $\pm$ s.d. (n = 3 independent experiments) (**Fig. 4b, Supplementary Fig. 27a, and Supplementary Fig. 28 in the revised manuscript**)

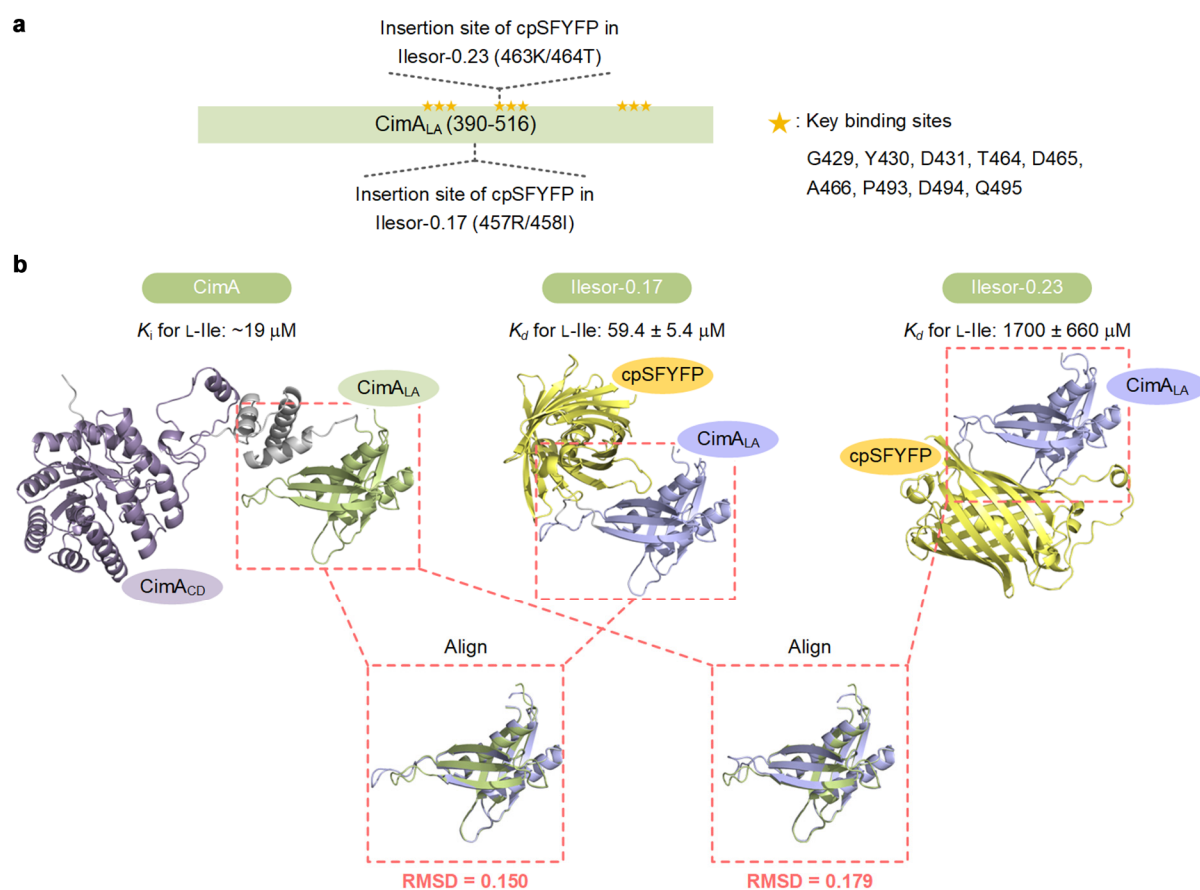


Figure 95 Analysis of the effect of cpSFYFP insertion sites on the affinity of the resulting biosensor variants. **(a)** Schematic representation of the cpSFYFP insertion sites in the CimA_{LA} domain of Ilesor-0.17 (a low- K_d L-isoleucine biosensor variant) and Ilesor-0.23 (a high- K_d variant). Key L-isoleucine binding sites were shown. **(b)** Structure 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. (**Supplementary Fig. 49e, f in the revised manuscript**)

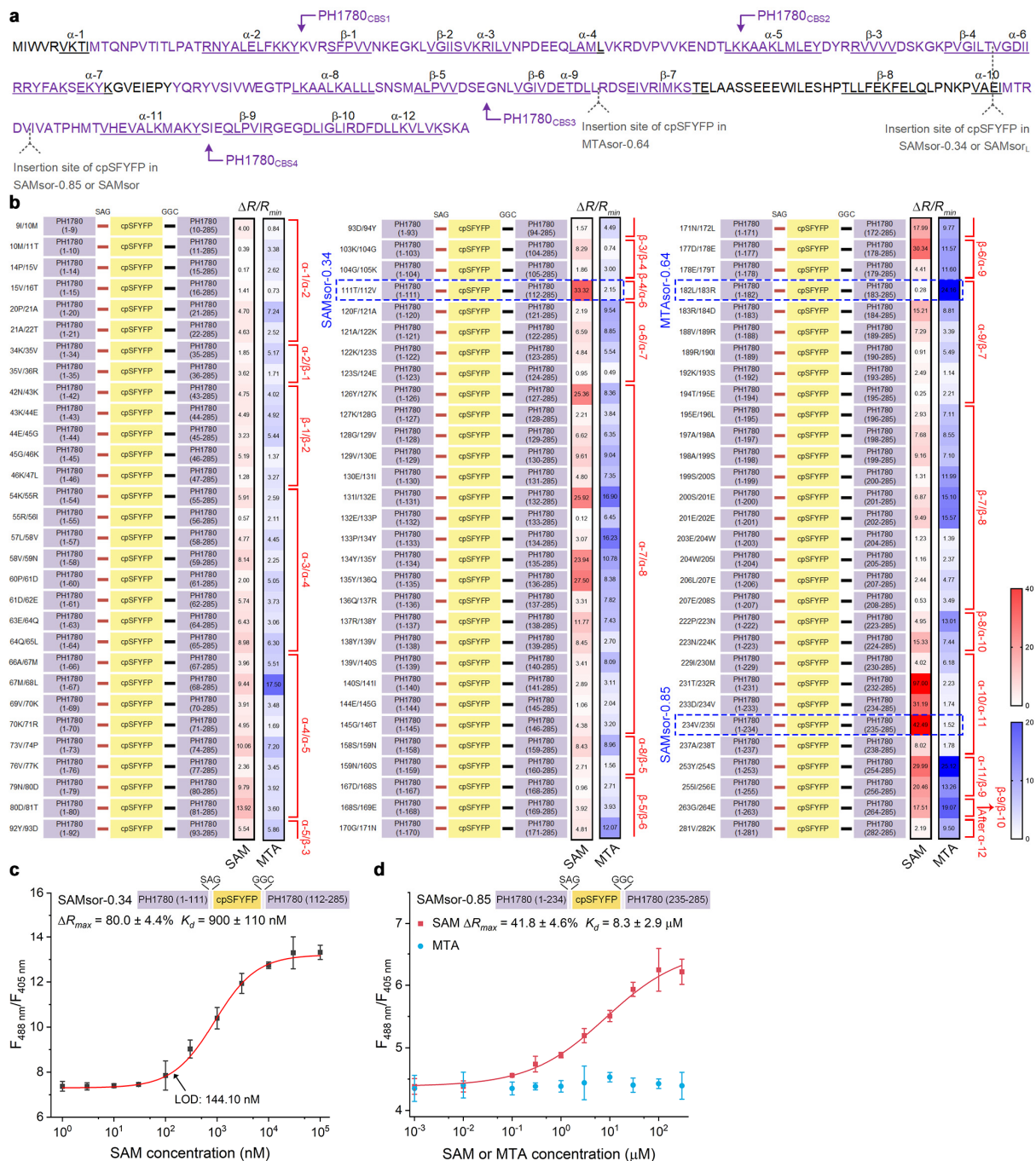


Figure 96 Construction of MTAsor-0.64, SAMsor-0.85 (precursor of SAMsor), and SAMsor-0.34 (precursor of SAMsor_L) based on insertion sites screening. **(a)** Schematic representations of the amino acid sequence and the predicted secondary structure of PH1780. The four CBS domains of PH1780 were shown 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 of the MTA biosensor MTAsor-0.64, SAM biosensor with high detection range SAMsor-0.85, and SAM biosensor with low detection range SAMsor-0.34 based on insertion sites screening. The response magnitudes ($\Delta R/R_{min}$) of different biosensor variants to 100 μM 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. **(c, d)** Dose-response curves of SAMsor-0.34 **(c)** and SAMsor-0.85 **(d)** for increasing concentrations of SAM. The low K_d value variant SAMsor-0.34 (precursor of SAMsor_L) together with the high K_d value variant SAMsor-0.85 (precursor of SAMsor) were submitted to further bioinformatics analysis. All data shown are means $\pm$ s.d. ($n = 3$ independent experiments). (**Fig. 5e, Fig. 7b, Supplementary Fig. 37a, and, Supplementary Fig. 39 in the revised manuscript**)

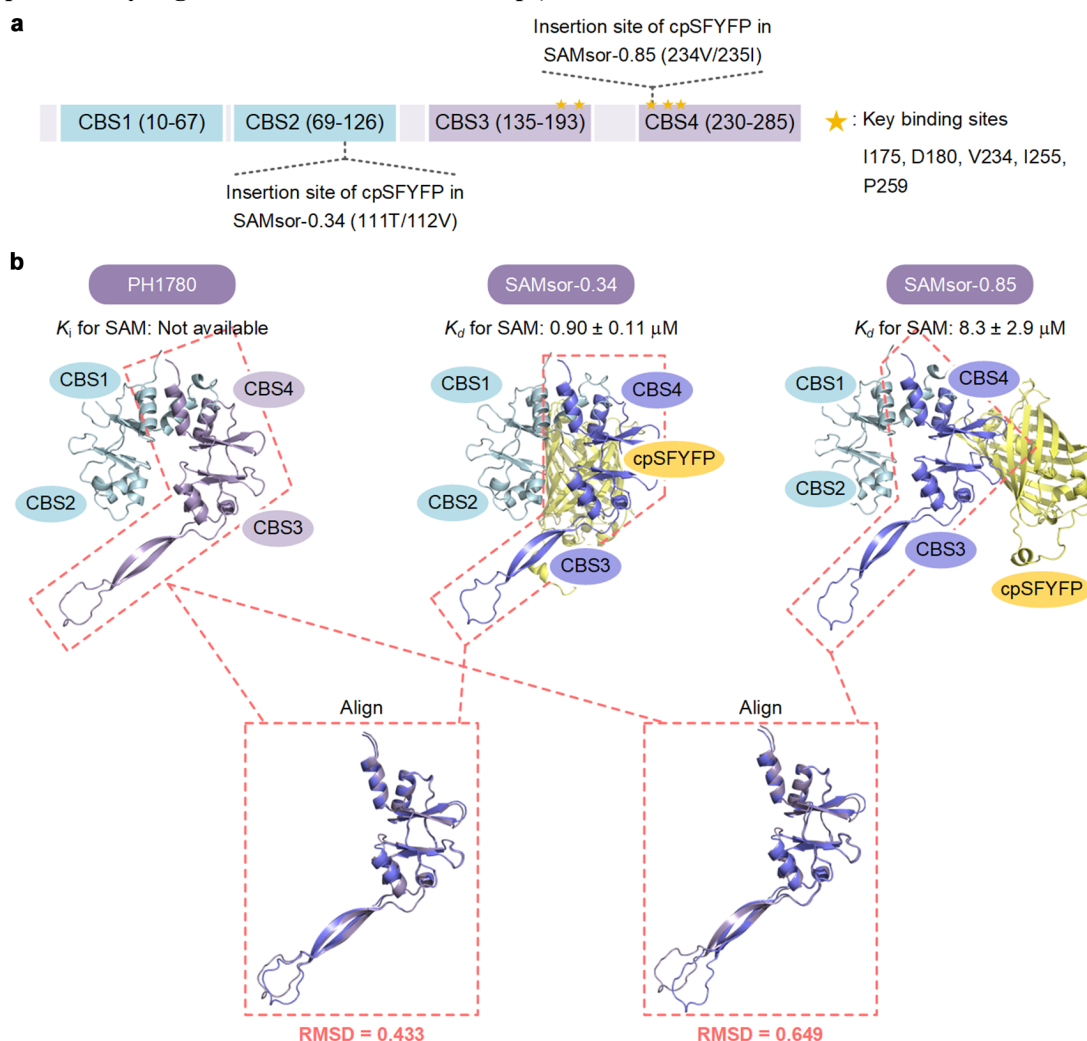


Figure 97 Analysis of the effect of cpSFYFP insertion sites on the affinity of the resulting biosensor variants. **(a)** Schematic representation of the cpSFYFP insertion sites in the PH1780 domain of SAMsor-0.34 (a low- K_d SAM biosensor variant) and SAMsor-0.85 (a high- K_d variant). Key SAM binding sites were shown. **(b)** Structure 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. (**Supplementary Fig. 49g, h in the revised manuscript**)

In conclusion, the relatively high K_d values of Phesor, Valsor, Ilesor, and SAMsor reflect a rational design based on the selection of the cpSFYFP insertion site. By

positioning cpSFYFP near to or distal from the ligand-binding pocket, the affinities of allosteric regulation domain-based GEFBs can be predictably modulated. This structure-function relationship provides a rational foundation for future engineering of GEFBs with predictable affinity, thereby expanding the toolbox for metabolic imaging and *in vitro* diagnostics across varying concentration scales.

Theoretically, other biosensor variants with different K_d values could also be further optimized via linker engineering. SAM is recognized as an important biomarker for evaluating human disease and health status. Elevated SAM levels in human body fluids are highly associated with the occurrence of diseases including digestive cancer, lung cancer, and pneumonia (**Fig. 98a**). The LOD of SAMsor for SAM was calculated to be 310 nM (**Fig. 87d**), which exceeds the reported serum SAM concentrations in healthy individuals (~73.5–120 nM) (*Metabolites*, 2022, 12:373). Fortunately, among the 90 insertion variants screened, we identified SAMsor-0.34, with cpSFYFP inserted between residues 111T/112V of PH1780, exhibited higher sensitivity to SAM (**Fig. 96c and Fig. 98b**). Dose-response curve analysis indicated that SAMsor-0.34 achieved a ΔR_{max} of $80.0 \pm 4.4\%$, a low K_d of 900 ± 110 nM, and a low LOD of 144.10 nM for SAM (**Fig. 96c and Fig. 98b**). Subsequently, we conducted a systematic optimization of the linker in SAMsor-0.34 and identified a more sensitive biosensor SAMsor_L with the N-terminal linker of “Leu-Glu” and the C-terminal linker of “Thr-Pro” from 2,715 mutants (**Fig. 98c**). SAMsor_L exhibited an increased ΔR_{max} of $128.5 \pm 1.1\%$, a lower K_d of 379 ± 41 nM, and a decreased LOD of 57.25 nM for SAM, making it suitable for SAM detection in human body fluids (**Fig. 98d**).

Next, we prepared authentic serum and urine samples from 13 healthy individuals and 15 patients with lung cancer. Quantification of SAM in these samples using SAMsor_L exhibited good correlation and consistency with HPLC measurements (**Fig. 98e-h**). The measured SAM concentrations in serum and urine from healthy individuals were 100 ± 40 nM and 11.9 ± 1.7 μ M, respectively, consistent with previously reported values (~73.5–120 nM in serum and ~10.2–22.1 μ M in urine; *Metabolites*, 2022, 12:373; *Electrophoresis*, 2020, 41:209–214) (**Fig. 98i, j**). Notably, serum SAM levels were significantly elevated in lung cancer patients, approximately 2.81-fold higher than those in healthy individuals (**Fig. 98i**). However, no significant differences were observed in urine SAM levels between the two

population groups (**Fig. 98j**). Receiver operating characteristic (ROC) analysis indicated that serum SAM could effectively differentiate patients with lung cancer from healthy subjects, with an area under the curve (AUC) of 0.759, while urine SAM could not (AUC = 0.585) (**Fig. 98k, l**). **These results demonstrate the feasibility of developing multiple biosensors with distinct affinities from a single allosteric regulatory domain.**

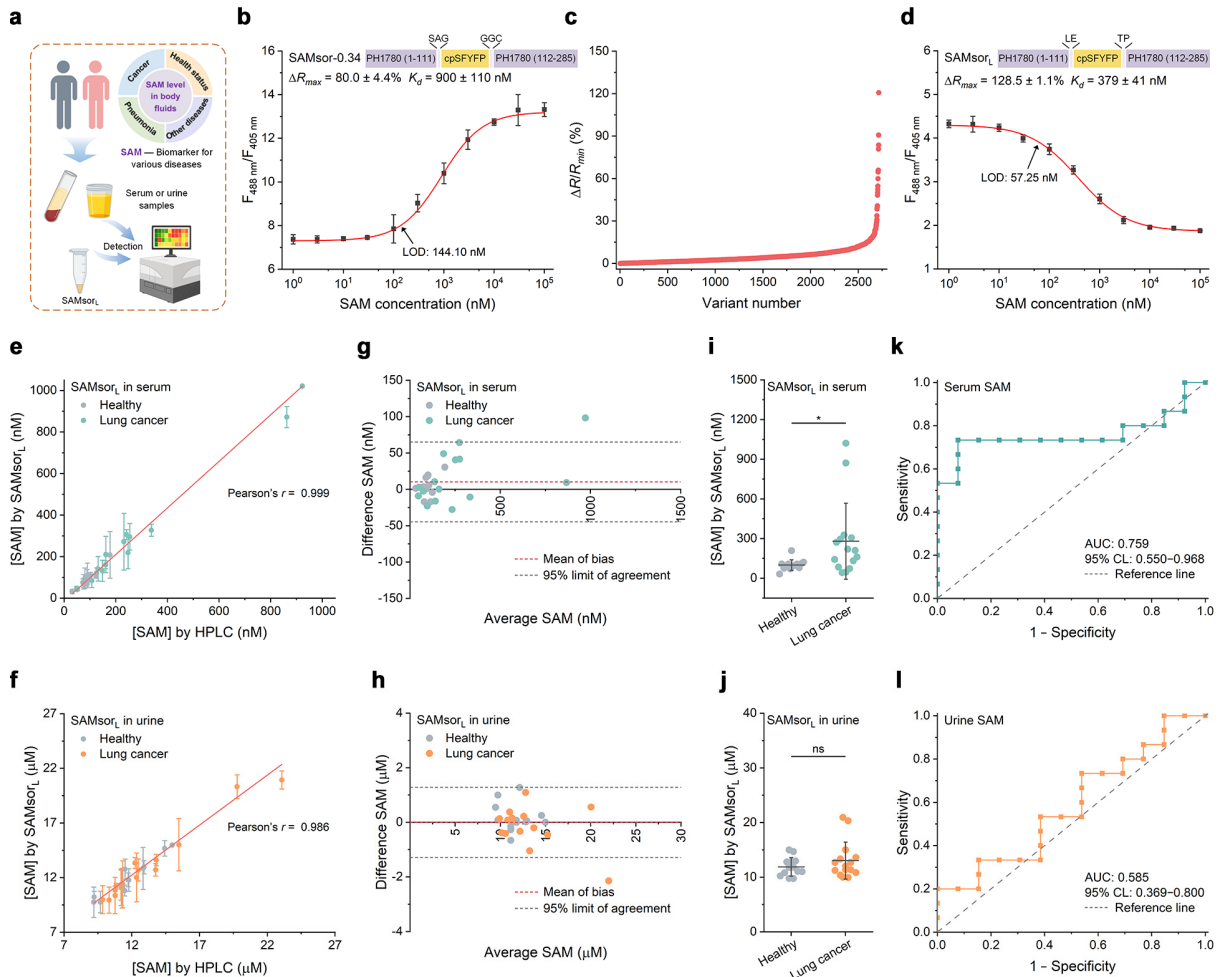


Figure 98 Optimization and application of the SAM biosensor SAMsor_L with low detection range. **(a)** Schematic representation of the workflow for quantitative detection of SAM in human serum and urine samples using SAMsor_L. **(b)** Dose-response curve of SAMsor-0.34 for increasing concentrations of SAM. The structural composition of SAMsor-0.34 was shown. **(c)** Optimization of SAMsor-0.34 by linker random mutagenesis combined with high-throughput screening. The response magnitudes of crude extracts of 2,715 linker mutants were determined in the presence of 10 μ M SAM. **(d)** Dose-response curve of SAMsor_L for increasing concentrations of SAM. The structural composition of SAMsor_L was shown. **(e, f)** Correlation analysis between SAMsor_L and HPLC for quantitative detection of SAM in serum **(e)** and urine **(f)** samples from healthy individuals and patients with lung cancer. Quantitative results measured by SAMsor_L were plotted against those obtained by HPLC. r , correlation coefficient. **(g, h)** Bland-Altman

analysis for SAM in serum **(g)** and urine **(h)** samples from healthy individuals and patients with lung cancer measured by SAMsor_L and HPLC. Bias (red dotted line) and 95% limits of agreement (gray dotted lines) were shown. **(i, j)** Analysis of differences in serum **(i)** and urine **(j)** SAM levels between healthy individuals and patients with lung cancer measured by SAMsor_L. **(k, l)** ROC curves of patients with lung cancer compared with healthy individuals based on serum **(k)** or urine **(l)** SAM levels. The area under the curve (AUC) and 95% confidence level (CL) were shown. All data shown are means $\pm$ s.d. (n = 3 independent experiments). *, $P < 0.05$; ns, no significant difference ($P \geq 0.05$); two-tailed Student's *t* test. **(Fig. 7 in the revised manuscript)**

Related data were presented in **Fig. 2a, c, Fig. 3b, d, Fig. 4b, d, Fig. 5e, Fig. 6c, Fig. 7, Supplementary Fig. 2a, Supplementary Fig. 4, Supplementary Fig. 5a, b, e, f, Supplementary Fig. 16, Supplementary Fig. 18a, b, e, f, Supplementary Fig. 27a, Supplementary Fig. 28, Supplementary Fig. 30a, b, e, f, Supplementary Fig. 37a, Supplementary Fig. 39, Supplementary Fig. 40a, b, e, f, Supplementary Fig. 49, and Supplementary Table 4** in the revised manuscript. Related descriptions were added in the “Results” section of the revised manuscript as follows:

For Phesor:

“Purified Phesor existed as a dimer both in the absence and presence of L-phenylalanine (**Supplementary Fig. 5a, b**). Dose-response curve analysis indicated that the detection range of Phesor encompasses the physiological concentrations of L-phenylalanine both in bacteria and in mammalian cells (**Fig. 2c**). Furthermore, it achieved a high ΔR_{max} ($4,030 \pm 40\%$), an appropriate K_d ($1,760 \pm 40 \mu\text{M}$; close to the value of $2.69 \pm 0.13 \text{ mM}$ determined by thermal shift assay [TSA]), and a Hill slope close to 1 for L-phenylalanine (**Fig. 2c, Supplementary Fig. 5e, and Supplementary Table 4**). As a ratiometric biosensor, Phesor displayed a robust response independent of its own concentration (**Supplementary Fig. 5f**).”

For Valsor:

“Valsor existed as a dimer and had two excitation peaks around 426 and 504 nm, with a single emission peak around 509 nm (405 nm excitation) or 518 nm (488 nm excitation) (**Supplementary Fig. 18a-d**). Dose-response curve analysis indicated that Valsor achieved a wide detection range, with an increased ΔR_{max} ($875 \pm 52\%$), an appropriate K_d ($2,170 \pm 110 \mu\text{M}$; close to the value of $4.46 \pm 0.50 \text{ mM}$ determined by TSA), and a Hill slope close to 1 for L-valine (**Fig. 3d, Supplementary Fig. 18e, and Supplementary Table 4**). In addition,

Valsor demonstrated a robust response independent of its own concentration, high specificity, good reversibility, and correctable pH-stability from pH 5.0 to 10.0 for L-valine detection (**Supplementary Fig. 17d-f, Supplementary Fig. 18f-j, and Supplementary Fig. 19a**)."

For Ilesor:

"Consistent with the quaternary structure of CimA_{LA}³², Ilesor exists as a mixture of dimers and tetramers both in the absence and presence of L-isoleucine (**Supplementary Fig. 30a, b**). It had two excitation peaks around 420 and 504 nm, with a single emission peak around 507 nm (405 nm excitation) or 517 nm (488 nm excitation) (**Supplementary Fig. 30c, d**). Dose-response curve analysis indicated that Ilesor achieved a suitable detection range, an increased ΔR_{max} ($1,325.3 \pm 7.1\%$), an appropriate K_d ($3,620 \pm 400 \mu\text{M}$; close to the value of $2.22 \pm 0.30 \text{ mM}$ determined by TSA), and a Hill slope close to 1 for L-isoleucine (**Fig. 4d, Supplementary Fig. 30e, and Supplementary Table 4**). In addition, Ilesor demonstrated a robust response independent of its own concentration, high specificity, good reversibility, and correctable pH-stability from pH 5.0 to 8.2 for L-isoleucine detection (**Supplementary Fig. 19a, Supplementary Fig. 29d-f, and Supplementary Fig. 30f-j**)."

For SAMsor:

"SAMsor existed as a dimer and had two excitation peaks around 420 and 504 nm, with a single emission peak around 514 nm (405 nm excitation) or 518 nm (488 nm excitation) (**Supplementary Fig. 40a-d**). Dose-response curve analysis indicated that SAMsor achieved an increased ΔR_{max} ($3,540 \pm 360\%$), an appropriate K_d ($204 \pm 21 \mu\text{M}$; close to the value of $0.295 \pm 0.061 \text{ mM}$ determined by TSA), a Hill slope close to 1, and a wide detection range suitable for detecting physiological concentrations of SAM in mammalian cells and bacteria (**Fig. 6c, Supplementary Fig. 40e, and Supplementary Table 2**). SAMsor displayed a robust response independent of its own concentration (**Supplementary Fig. 40f**)."

For SAMsor_L:

"SAM is also recognized as an important biomarker for evaluating human disease and health status³⁶. Elevated SAM levels in body fluids are highly associated with the occurrence of diseases including digestive cancer, lung cancer, and pneumonia (**Fig. 7a**)^{37,38}. The limit of detection (LOD) of SAMsor for SAM was calculated to be 310 nM (**Fig. 6c**), which exceeds the reported serum SAM concentrations in healthy individuals ($\sim 73.5\text{--}120 \text{ nM}$)

(**Supplementary Table 2**). Fortunately, we found that the 34th variant (denoted as SAMsor-0.34, with cpSFYFP inserted between residues 111T/112V of PH1780) exhibited higher sensitivity to SAM among the 90 insertion variants of the SAM biosensor (**Fig. 7b**, **Supplementary Fig. 37b**, and **Supplementary Fig. 39**). Dose-response curve analysis indicated that SAMsor-0.34 achieved a ΔR_{max} of $80.0 \pm 4.4\%$, a low K_d of 900 ± 110 nM, and a low LOD of 144.10 nM for SAM (**Fig. 7b**). Then, we conducted a systematic optimization of the linker in SAMsor-0.34 and identified a more sensitive biosensor SAMsor_L with the N-terminal linker of “Leu-Glu” and the C-terminal linker of “Thr-Pro” from 2,715 mutants (**Fig. 7c**). SAMsor_L exhibited an increased ΔR_{max} of $128.5 \pm 1.1\%$, a lower K_d of 379 ± 41 nM, and a decreased LOD of 57.25 nM for SAM (**Fig. 7d**).

We prepared authentic serum and urine samples from 13 healthy individuals and 15 patients with lung cancer. Quantification of SAM in these samples using SAMsor_L exhibited good correlation and consistency with HPLC measurements, and the results were unaffected by the presence of different SAM analogs (**Fig. 7e-h** and **Supplementary Fig. 48**). The measured SAM concentrations in serum and urine from healthy individuals were 100 ± 40 nM and 11.9 ± 1.7 μ M, respectively, consistent with previously reported values (**Fig. 7i, j** and **Supplementary Table 2**). Notably, serum SAM levels were significantly elevated in lung cancer patients, approximately 2.81-fold higher than those in healthy individuals (**Fig. 7i**). However, no significant differences were observed in urine SAM levels between the two population groups (**Fig. 7j**). Receiver operating characteristic (ROC) analysis indicated that serum SAM could effectively differentiate patients with lung cancer from healthy subjects, with an area under the curve (AUC) of 0.759, while urine SAM could not (AUC = 0.585) (**Fig. 7k, l**). These results demonstrate that GEFBs with adjustable affinities can be derived from the same allosteric regulatory domain. Furthermore, the SAM biosensor SAMsor_L is applicable for sensitive and precise detection of SAM in human body fluids.”

In addition, a brief discussion regarding the structural perturbation-induced K_d discrepancies was added in the “Discussion” section of the revised manuscript as follows:

“In this study, we demonstrated the feasibility of utilizing regulatory domains of allosteric enzymes as biorecognition elements for GEFBs development. By coupling the ACT domain from homomultimeric enzyme (PheA_{ACT}), ACT-containing regulatory subunit from

heteromultimeric enzyme (IlvH), or LA domain from homomultimeric enzyme (CimA_{LA}) with cpSFYFP, we developed three biosensors for detection of L-phenylalanine, L-valine, and L-isoleucine, respectively. We also confirmed that the multi-ligand-binding PH1780 can serve as a biorecognition element to derive diverse specific biosensors, and then constructed two SAM-specific biosensors with complementary detection ranges based on PH1780 and cpSFYFP. These biosensors assembled into proper quaternary structures consistent with those of the native PheA_{ACT} domain, IlvH, CimA_{LA} domain, and CBS domain^{13,15,21,25,29}. Each ligand molecule bound independently to individual monomers within the multimeric biosensors (hill slope close to 1). These results demonstrate that the developed biosensors preserved the intrinsic ligand-binding characteristics of their native allosteric regulatory domains. The methodology for developing GEFBs based on allosteric regulatory domains could be summarized in four steps: (i) selection of specific biorecognition elements, (ii) identification of optimal cpSFYFP insertion sites within loop regions to obtain a variant with obvious responsiveness and suitable affinity, (iii) optimization of linkers to maximize responsiveness, and (iv) functional testing (**Fig. 1a**). Notably, the affinities of allosteric regulatory domain-based GEFBs can be strategically modulated by shifting the insertion site of cpSFYFP. Besides SAMsor-0.34, multiple insertion variants with lower K_d values than Phesor, Valsor, and Ilesor were identified, such as Phesor-0.04 ($57 \pm 12 \mu\text{M}$), Valsor-0.01 ($35 \pm 29 \mu\text{M}$), and Ilesor-0.17 ($59.4 \pm 5.4 \mu\text{M}$) (**Supplementary Fig. 4, Supplementary Fig. 16, and Supplementary Fig. 28**). The higher affinities of these variants might be attributed to the insertion site of cpSFYFP being positioned distal to the binding pocket, which minimizes structural disruption and maintains higher architectural fidelity of the biorecognition element to its native conformation (**Supplementary Fig. 49**). This structure-function relationship provides a rational foundation for future GEFB engineering, thereby expanding the toolbox for metabolic imaging and *in vitro* diagnostics across varying concentration scales.”

Related experimental methods were added in the “Supplementary Methods” section of the revised manuscript as follows:

“Supplementary Methods

Characterization of Phesor, Valsor, Ilesor, and SAMsor *in vitro*

Oligomeric state analysis. To determine the native molecular weight of Phesor, Valsor,

Ilesor, and SAMsor in the absence or presence of target ligands, gel-filtration chromatography was performed using a Superdex 200 10/300 GL column (GE Healthcare, USA) and standard proteins including ferritin (440 kDa), aldolase (158 kDa), conalbumin (75 kDa), ovalbumin (43 kDa), and aprotinin (6.5 kDa). The elution buffer consisted of 50 mM H₃PO₄ and 150 mM NaCl (pH 7.2) with or without the addition of 1 mM L-phenylalanine, 1 mM L-valine, 1 mM L-isoleucine, or 100 μ M SAM. The oligomeric states of these biosensors were inferred by comparison of the native molecular weight with the theoretical molecular weight of the protein monomers.

Affinity analysis through thermal shift assay (TSA). For analysis of the binding properties of Phesor, Valsor, Ilesor, or SAMsor to target ligands by TSA, 6 μ M purified different biosensors, 5 $\times$ SYPRO Orange, and increasing concentrations of L-phenylalanine (0–50 mM), L-valine (0–70 mM), L-isoleucine (0–150 mM), or SAM (0–20 mM) were mixed in 50 μ L TSA buffer (20 mM Na₂HPO₄ and 150 mM NaCl, pH 7.4). 25 μ L of the mixture was then transferred into a 96-well white PCR plate (Roche, Switzerland). Ligand binding-induced thermal stability changes in these biosensors were analyzed using a LightCycler 480 system (Roche, Switzerland) with the following instrument settings: a temperature ramp from 25 to 95 °C at a heating rate of 0.05 °C s⁻¹, excitation at 533 nm, and emission at 580 nm. The T_m values were determined according to the derivative melt curves by calculating the first derivative of the raw fluorescence data at each temperature. The Δ T_m values were calculated by subtracting T_m values of control group (without any target ligands) from T_m values of experimental group. The Δ T_m values were then fitted to the ligand concentrations using OriginPro 2019 (OriginLab, USA) according to the following equation to calculate K_d value:

$$f([\text{Ligand}]) = \max + \frac{\min - \max}{1 + ([\text{Ligand}] / K_d)^p} \quad (2)$$

where [Ligand] and K_d represented ligand concentration and apparent dissociation constant, respectively.”

We hope these clarifications and the newly added data satisfactorily address your concerns. We stand ready to perform further experiments should you require additional evidence to support our conclusions.

2. I found it frustrating that I could not be certain of the sequence of many of the constructs used in the study - in some instances it wasn't precisely clear what the engineered proteins were or what had been optimised through random mutagenesis.

Response: We appreciate your constructive suggestions and sincerely apologize for the lack of detail in our original descriptions. In accordance with your advice, we have included four new supplementary figures in the revised manuscript to clearly illustrate the step-by-step development of Phesor, Valsor, Ilesor, SAMsor, and SAMsor_L. These figures explicitly depict the amino acid sequences of the final biosensors, their intermediate variants, the original biorecognition elements, and the improvements in detection properties before and after optimization (**Fig. 99, Fig. 100, Fig. 101, and Fig. 102**).

Taking the L-phenylalanine biosensor Phesor as an example, we first inserted cpSFYFP between residues 365I/366T of the PheA_{ACT} domain with an N-terminal linker of “Ser-Ala-Gly” and a C-terminal linker of “Gly-Gly-Cys”, generating the intermediate variant Phesor-0.38. This variant was subsequently optimized through random linker mutagenesis, yielding the final biosensor, Phesor, which features an N-terminal linker of “Ala-Arg-Leu” and a C-terminal linker of “Gln-His-Leu”. This optimization resulted in a dramatic enhancement in sensitivity, with the response magnitude (ΔR_{max}) increasing from $67.2 \pm 2.9\%$ (Phesor-0.38) to $4,030 \pm 40\%$ (Phesor) (**Fig. 99**).

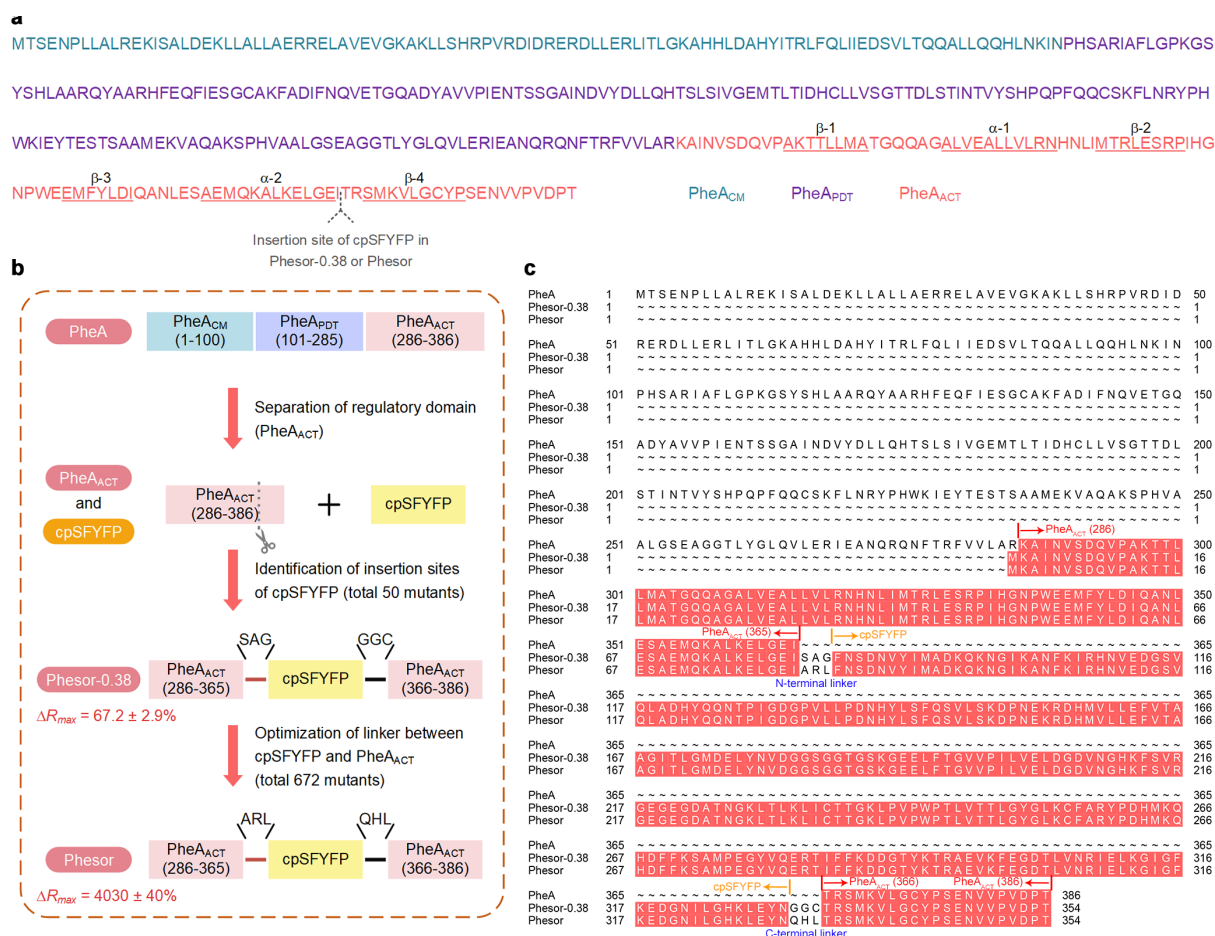


Figure 99 Workflow for the development of L-phenylalanine biosensor Phesor. **(a)** Schematic representations of the amino acid sequence and the predicted secondary structure of PheA. The chorismate mutase (PheA_{CM}) domain, prephenate dehydratase (PheA_{PDT}) domain, and ACT (PheA_{ACT}) domain of 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 residues 286K-365I/366T-386T of the PheA_{ACT} with an N-terminal linker of “Ala-Arg-Leu” and a C-terminal linker of “Gln-His-Leu”. **(c)** Sequence alignment between PheA, Phesor-0.38, and Phesor. The PheA_{ACT} region, the cpSFYFP region, and the N- and C-terminal linkers were indicated. (**Supplementary Fig. 2 in the revised manuscript**)

We have provided similarly detailed illustrations for the development processes of Valsor, Ilesor, SAMsor, and SAMsor_L, including their amino acid sequences and the improvements in detection properties before and after optimization (**Fig. 100, Fig. 101, and Fig. 102**).

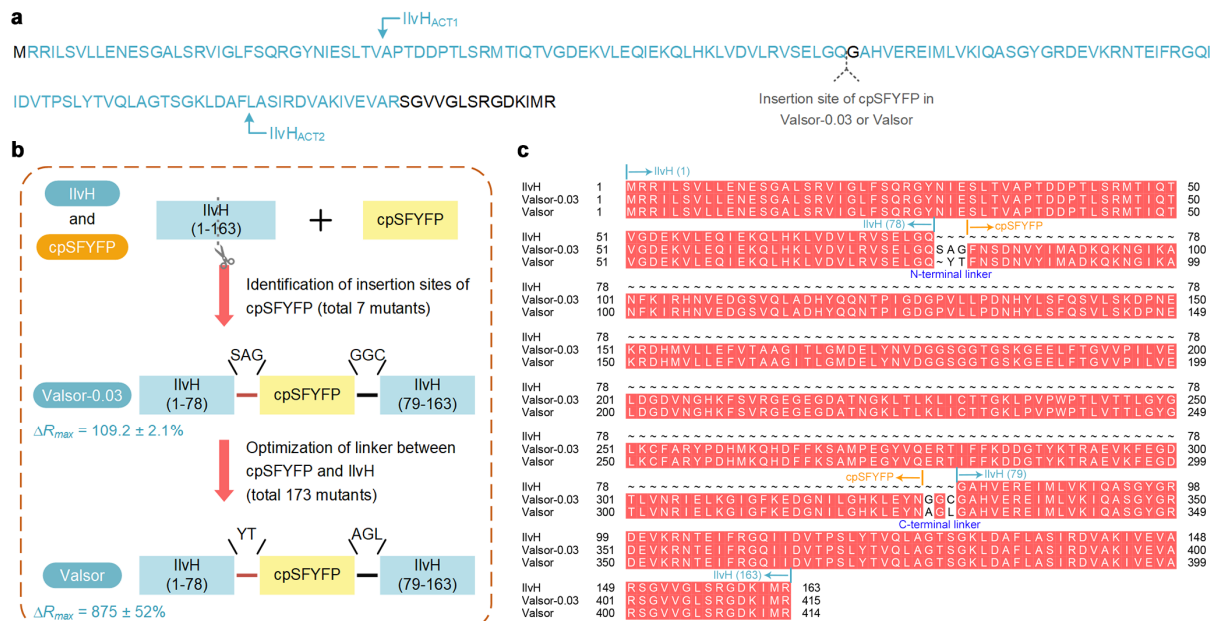


Figure 100 Workflow for the development of L-valine biosensor Valsor. **(a)** Schematic representation of the amino acid sequence of IlvH. The two ACT domains of IlvH were shown in cyan. **(b)** Schematic representation of the construction and optimization of Valsor. The final L-valine biosensor, Valsor, was constructed by inserting cpSFYFP between residues 1M-78Q/79G-163R of the IlvH with an N-terminal linker of “Tyr-Thr” and a C-terminal linker of “Ala-Gly-Leu”. **(c)** Sequence alignment between IlvH, Valsor-0.03, and Valsor. The IlvH region, the cpSFYFP region, and the N- and C-terminal linkers were indicated. (Supplementary Fig. 15 in the revised manuscript)

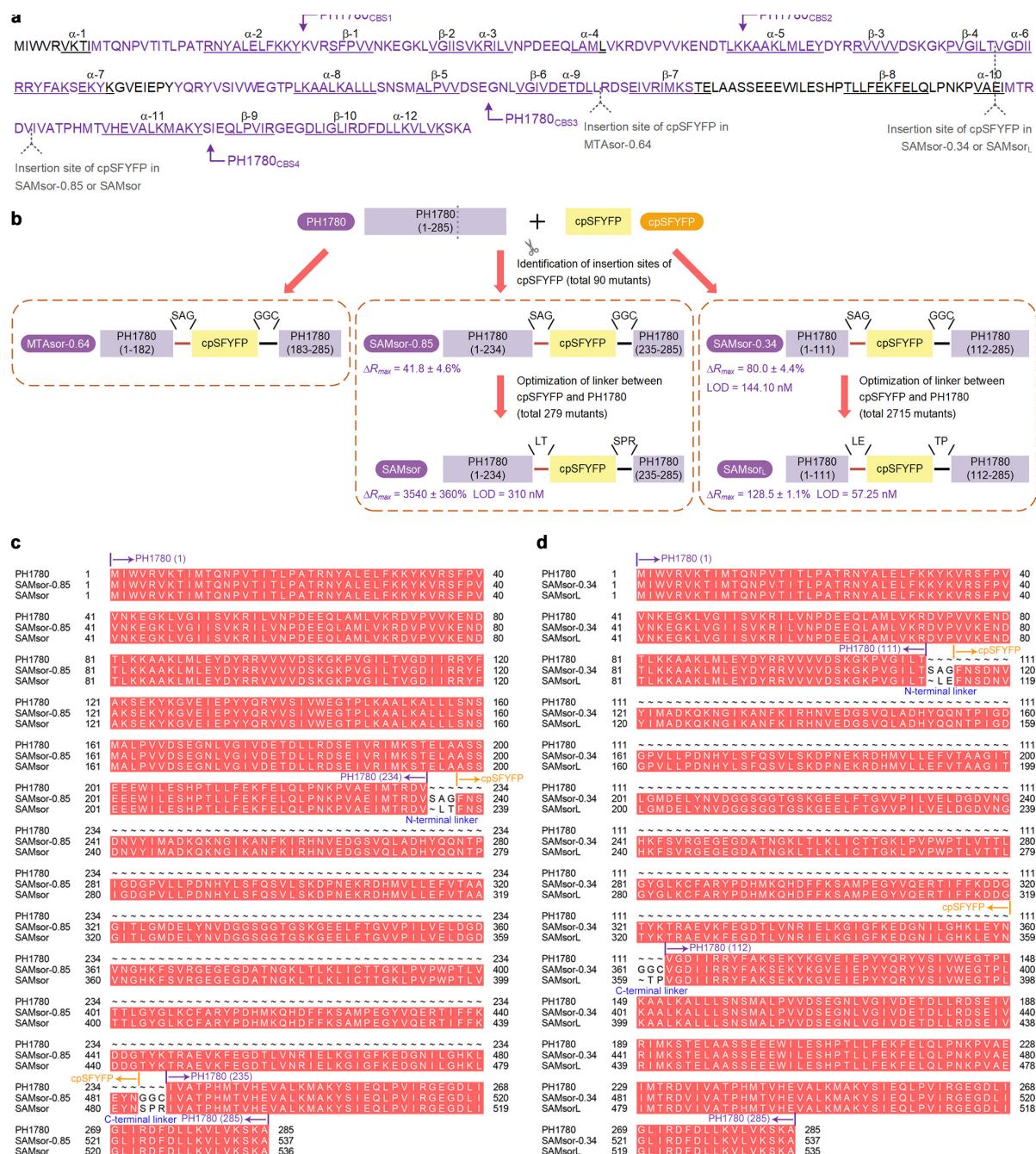


Figure 102 Workflow for the development of SAM biosensors SAMsor and SAMsor_L. **(a)** Schematic representations of the amino acid sequence and the predicted secondary structure of PH1780. The four CBS domains of PH1780 were shown in purple. The predicted α -helices and β -sheets of PH1780 were indicated 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 residues 1M-182L/183R-285A of the 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 residues 1M-234V/235I-285A of the 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 residues 1M-111T/112V-285A of the PH1780 with an N-terminal linker of “Leu-Glu” and a C-terminal linker of “Thr-Pro”. **(c)** Sequence alignment between PH1780, SAMsor-0.85, and SAMsor. The PH1780 region, the cpSFYFP region, and the N- and C-terminal linkers were indicated. **(d)** Sequence alignment between PH1780, SAMsor-0.34, and SAMsor_L. The PH1780 region, the cpSFYFP region, and the N- and C-terminal linkers were indicated. **(Supplementary Fig. 37 in the revised manuscript)**

In addition, to provide an at-a-glance summary, we have added a new table to the revised manuscript (**Table 6**). This table comprehensively summarizes the key physicochemical properties of the final biosensors, including the maximum fluorescence ratio change (ΔR_{max}), affinity (K_d), limit of detection (LOD), oligomeric state, excitation peak, emission peak, and acid dissociation constant (pK_a).

Table 6 Detection properties of Phesor, Valsor, Ilesor, SAMsor, SAMsor_L, and cpSFYFP. (Supplementary Table 4 in the revised manuscript)

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)	9.03	8.68	9.08	8.87	/	8.54
pK _a (with ligand) ^b	8.23	8.50	8.72	7.72	/	8.49 (L-phenylalanine) 8.49 (L-valine) 8.69 (L-isoleucine) 8.60 (SAM)

^aThe limit of detection (LOD) is calculated by interpolating the average background counts + (or -) 3 × standard deviation value (*Nat. Commun.*, 2021, 12:3619).

^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.

Related data were presented in **Supplementary Fig. 2, Supplementary Fig. 15, Supplementary Fig. 27, Supplementary Fig. 37, and Supplementary Table 4** in the revised manuscript. In addition, detailed information regarding the optimization process of each biosensor, particularly the linker sequences and detection performance before and after random linker mutagenesis, has been added in the “Results” section of the revised manuscript as follows:

For Phesor:

“Firstly, we constructed 50 biosensor variants by inserting cpSFYFP between amino acid residues located in the loop region of the PheA_{ACT} domain with an N-terminal linker of “Ser-Ala-Gly” and a C-terminal linker of “Gly-Gly-Cys” (**Supplementary Fig. 2 and Supplementary Fig. 4**). The 38th variant (denoted as Phesor-0.38, with cpSFYFP inserted between residues 365I/366T of the PheA_{ACT} domain) exhibited a higher maximum fluorescence ratio change (ΔR_{max}) of $67.2 \pm 2.9\%$ and an appropriate apparent dissociation constant (K_d) of $440 \pm 80 \mu\text{M}$, making it suitable for L-phenylalanine detection both in living cells and in human body fluids (**Fig. 2a and Supplementary Table 2**). Subsequently, we conducted random mutagenesis and high-throughput screening for linkers connecting the cpSFYFP and the PheA_{ACT} domain. Among a total of 672 mutants, the biosensor with the N-terminal linker of “Ala-Arg-Leu” and the C-terminal linker of “Gln-His-Leu” possessed the highest response magnitude to L-phenylalanine (**Fig. 2b and Supplementary Fig. 2b, c**). This biosensor was denoted as Phesor. Its amino acid sequence is provided in **Supplementary Table 3.**”

For Valsor:

“We constructed seven biosensor variants by inserting cpSFYFP into the following locations: the N-terminus or C-terminus of the intact IlvH, the middle of the two ACT domains of IlvH, the N-terminus or C-terminus of the separated IlvH_{ACT1} domain, or the N-terminus or C-terminus of the separated IlvH_{ACT2} domain (**Supplementary Fig. 16**). The variant Valsor-0.03, created by inserting cpSFYFP into the middle of the two ACT domains of intact IlvH, possessed a higher ΔR_{max} of $109.2 \pm 2.1\%$ and an appropriate K_d of $414 \pm 43 \mu\text{M}$ for L-valine (**Fig. 3b**). Subsequently, we systematically optimized the linker in Valsor-0.03 and identified a high-response magnitude biosensor, Valsor, with the N-terminal

linker of “Tyr-Thr” and the C-terminal linker of “Ala-Gly-Leu”, from 173 mutants (**Fig. 3c and Supplementary Fig. 15b, c).**”

For Ilesor:

“We constructed 36 biosensor variants by inserting cpSFYFP between amino acid residues located in the loop region of the Cim_{ALA} (**Supplementary Fig. 27a and Supplementary Fig. 28**). The 23rd variant (denoted as Ilesor-0.23, with cpSFYFP inserted between residues 463K/464T of the Cim_{ALA}) exhibited a higher ΔR_{max} of $97.0 \pm 2.4\%$ and an appropriate K_d of $1,700 \pm 660 \mu\text{M}$ for L-isoleucine (**Fig. 4b**). A high-response magnitude mutant with the N-terminal linker of “Phe-Arg-Thr” and the C-terminal linker of “Pro-His-Leu”, denoted as Ilesor, was then screened from 375 mutants through linker random mutagenesis (**Fig. 4c and Supplementary Fig. 27b, c).**”

For SAMsor:

“The 85th variant (denoted as SAMsor-0.85, with cpSFYFP inserted between residues 234V/235I of PH1780) showed high selectivity for SAM (**Fig. 5d**). Subsequently, we performed a systematic optimization of the SAM biosensor SAMsor-0.85 (**Supplementary Fig. 37b**). A high-response magnitude mutant with the N-terminal linker of “Leu-Thr” and the C-terminal linker of “Ser-Pro-Arg”, denoted as SAMsor, was screened from 279 mutants through linker random mutagenesis (**Fig. 6b and Supplementary Fig. 37b, c).**”

We hope these clarifications address your concerns.

3. These AA binding domains do rely on the appropriate quaternary structure to form binding sites. There is no comment on this or assessment of quaternary structure of any of the sensors. As quaternary structure for some of these relies on the catalytic domain interfaces is it possible that appropriate quaternary structures have not assembled - or does this relate to the poor dissociation constants? I'm concerned about the impact of protein conc on relative fluorescence here and would like to see results that demonstrate predictable response.

Response: We have combined our response to this comment with our response to **Comment 1**, where we have comprehensively addressed the assessment of quaternary structures (**Fig. 88**) and the effects of protein concentration on fluorescence response (**Fig. 89**). We deeply appreciate your constructive suggestions. These insights are critical not only

for validating our current biosensors but also for guiding the rational design of future allosteric enzyme-derived GEFBs with adaptable detection ranges across diverse biological scenarios.

4. I had some concerns over the use of normalised florescence data - while this was useful for some presentations - it felt like it was hiding the absolute changes that should be available for inspection in the supplementary material.

Response: Thanks for your good suggestion. We fully agree that the absolute fluorescence values are crucial for a comprehensive and transparent interpretation of the results. Given that the revised manuscript already contains a substantial number of Supplementary Figures (49 in total), we have replaced the majority of the normalized fluorescence data with absolute (non-normalized) values throughout the revised manuscript.

We have retained normalized data only in four specific instances involving dynamic monitoring in human cells. In these figures, we present the cpSFYFP-corrected fluorescence ratios to account for potential intracellular pH fluctuations (as shown in **Fig. 103**, **Fig. 105**, **Fig. 107**, and **Fig. 109** below). Crucially, to fully address your concern, the corresponding original non-normalized datasets for these specific figures have now been added to the “Supplementary Information” file (as shown in **Fig. 104**, **Fig. 106**, **Fig. 108**, and **Fig. 110** below).

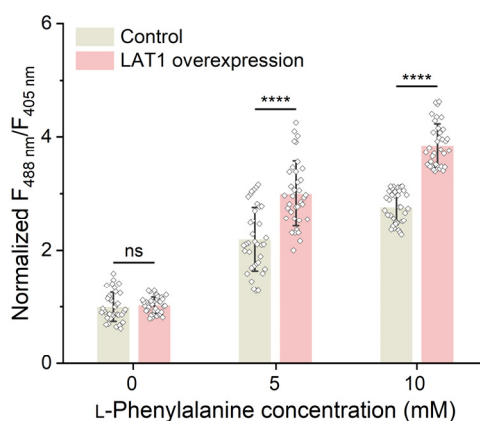


Figure 103 Analysis of the function of LAT1 in the uptake of L-phenylalanine in HEK293FT cells using Phesor. Data were corrected by cpSFYFP. All data shown are means $\pm$ s.d. ($n = 36$ cells). ****, $P < 0.0001$; ns, no significant difference ($P \geq 0.05$); one-way ANOVA test with Tukey’s Multiple Comparison Test. (Fig. 2i in the revised manuscript)

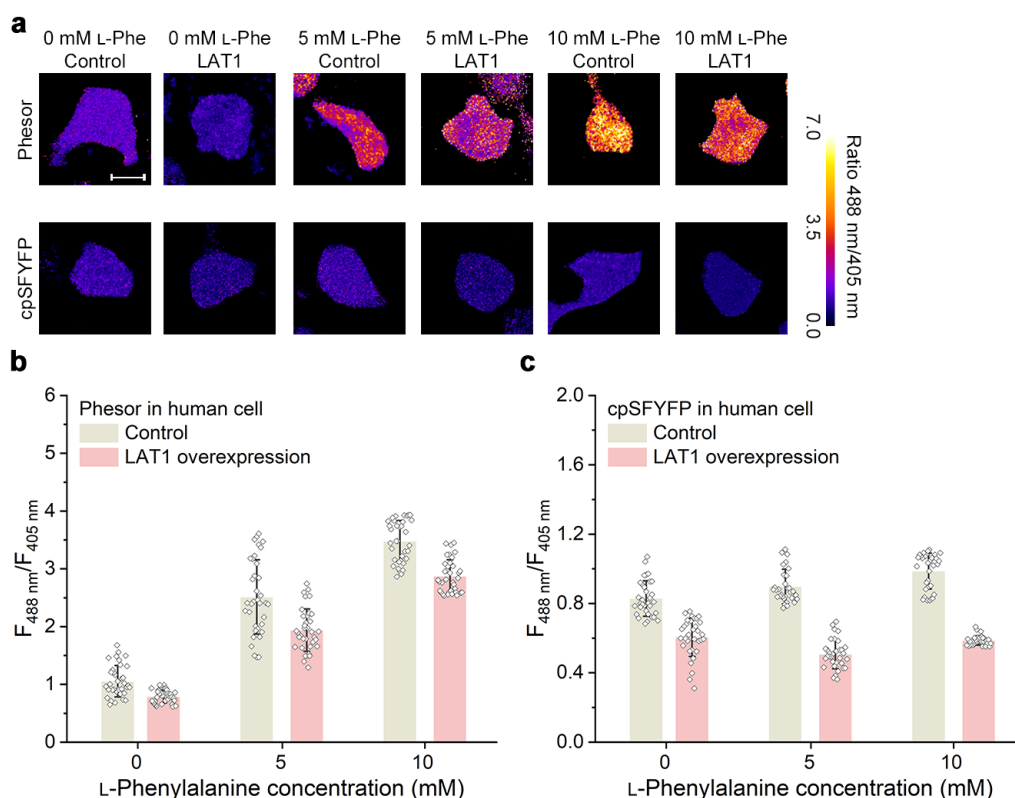


Figure 104 Analysis of the function of LAT1 in the uptake of L-phenylalanine in HEK293FT cells using Phesor. **(a)** Ratiometric images of single HEK293FT cell expressing Phesor or cpSFYFP in response to L-phenylalanine addition and LAT1 overexpression. Cells were treated with 0, 5, or 10 mM L-phenylalanine with or without LAT1 overexpression, and the fluorescence ratios were determined after 20 h. Scale bar, 10 μ m. **(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. 103**. All data shown are means $\pm$ s.d. (n = 36 cells for **b**; n = 33 cells for **c**).

(Supplementary Fig. 10 in the revised manuscript)

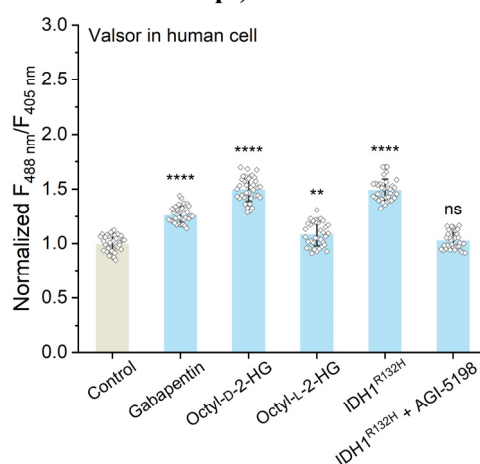


Figure 105 Analysis of the effects of different BCAT inhibitors on intracellular L-valine levels in HEK293FT cells using Valsor. The concentrations of gabapentin, octyl-D-2-HG (octyl-D-2-

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. Data were corrected by cpSFYFP. All data shown are means $\pm$ s.d. ($n = 36$ cells). **, $P < 0.01$; ****, $P < 0.0001$; ns, no significant difference ($P \geq 0.05$); one-way ANOVA test with Tukey's Multiple Comparison Test. (**Fig. 3i in the revised manuscript**)

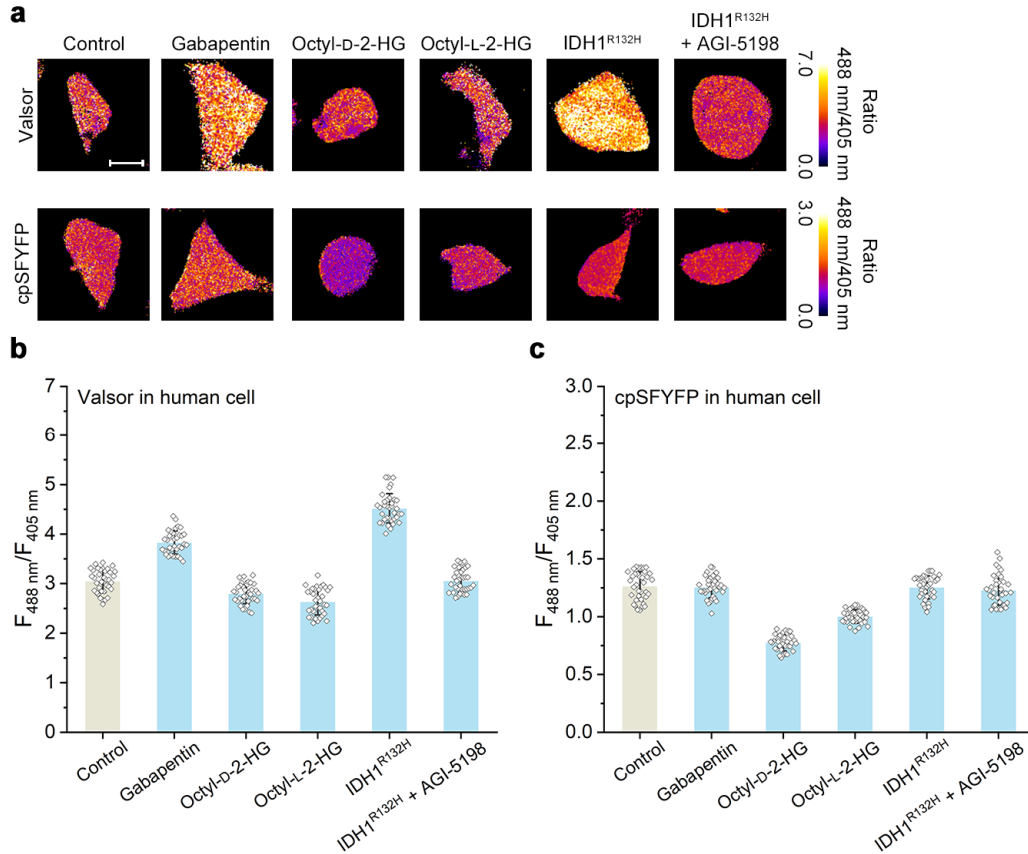


Figure 106 Analysis of the effects of different BCAT inhibitors on intracellular L-valine levels in HEK293FT cells using Valsor. **(a)** Ratiometric images of single HEK293FT cell expressing Valsor or cpSFYFP in response to different inhibitors treatment. The concentrations of gabapentin, octyl-D-2-HG (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. **(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. 105**. All data shown are means $\pm$ s.d. ($n = 36$ cells). (**Supplementary Fig. 23 in the revised manuscript**)

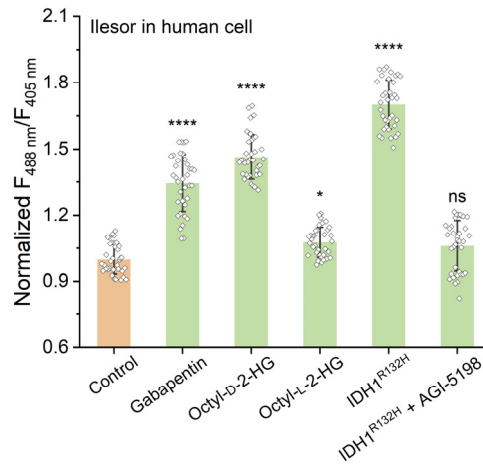


Figure 107 Analysis of the effects of different BCAT inhibitors on intracellular L-isoleucine levels in HEK293FT cells using Ilesor. Data were corrected by cpSFYFP. All data shown are means $\pm$ s.d. ($n = 36$ cells). **, $P < 0.01$; ****, $P < 0.0001$; ns, no significant difference ($P \geq 0.05$); one-way ANOVA test with Tukey's Multiple Comparison Test. (**Fig. 4i in the revised manuscript**)

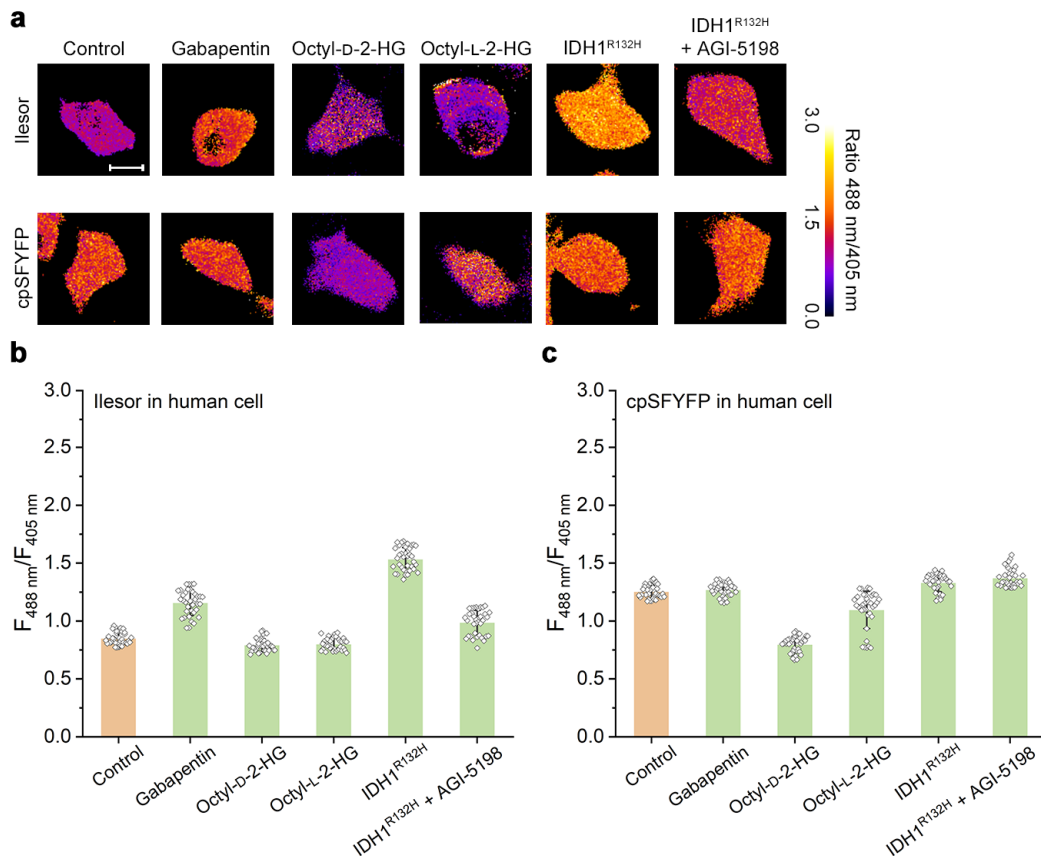


Figure 108 Analysis of the effects of different BCAT inhibitors on intracellular L-isoleucine levels in HEK293FT cells using Ilesor. **(a)** Ratiometric images of single HEK293FT cell expressing Ilesor or cpSFYFP in response to different inhibitors treatment. The concentrations of gabapentin, octyl-D-2-HG, 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. **(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. 107**. All data shown are means $\pm$ s.d. ($n = 36$ cells).

(Supplementary Fig. 33 in the revised manuscript)

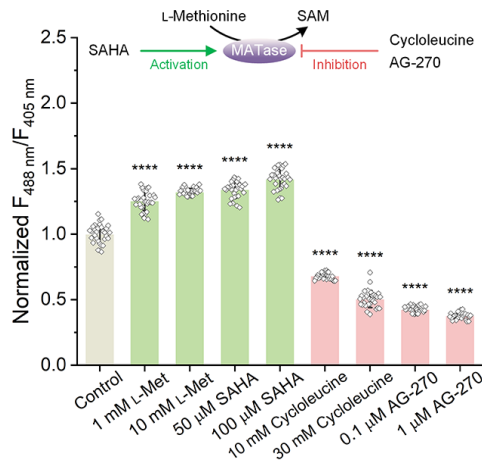


Figure 109 Analysis of the effects of different MATase activator or inhibitors on intracellular SAM levels in HEK293FT using SAMsor. Data were corrected by cpSFYFP. All data shown are means $\pm$ s.d. ($n = 36$ cells). ****, $P < 0.0001$; one-way ANOVA test with Tukey's Multiple Comparison Test. (**Fig. 6j** in the revised manuscript)

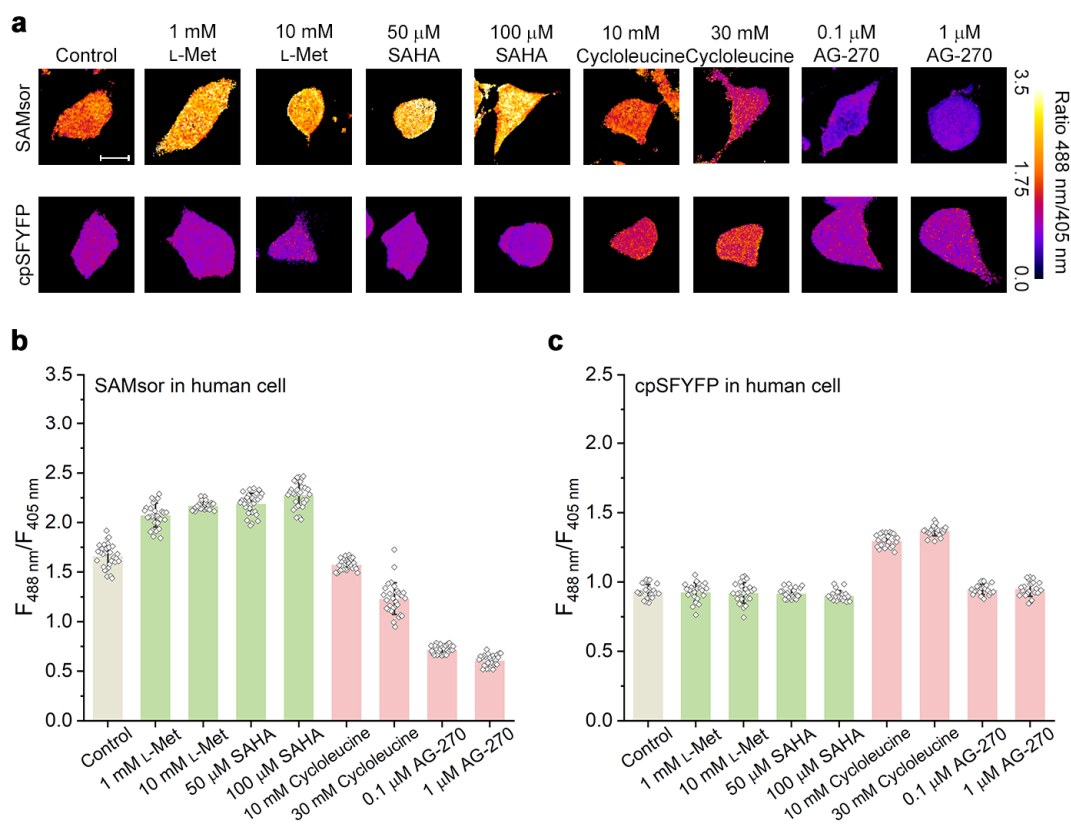


Figure 110 Analysis of the effects of different MATase activator or inhibitors on intracellular SAM levels in HEK293FT using SAMsor. (a) Ratiometric images of single HEK293FT cell expressing SAMsor or cpSFYFP in response to different MATase activator or inhibitors treatment. The fluorescence ratios were

determined 20 h after treatment with different activator or inhibitors. Scale bar, 10 μm . **(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. 109**. All data shown are means $\pm$ s.d. (n = 30 cells for **b**; n = 23 cells for **c**). (**Supplementary Fig. 45 in the revised manuscript**)

Related data were presented in **Fig. 2i**, **Fig. 3i**, **Fig. 4i**, **Fig. 6j**, **Supplementary Fig. 10**, **Supplementary Fig. 23**, **Supplementary Fig. 33**, and **Supplementary Fig. 45** in the revised manuscript. We believe these modifications significantly enhance the transparency of our data.

5. Repeat experiments - I was unsure of the nature of the replicates given in some places - in particular for the cellular work were these biological replicates?

Response: Thanks for your good comment. In all cellular experiments, the reported sample sizes (n) represent the total number of individual cells analyzed from three independent biological replicates (three independent experiments). This quantification strategy aligns with established statistical standards widely utilized in similar studies (*Dev. Cell*, 2020, 53:240–252; *Cell Metab.*, 2023, 35:200–211). For instance, in **Fig. 111**, the value n = 25 corresponds to 25 individual cells analyzed across three independent experiments.

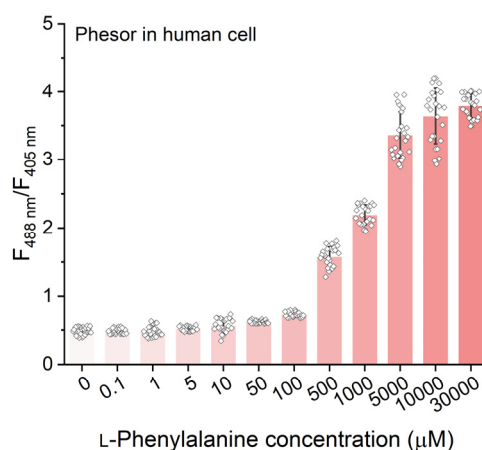


Figure 111 Gradient responses of Phesor expressed in permeabilized HEK293FT cells to increasing concentrations of L-phenylalanine addition. All data shown are means $\pm$ s.d. (n = 25 cells). (**Fig. 2h in the revised manuscript**)

Related data were presented in **Fig. 2h** in the revised manuscript. In addition, to improve the clarity and rigor of our statistical methods, we have included a brief description in the

“Methods” section of the revised manuscript as follows:

“Sample numbers (n) and experimental repeats are indicated in the figure legends. Similar results were obtained from at least three independent experiments in all experiments. For human cell studies, the n values represent the number of individual cells analyzed from three independent experiments.”

6. All the measurements must be reported to the appropriate significant figures that correspond to the accuracy of the measurement. Sometimes 5 or 6 sf are used in measurements or errors! Reporting figures such as 1659.85 ± 110.02 or 444.08 ± 97.38 just suggests that the accuracy of the data is not well understood. Sometimes also % values in the text are reported without errors which makes the excess significant figures even more misleading.

Response: Thanks for your constructive comment. We fully agree that the significant figures originally reported did not accurately reflect the precision of our measurements. Accordingly, we have thoroughly revised all numerical values and error estimates throughout the manuscript. For example, the value 1659.85 ± 110.02 has been corrected to 1660 ± 110 .

In addition, all percentage values in the revised manuscript are now reported with their corresponding standard deviations. For example, the value 24.0% has been corrected to $24.0 \pm 1.3\%$.

We believe these revisions significantly improve the accuracy and clarity of our data presentation, and hope they meet your satisfaction.

Reviewers' comments:

Reviewer #1 (Remarks to the Author):

I would like to thank the authors for their comprehensive response and the immense effort put into revising the manuscript. The addition of extensive new data, particularly the development of the high-affinity SAMsor_L, the detailed subcellular imaging, and the validation with authentic clinical samples, has significantly strengthened the work.

Regarding my previous Major Concern 1 (Suitability for intracellular imaging), the new subcellular localization experiments convincingly demonstrate that these sensors can resolve metabolite fluctuations in specific organelles. This effectively highlights the unique value of these genetically encoded sensors over traditional methods, addressing my concerns regarding spatiotemporal resolution. Similarly, my concerns regarding the manuscript's structure and the generalizability of the design strategy (previous Major Concerns 3 and 4) have been well addressed. The inclusion of a unified workflow schematic successfully articulates the design pipeline, enhancing the logical flow. I also appreciate the detailed analysis of insertion sites relative to secondary structures and ligand-binding pockets. This analysis provides valuable insights into the structure-function relationship—specifically how the distance between the insertion site and the binding pocket modulates affinity—thereby offering a clearer guide for future engineering efforts.

However, regarding the validation of biosensors for *in vitro* detection, I have a remaining specific concern regarding the characterization of the results. Specifically, the claim that the current L-phenylalanine sensor (Phesor) is suitable for “rapid” and “point-of-care diagnostics” is not supported by the methods described.

In the Discussion (Lines 515–519), the authors state: “Compared to traditional HPLC- or mass spectrometry-based methods, Phesor is easy to handle, cost-effective, rapid, and compatible with high-throughput assays (Supplementary Table 5), making it an ideal tool for point-of-care diagnostics of L-phenylalanine-related diseases.” However, a close examination of the “Preparation of human body fluid samples” section (Lines 792–800) reveals that the sample preparation for dried blood spots involves “sonicating for 20 min,” “stirring overnight,” and “completely drying in a vacuum centrifuge” before resuspension. A workflow

requiring overnight stirring and bulky equipment fundamentally contradicts the definition of “rapid” and “point-of-care diagnostics”. It appears that this laborious processing is necessary to concentrate the samples because the selected Phesor variant ($K_d \approx 1.7$ mM) has a relatively low affinity compared to the physiological phenylalanine levels in healthy or mild-PKU blood samples (~ 50 – 200 μ M). I note that the authors identified a high-affinity variant during screening (Phesor-0.04, $K_d \approx 57$ μ M) but chose to optimize the lower-affinity Phesor-0.38 variant. While the resulting Phesor ($K_d \approx 1.7$ mM) has a high dynamic range suitable for intracellular imaging of elevated metabolite levels, its use for serum detection necessitates the extensive sample pre-concentration mentioned above.

While the experimental validation is now sufficient and demonstrates the sensor’s robustness in complex matrices, the textual claims regarding “easy to handle” and “point-of-care diagnostics” in the Discussion (specifically Lines 515–519) are not supported by the described workflow and should be rephrased. The current workflow does not offer a timeline advantage over traditional HPLC methods, and acknowledging this limitation would provide a more balanced view for the readers.

Minor Issue:

On page 55 of the Supplementary Information file, Supplementary Figure 37 appears to be displayed incompletely or cut off. Please check and correct this in the final version.

In summary, the manuscript is now of high quality and fits the scope of Nature Communications. With the textual modifications suggested above to clarify the practical positioning of the *in vitro* assay, I support the publication of this work.

Reviewer #2 (Remarks to the Author):

The authors have thoroughly revised the manuscript in response to the reviewers’ comments. The revisions have substantially improved the quality, clarity, and rigor of the study. The reviewer is supportive of publication in Nature Communications. However, several points should be further addressed to strengthen the manuscript.

Major issue #4.

The authors now convincingly demonstrate the complete reversibility of SAMsor using purified protein with additional elution steps. This represents an important technical

improvement. However, the process leading to this refinement, as well as the authors' speculation that the originally observed incomplete reversibility may have resulted from residual SAM remaining in the assay system, would be highly valuable information for end users.

To provide transparency and practical guidance, the original dataset (Fig. 6e in the initial submission) should be retained in the revised manuscript, accompanied by a clear explanation of the experimental optimization and the rationale for the updated results. Including both versions would enhance the manuscript's instructional value and reproducibility.

Major issue #5.

The reported pK_a values of each biosensor in the presence and absence of ligand are very similar. It is important to clarify whether this observation is consistent with the large ratiometric response (F_{488}/F_{405}) observed around pH ~7 (Fig. 3e).

If the pK_a values remain largely unchanged upon ligand binding, this would suggest that the primary mechanism of fluorescence modulation is unlikely to arise from a shift in the equilibrium between the neutral and anionic chromophore states. Instead, the response may predominantly reflect changes in extinction coefficient, quantum yield, chromophore environment, or other structural factors.

To clarify this point, the authors should present full pH titration curves for each biosensor in both the ligand-bound and ligand-free states (currently referenced in Fig. 3h), rather than reporting only the ligand-bound state. These curves would allow readers to directly evaluate whether ligand binding alters the protonation equilibrium or instead modulates fluorescence through alternative mechanisms.

An expanded discussion addressing the likely molecular basis of the fluorescence response in these four biosensors would further strengthen the mechanistic insight of the study.

Reviewer #3 (Remarks to the Author):

The authors have thoroughly addressed the concerns raised in the first round of review. I particularly appreciate the addition of applying GEFBs in both clinical and bioprocess contexts, as well as the development of sensor variants with compartment-specific

localization. These revisions have significantly strengthened the manuscript. That said, I still have several comments that could further improve the clarity of the manuscript and enhance the presentation of the tools' capabilities.

Major comments:

1. The authors used degenerate primers for random linker mutations. The authors should provide more details on the nature of these mutations (such as linker length and amino acid compositions) and the theoretical diversity of a mutation library. For most GEFBs, only several hundred variants were screened, whereas six randomized linker residues could in principle generate up to 20^6 possible combinations.

2. The authors demonstrate that the ratiometric GEFB responses are independent of sensor concentration. To further support this point, I suggest including the corresponding single-channel fluorescence responses for comparison.

3. What is the difference between ΔR_{max} and $\Delta R/R_{min}$, as both metrics are frequently used throughout the manuscript? I also notice that some sensor variants exhibit negatively-going responses, such as Phesor-0.38, Valsor-0.03, MTAsor-0.64 and SAMsorL. For such sensors, reporting ΔR_{max} and $\Delta R/R_{min}$ may be less appropriate, particularly for imaging in living cells or for quantifying exogenous ligands, since achieving R_{min} requires saturate ligand concentrations, which are usually hard to reach. I therefore suggest presenti $\Delta R/R_0$ (R without ligands) instead of ΔR_{max} in the dose-response curves like Fig. 7d and in high-throughput screening results like Fig. 7c.

4. The authors have increased the utility of these tools by generating GEFB variants targeted to different subcellular compartments, which revealed lower amino acid/SAM levels in mitochondria compared with the cytosol and nucleus. To further highlight the advantage of high spatiotemporal resolution, I suggest using these compartment-localized sensors to monitor spatial and temporal differences in drug-induced amino acid fluctuations through time-lapse imaging. For example, the subcellularly targeted Ilesor and Valsor could be used to examine valine and isoleucine dynamics in response to gabapentin or octyl-D-2-HG, or different SAMsor variants could be applied to track SAHA- and AG-270-regulated SAM synthesis. Such experiments could potentially provide additional insight into the compartment-specific and kinetic features of valine/isoleucine/SAM metabolism.

5. In the table comparing detection performance between HPLC and biosensors, the authors say that HPLC needs $> 100 \mu\text{L}$ sample, which is unusual given the very high sensitivity of HPLC. I suggest revisiting this point for accuracy. Additionally, it may be more informative for readers to compare the concentration detection ranges (or limits of detection) of these approaches, rather than focusing on sample volume requirements.

Minor comments:

1. In Fig. 1b, do the colors represent different secondary structures? If so, this should be explained in the figure legend.

2. In line 97, “feedback inhibits the activities of Phe_{ACM} and Phe_{APDT}” should be “act as a feedback inhibitor of Phe_{ACM} and Phe_{APDT} activities”.

3. For the high-throughput screening results (e.g., Fig. 2b, 3c, 4c, 6b and 7c), it would improve clarity to label the starting and final versions. For example, in Fig. 2b, the variants corresponding to Phesor-0.38 and the final Phesor could be explicitly marked.

4. In line 376-379, the descriptions of “diminished” and “abolished” response appear to be reversed, as mutated MTAsor-0.64 still show some response to MTA. Besides, the two panels lack statistic comparison (at least no significance indicators are labeled), and therefore stating “significantly” is not appropriate.

5. In Fig. 6e, the authors use $100 \mu\text{M}$ MTA and $10 \mu\text{M}$ SAH to demonstrate the selectivity of SAMor. However, even $10 \mu\text{M}$ MTA induce a modest response. According to Supplementary Fig. 40, the physiological concentrations of MTA, adenosine and SAH fall within a similar range. Therefore, it will be more appropriate to use SAH at $100 \mu\text{M}$ in Fig. 6e for a more balanced comparison.

6. In heatmaps like supplementary Fig. 4a, the legend of ΔR_{max} is missing a “%” symbol. In addition, I can not find such heatmaps for Valsor development.

7. The upper parts of some supplementary figures including Fig. 2, 4 and 37 appear to be cropped or incomplete. For example, the label “ ΔR_{max} ” in supplementary Fig. 4 is partially obscured.

8. Using structural modeling, the authors found that the low- K_d variants tend to have cpSFYFP insertion sites distal to the ligand-binding pockets. It will be clearer to highlight the ligand-binding sites in the structures. In addition, the figure legends should specify whether

these structures were obtained from experimental PDB entries or predicted (e.g., by AF3).

9. The authors state that PLIP website was used for binding site prediction for PheA.

Was the same approach applied to the other sensors as well?

10. Compared with the 12-hour permeabilization shown in Fig. 4h of the original manuscript, the signal kinetics after 24-hour permeabilization in current Fig. 4e seems even slower. How to explain this unexpected result?

11. Alphafold3 is not a simple homology modeling tool. Please use the term “structural modeling” or “structural prediction” instead of “homology modeling”.

Reviewer #4 (Remarks to the Author):

This manuscript has undergone a thorough revision in response to the reviewers comments. The manuscript has undergone considerable change addressing my primary concerns about the data that had been presented. I believe appropriate data is now included in both the main manuscript and the supplementary information to allow for publication.

Responses to the reviewers' comments point-by-point:

We sincerely thank the reviewers for their constructive comments and thoughtful suggestions, which have been very helpful in improving our manuscript. We have carefully considered all comments and revised the manuscript accordingly. A detailed point-by-point response to each comment is provided below:

To Reviewer #1:

I would like to thank the authors for their comprehensive response and the immense effort put into revising the manuscript. The addition of extensive new data, particularly the development of the high-affinity SAMsor_L, the detailed subcellular imaging, and the validation with authentic clinical samples, has significantly strengthened the work.

Regarding my previous Major Concern 1 (Suitability for intracellular imaging), the new subcellular localization experiments convincingly demonstrate that these sensors can resolve metabolite fluctuations in specific organelles. This effectively highlights the unique value of these genetically encoded sensors over traditional methods, addressing my concerns regarding spatiotemporal resolution. Similarly, my concerns regarding the manuscript's structure and the generalizability of the design strategy (previous Major Concerns 3 and 4) have been well addressed. The inclusion of a unified workflow schematic successfully articulates the design pipeline, enhancing the logical flow. I also appreciate the detailed analysis of insertion sites relative to secondary structures and ligand-binding pockets. This analysis provides valuable insights into the structure-function relationship—specifically how the distance between the insertion site and the binding pocket modulates affinity—thereby offering a clearer guide for future engineering efforts.

Response: We sincerely appreciate your positive evaluation of our revised manuscript and are encouraged by your recognition of our efforts. Your constructive critiques in the initial review were instrumental in enhancing the practical applicability and novelty of our work. We have carefully addressed your remaining concerns point-by-point below, and we hope the updated version is to your satisfaction.

However, regarding the validation of biosensors for in vitro detection, I have a remaining specific concern regarding the characterization of the results. Specifically, the claim that the current L-phenylalanine sensor (Phesor) is suitable for “rapid” and “point-of-care diagnostics” is not supported by the methods described.

In the Discussion (Lines 515–519), the authors state: “Compared to traditional HPLC- or mass spectrometry-based methods, Phesor is easy to handle, cost-effective, rapid, and compatible with high-throughput assays (Supplementary Table 5), making it an ideal tool for point-of-care diagnostics of L-phenylalanine-related diseases.” However, a close examination of the “Preparation of human body fluid samples” section (Lines 792–800) reveals that the sample preparation for dried blood spots involves “sonicating for 20 min,” “stirring overnight,” and “completely drying in a vacuum centrifuge” before resuspension. A workflow requiring overnight stirring and bulky equipment fundamentally contradicts the definition of “rapid” and “point-of-care diagnostics”. It appears that this laborious processing is necessary to concentrate the samples because the selected Phesor variant ($K_d \approx 1.7 \text{ mM}$) has a relatively low affinity compared to the physiological phenylalanine levels in healthy or mild-PKU blood samples ($\sim 50\text{--}200 \text{ }\mu\text{M}$). I note that the authors identified a high-affinity variant during screening (Phesor-0.04, $K_d \approx 57 \text{ }\mu\text{M}$) but chose to optimize the lower-affinity Phesor-0.38 variant. While the resulting Phesor ($K_d \approx 1.7 \text{ mM}$) has a high dynamic range suitable for intracellular imaging of elevated metabolite levels, its use for serum detection necessitates the extensive sample pre-concentration mentioned above.

While the experimental validation is now sufficient and demonstrates the sensor’s robustness in complex matrices, the textual claims regarding “easy to handle” and “point-of-care diagnostics” in the Discussion (specifically Lines 515–519) are not supported by the described workflow and should be rephrased. The current workflow does not offer a timeline advantage over traditional HPLC methods, and acknowledging this limitation would provide a more balanced view for the readers.

Response: We sincerely appreciate your constructive comments. As you correctly noted, it is inappropriate to broadly describe the Phesor-based clinical diagnostic workflow as “rapid”, “point-of-care”, and “easy to handle”, particularly for dried blood spot (DBS) samples.

According to current clinical guidelines, the management of phenylketonuria necessitates large-scale newborn screening followed by clinical confirmation. DBS samples are pivotal in newborn screening due to their convenience for home-based sampling, transport, and storage. In contrast, clinical point-of-care testing typically relies on the direct analysis of liquid matrices, such as blood, serum, or urine samples (*GeneReviews*, 2025, NBK1504; *Nat. Rev. Dis. Primers*, 2021, 7:36; *Orphanet J. Rare Dis.*, 2017, 12:162).

In this study, we demonstrated the clinical applicability of Phesor using human serum, urine, and DBS samples. Traditional methods for serum and urine analysis, such as HPLC or mass spectrometry, require laborious deproteinization processes and entail a sequential 30-min analysis per sample. In contrast, the Phesor assay merely requires mixing a 10 μ L liquid sample (without laborious pretreatments, such as deproteinization, concentration, or centrifugation) with 10 μ L of purified biosensor in a 384-well plate, followed by a direct fluorescence readout within 2 min. This streamlined and high-throughput workflow highlights the suitability of Phesor for point-of-care applications in these liquid matrices.

However, for DBS samples, efficiently extracting L-phenylalanine from the filter paper matrix is an inherently laborious process. In our protocol, the blood spot areas (~ 0.7 cm in diameter, containing ~ 25 – 75 μ L of blood) were completely excised and immersed in 500 μ L of Tris-HCl buffer (pH 7.4). The mixture was sonicated for 20 min, vortexed for 2 min, and stirred overnight, followed by centrifugation at $12,000 \times g$ for 10 min to collect the supernatant. These cumbersome extraction steps are important for maximizing L-phenylalanine recovery. Subsequently, the resulting supernatant was completely dried using a vacuum centrifuge at 45°C and resuspended in an appropriate volume of 50 mM Tris-HCl (pH 7.4) to ensure precise quantification by Phesor.

As you correctly noted, the Phesor assay does not circumvent the time-consuming L-phenylalanine extraction from DBS required by traditional HPLC methods. Nevertheless, for the downstream quantification, HPLC mandates a sequential 30-min analysis per sample. In contrast, the Phesor assay merely requires mixing a 10 μ L aliquot with 10 μ L of purified biosensor in a 384-well plate to achieve a rapid, parallel fluorescence readout within 2 min. This substantial advantage in throughput effectively mitigates the labor and equipment constraints of large-scale testing.

Furthermore, we strongly agree with your insightful comment that the current biosensor, Phesor ($K_d \approx 1.7$ mM; LOD ≈ 48.52 μ M), features a relatively high detection range ideal for intracellular applications. To ensure highly precise quantification in clinical DBS samples, we incorporated the above-mentioned pre-analytical enrichment step (drying and resuspension). We previously developed SAMsor_L, a highly sensitive SAM biosensor variant tailored for clinical applications, by combining rational conformational coupling with random mutation screening. Following a similar design rationale, engineering a sensitive L-phenylalanine biosensor by optimizing the high-affinity variant Phesor-0.04 ($K_d \approx 57$ μ M) would effectively streamline the workflow for large-scale, DBS-based newborn screening. Therefore, we have explicitly integrated your valuable perspective into the “Discussion” section of the revised manuscript as a key avenue for future research. Concurrently, we have rectified the broad descriptions (i.e., “rapid”, “point-of-care”, and “easy to handle”) to present a more objective and rigorous description of the Phesor-based clinical diagnostic workflow, as follows:

“In addition, L-phenylalanine is a critical biomarker for phenylketonuria diagnosis and therapeutic monitoring²⁰. Compared with traditional HPLC- or mass spectrometry-based methods, Phesor provides a point-of-care, easy-to-handle, cost-effective, and high-throughput alternative for clinical serum and urine testing (**Supplementary Table 5**). However, due to the relatively high detection range of Phesor, additional sample enrichment was incorporated to ensure the precise quantification of L-phenylalanine from dried blood spots. Future development of a higher-affinity variant based on Phesor-0.04 would effectively streamline the workflow for large-scale, dried blood spot-based newborn screening²⁰.”

Accordingly, we have carefully examined the manuscript to ensure precise phrasing throughout. Furthermore, we have provided a detailed step-by-step DBS processing workflow in the “Methods” section of the revised manuscript as follows:

“Preparation of human body fluid samples and bacterial fermentation medium samples

Dried blood spot samples from healthy children and children with phenylketonuria were prepared by spotting ~ 25 – 75 μ L of whole blood onto filter paper cards. To maximize the recovery of L-phenylalanine from the matrix, the blood spot areas were completely excised, divided into small fragments, and immersed in 500 μ L of Tris-HCl buffer (pH 7.4). The

mixture was sonicated for 20 min, vortexed for 2 min, and stirred overnight, followed by centrifugation at $12,000 \times g$ for 10 min to collect the supernatant. To concentrate the samples for Phosor detection, the resulting supernatant was then completely dried in a vacuum centrifuge (Concentrator Plus, Eppendorf, Germany) at 45 °C, resuspended in an appropriate volume of 50 mM Tris-HCl (pH 7.4), and stored at $-80\text{ }^{\circ}\text{C}$.”

We sincerely hope these modifications satisfactorily address your concerns. If additional clarifications or experimental data are desired, we would be pleased to continue refining our manuscript.

Minor Issue:

On page 55 of the Supplementary Information file, Supplementary Figure 37 appears to be displayed incompletely or cut off. Please check and correct this in the final version.

Response: We sincerely appreciate your careful review. We have corrected the cropping error in Supplementary Fig. 37 and thoroughly reviewed all other figures to ensure their proper formatting in the updated Supplementary Information.

In summary, the manuscript is now of high quality and fits the scope of Nature Communications. With the textual modifications suggested above to clarify the practical positioning of the in vitro assay, I support the publication of this work.

Response: We sincerely appreciate the time and effort you have dedicated to reviewing our manuscript. We hope that our responses and the corresponding revisions have satisfactorily addressed your remaining concerns. Thank you again for your constructive feedback and for your support of this work.

To Reviewer #2:

The authors have thoroughly revised the manuscript in response to the reviewers' comments. The revisions have substantially improved the quality, clarity, and rigor of the study. The reviewer is supportive of publication in Nature Communications. However, several points should be further addressed to strengthen the manuscript.

Response: We sincerely appreciate your positive evaluation of the revised manuscript. We have carefully considered your remaining suggestions and have addressed them point-by-point below to further enhance the manuscript.

Major issue #4.

The authors now convincingly demonstrate the complete reversibility of SAMs using purified protein with additional elution steps. This represents an important technical improvement. However, the process leading to this refinement, as well as the authors' speculation that the originally observed incomplete reversibility may have resulted from residual SAM remaining in the assay system, would be highly valuable information for end users.

To provide transparency and practical guidance, the original dataset (Fig. 6e in the initial submission) should be retained in the revised manuscript, accompanied by a clear explanation of the experimental optimization and the rationale for the updated results. Including both versions would enhance the manuscript's instructional value and reproducibility.

Response: We sincerely appreciate your constructive suggestion. We have now included the original single-elution data alongside the optimized multiple-elution data in the revised Supplementary Information (Supplementary Fig. 40n, o). For your convenience, these results are presented as **Fig. 1** below.

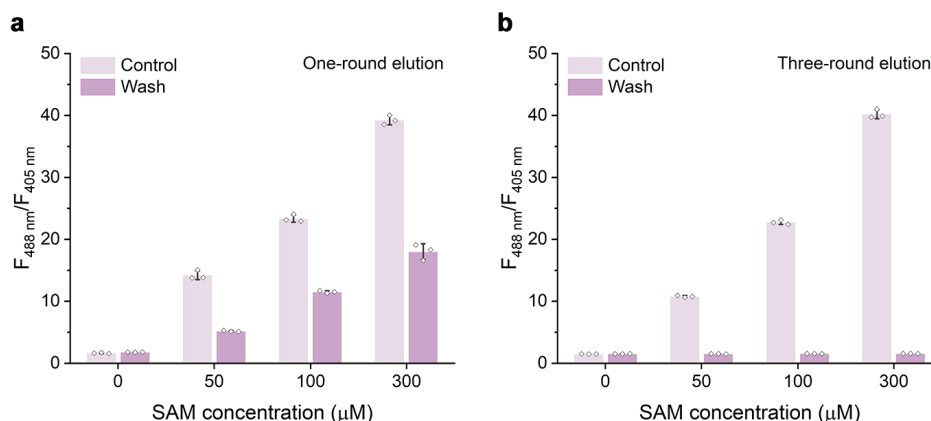


Figure 1 Reversibility analysis of SAMsor. **(a, b)** Fluorescence ratios of SAMsor after SAM addition and subsequent removal through one **(a)** or three **(b)** elution procedures. All data shown are means $\pm$ s.d. ($n = 3$ independent experiments). (**Supplementary Fig. 40n, o in the revised manuscript**)

The rationale for this experimental optimization has been incorporated into the “Results” section of the revised manuscript as follows:

“SAMsor also exhibited reversible SAM binding, although its low limit of detection (LOD, 310 nM) necessitated two additional elution steps to completely remove detectable SAM from the system (**Fig. 6c** and **Supplementary Fig. 40n-p**).”

Furthermore, we have detailed the optimized procedure in the “Supplementary Methods” section of the revised manuscript to ensure transparency and reproducibility:

“Characterization of Phesor, Valsor, Ilesor, and SAMsor *in vitro*”

Reversibility analysis. Purified Phesor, Valsor, and Ilesor were first mixed with different concentrations of target ligands, after which the ligands were eluted by centrifugation at $3,800 \times g$ for 30 min at 4 °C using a 10-kDa ultrafiltration centrifuge tube. The sample volume was then replenished to the initial level using ligand-free 50 mM Tris-HCl (pH 7.4). For SAMsor, given its significantly lower limit of detection (LOD), the same elution process was performed three times to completely remove detectable SAM from the retentate. The reversibility of the biosensors was analyzed by measuring the changes in the fluorescence ratio before and after ligand elution.”

We hope these revisions adequately address your concerns.

Major issue #5.

The reported pK_a values of each biosensor in the presence and absence of ligand are

very similar. It is important to clarify whether this observation is consistent with the large ratiometric response (F_{488}/F_{405}) observed around pH ~7 (Fig. 3e).

If the pK_a values remain largely unchanged upon ligand binding, this would suggest that the primary mechanism of fluorescence modulation is unlikely to arise from a shift in the equilibrium between the neutral and anionic chromophore states. Instead, the response may predominantly reflect changes in extinction coefficient, quantum yield, chromophore environment, or other structural factors.

To clarify this point, the authors should present full pH titration curves for each biosensor in both the ligand-bound and ligand-free states (currently referenced in Fig. 3h), rather than reporting only the ligand-bound state. These curves would allow readers to directly evaluate whether ligand binding alters the protonation equilibrium or instead modulates fluorescence through alternative mechanisms.

An expanded discussion addressing the likely molecular basis of the fluorescence response in these four biosensors would further strengthen the mechanistic insight of the study.

Response: We sincerely appreciate your insightful comments regarding the molecular mechanisms of the biosensors. As suggested, we have now included the full pH titration curves for all four biosensors in both ligand-bound and ligand-free (apo) states. As shown in **Fig. 2**, the pK_a values were determined as follows: 8.92 (apo) and 8.10 (with 1 mM L-phenylalanine) for Phesor; 8.56 (apo) and 8.20 (with 1 mM L-valine) for Valsor; 9.14 (apo) and 8.74 (with 1 mM L-isoleucine) for Ilesor; and 8.99 (apo) and 7.84 (with 200 μ M SAM) for SAMsor. Notably, to ensure consistency in data presentation throughout the manuscript, these titration curves were replotted and the pK_a values recalculated using OriginPro. Although this software transition resulted in minor numerical variations compared to the initial values derived using GraphPad Prism, the relative pK_a shifts between the apo and ligand-bound states remained nearly unchanged.

Furthermore, while the observed pK_a shifts were relatively minor, significant ratiometric responses were consistently achieved at our standard assay pH (~7.4). Specifically, the $F_{488\text{ nm}}/F_{405\text{ nm}}$ ratio increased 15.29-fold for Phesor (with 1 mM L-phenylalanine), 3.47-fold for Valsor (with 1 mM L-valine), 4.28-fold for Ilesor (with 1 mM L-isoleucine), and 20.06-fold

for SAMsor (with 200 μ M SAM) (Fig. 2).

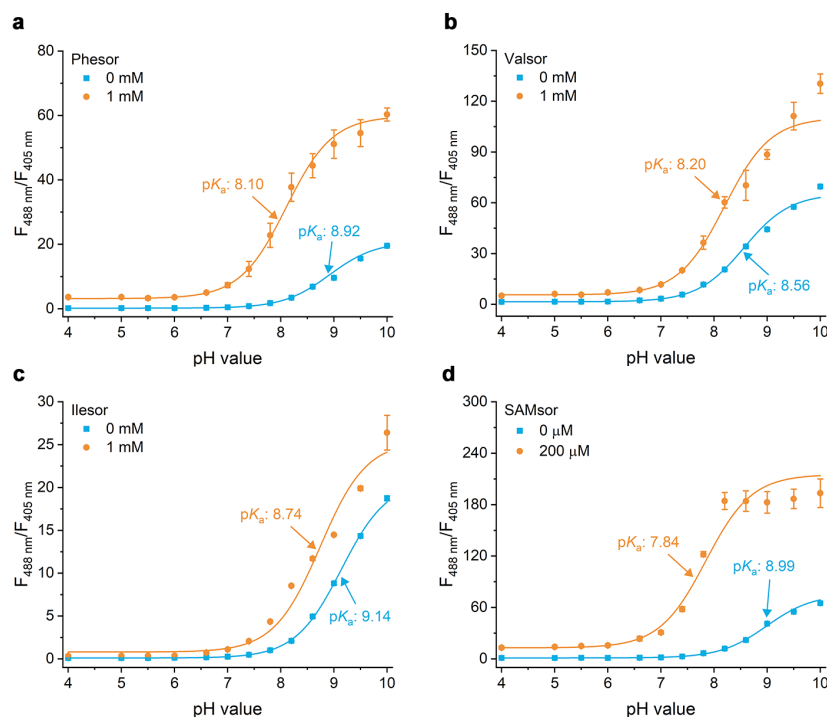


Figure 2 pK_a analysis of Phesor, Valsor, Ilesor, and SAMsor. **(a-d)** Determination of pK_a values of Phesor **(a)**, Valsor **(b)**, Ilesor **(c)**, and SAMsor **(d)** in the absence or presence of target ligands. All data shown are means $\pm$ s.d. ($n = 3$ independent experiments). (Supplementary Fig. 5f, Supplementary Fig. 18f, Supplementary Fig. 30f, and Supplementary Fig. 40f in the revised manuscript)

The fluorescence response of cpFP-based biosensors is generally driven by shifts in the chromophore protonation equilibrium (reflected by pK_a changes), as well as variations in the extinction coefficient (ϵ) and quantum yield (Φ) (*Nat. Chem. Biol.*, 2021, 17:509–518). As you correctly noted, the modest pK_a shifts indicate that the equilibrium between the neutral and anionic chromophore states remains largely unchanged upon ligand binding. Consequently, the substantial ratiometric responses ($F_{488\text{ nm}}/F_{405\text{ nm}}$) may be attributed to asymmetric changes in the intrinsic photophysical properties (ϵ and Φ) of these two states. To verify this, we determined ϵ , Φ , and overall brightness for each biosensor both in the absence and presence of their target ligands.

Table 1 Detection properties of Phesor, Valsor, Ilesor, SAMsor, SAMsor_L, and cpSFYFP. (Supplementary Table 4 in the revised manuscript)

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^[46].

^bThe $\text{p}K_{\text{a}}$ 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 .

As detailed in **Table 1**, ligand binding induced significant changes in these photophysical properties. Specifically, upon binding 1 mM L-phenylalanine, the ϵ of Phesor increased 15.46-fold at 490 nm, while decreasing 2.13-fold at 416 nm. Although Φ slightly decreased at both excitation wavelengths, the combined effect yielded a 12.78-fold increase in overall brightness under 490 nm excitation, alongside a 3.10-fold decrease under 416 nm excitation.

This photophysical modulation was consistently observed across the other three biosensors (**Table 1**). Notably, even Valsor and Ilesor, which exhibited the smallest pK_a shifts, showed substantial ligand-induced changes. For example, under 504 nm excitation, Valsor exhibited a 3.1-fold increase in ϵ , leading to a proportional 3.1-fold increase in overall brightness. For Ilesor, concurrent increases in both ϵ (3.1-fold) and Φ (3.4-fold) under 504 nm excitation collectively resulted in a 10.5-fold increase in overall brightness.

Related data were presented in **Supplementary Fig. 5f**, **Supplementary Fig. 18f**, **Supplementary Fig. 30f**, and **Supplementary Fig. 40f**, and **Supplementary Table 4** in the revised manuscript. Related descriptions were added in the “Results” section of the revised manuscript as follows:

For Phesor:

“Dose-response curve analysis indicated that the detection range of Phesor encompasses the physiological concentrations of L-phenylalanine both in bacteria and in mammalian cells (**Fig. 2c**). Furthermore, it achieved a high ΔR_{max} ($4,030 \pm 40\%$), an appropriate K_d ($1,760 \pm 40 \mu\text{M}$; close to the value of $2.69 \pm 0.13 \text{ mM}$ determined by thermal shift assay [TSA]), and a Hill slope close to 1 for L-phenylalanine (**Fig. 2c**, **Supplementary Fig. 5e**, and **Supplementary Table 4**). The pK_a value of Phesor was determined as 8.92, and decreased to 8.10 upon L-phenylalanine binding (**Supplementary Fig. 5f**). Its extinction coefficients, quantum yields, and brightness in both apo and ligand-bound states are summarized in **Supplementary Table 4**.”

For Valsor:

“Dose-response curve analysis indicated that Valsor achieved a wide detection range, with an increased ΔR_{max} ($875 \pm 52\%$), an appropriate K_d ($2,170 \pm 110 \mu\text{M}$; close to the value of $4.46 \pm 0.50 \text{ mM}$ determined by TSA), and a Hill slope close to 1 for L-valine (**Fig. 3d**,

Supplementary Fig. 18e, and Supplementary Table 4). The pK_a value of Valsor was determined as 8.56, and decreased to 8.20 upon L-valine binding (**Supplementary Fig. 18f**). Its photophysical properties are summarized in **Supplementary Table 4.**”

For Ilesor:

“Dose-response curve analysis indicated that Ilesor achieved a suitable detection range, an increased ΔR_{max} ($1,325.3 \pm 7.1\%$), an appropriate K_d ($3,620 \pm 400 \mu\text{M}$; close to the value of $2.22 \pm 0.30 \text{ mM}$ determined by TSA), and a Hill slope close to 1 for L-isoleucine (**Fig. 4d, Supplementary Fig. 30e, and Supplementary Table 4**). The pK_a value of Ilesor was determined as 9.14, and decreased to 8.74 upon L-isoleucine binding (**Supplementary Fig. 30f**). Its photophysical properties are summarized in **Supplementary Table 4.**”

For SAMsor:

“Dose-response curve analysis indicated that SAMsor achieved an increased ΔR_{max} ($3,540 \pm 360\%$), an appropriate K_d ($204 \pm 21 \mu\text{M}$; close to the value of $0.295 \pm 0.061 \text{ mM}$ determined by TSA), a Hill slope close to 1, and a wide detection range suitable for detecting physiological concentrations of SAM in mammalian cells and bacteria (**Fig. 6c, Supplementary Fig. 40e, and Supplementary Table 4**). The pK_a value of SAMsor was determined as 8.99, and decreased to 7.84 upon SAM binding (**Supplementary Fig. 40f**). Its photophysical properties are summarized in **Supplementary Table 4.**”

Mechanistically, in cpFP-based biosensors, changes in pK_a , ϵ , and Φ typically arise from ligand-induced alterations in the local chromophore microenvironment. For example, structural studies of classic cpFP-based biosensors, such as GCaMP2 (a Ca^{2+} biosensor) and G-Flamp1 (a cAMP biosensor), have demonstrated that ligand binding triggers domain rearrangements that remodel the local hydrogen-bonding network and restrict solvent access to the chromophore. These microenvironmental alterations collectively drive the shifts in pK_a , ϵ , and Φ , leading to the observed fluorescence signal changes (*Nat. Commun.*, 2022, 13:5363; *J. Biol. Chem.*, 2009, 284:6455–6464).

High-resolution structural analyses will be required to elucidate how conformational changes remodel the chromophore microenvironment to drive the photophysical shifts observed in the four as-designed biosensors. Following your suggestion, we have incorporated a detailed discussion on the molecular basis of the fluorescence response into

the “Discussion” section of the revised manuscript and outlined future structural studies, as follows:

“... Furthermore, significant ligand-induced fluorescence changes were observed in the as-designed biosensors. Analysis of pK_a and photophysical parameters reveals that changes in extinction coefficients and quantum yields, rather than altered chromophore protonation equilibria, may primarily account for this fluorescence behavior (**Supplementary Table 4**)³⁹. Future high-resolution structural analyses will be required to elucidate how conformational changes remodel the chromophore microenvironment to drive these photophysical shifts^{40,41}.”

We hope these additional data and expanded discussions fully address your concerns, and we remain available for any further clarification if required.

To Reviewer #3:

The authors have thoroughly addressed the concerns raised in the first round of review. I particularly appreciate the addition of applying GEFBs in both clinical and bioprocess contexts, as well as the development of sensor variants with compartment-specific localization. These revisions have significantly strengthened the manuscript. That said, I still have several comments that could further improve the clarity of the manuscript and enhance the presentation of the tools' capabilities.

Response: We sincerely appreciate your positive evaluation of our revised manuscript. We are encouraged that the new data on clinical applications and subcellular localization have adequately addressed your initial concerns. We are also particularly grateful for your further suggestions to improve the clarity of our manuscript. In accordance with these comments, we have thoroughly revised the manuscript to provide comprehensive experimental descriptions and rigorous data presentation. We hope these updates fully address your remaining questions. Please find our point-by-point responses below.

Major comments:

1. The authors used degenerate primers for random linker mutations. The authors should provide more details on the nature of these mutations (such as linker length and amino acid compositions) and the theoretical diversity of a mutation library. For most GEFBs, only several hundred variants were screened, whereas six randomized linker residues could in principle generate up to 20^6 possible combinations.

Response: We sincerely appreciate your insightful suggestion. In this study, we systematically optimized the sensitivity of the GEFBs through random mutagenesis and high-throughput screening of the linkers connecting the allosteric regulatory domain (biorecognition element) and the cpSFYFP reporter. To randomize the amino acid composition of these linkers while minimizing the occurrence of stop codons, we employed NNB and VNN degenerate codons in the forward and reverse primers, respectively, enabling the incorporation of all 20 standard amino acids. Furthermore, to screen for optimal conformational coupling between the biorecognition and reporter elements, we combinatorially varied the linker lengths at both termini. Specifically, the library was

constructed by pairing N-terminal linkers (comprising 0, 1, 2, or 3 random amino acids) with C-terminal linkers (comprising 0, 1, 2, or 3 random amino acids).

Taking the linker randomization of Phesor-0.38 as an example, a set of four specific primers (Phesor-0.38-0N-mutation, Phesor-0.38-1N-mutation, Phesor-0.38-2N-mutation, and Phesor-0.38-3N-mutation) was used to introduce linkers comprising 0, 1, 2, or 3 random amino acids at the N-terminus (**Table 2**). Concurrently, a corresponding set of four primers (Phesor-0.38-0C-mutation, Phesor-0.38-1C-mutation, Phesor-0.38-2C-mutation, and Phesor-0.38-3C-mutation) was employed to introduce linkers of 0, 1, 2, or 3 random amino acids at the C-terminus (**Table 2**). The theoretical diversity generated by this combinatorial approach can be calculated as the product of the possible variations at both termini: $(20^0 + 20^1 + 20^2 + 20^3) \times (20^0 + 20^1 + 20^2 + 20^3)$. This yields a theoretical library size of $\sim 7.09 \times 10^7$ possible sequence combinations. Linker mutations for the L-valine, L-isoleucine, and SAM biosensors were performed using the same procedure.

Table 2 Primers used for linker random mutation in Phesor-0.38 (**Supplementary Table 8 in the revised manuscript**)

Primer	Sequence (5'-3')
Phesor-0.38-0N-mutation	AAGAGTTAGGGGAAATCTTCAACAGCGACAACGTCTATATC
Phesor-0.38-1N-mutation	AAGAGTTAGGGGAAATC NNB TTCAACAGCGACAACGTCTATATC
Phesor-0.38-2N-mutation	AAGAGTTAGGGGAAATC NNBNNB TTCAACAGCGACAACGTCTATATC
Phesor-0.38-3N-mutation	AAGAGTTAGGGGAAATC NNBNNBNNB TTCAACAGCGACAACGTCTATATC
Phesor-0.38-0C-mutation	CTTCATTGAACGGGTGTTGTACTCCAGCTTGTGCCCCA
Phesor-0.38-1C-mutation	CTTCATTGAACGGGT VNN GTTGTACTCCAGCTTGTGCCCCA
Phesor-0.38-2C-mutation	CTTCATTGAACGGGT VNNVNN GTTGTACTCCAGCTTGTGCCCCA
Phesor-0.38-3C-mutation	CTTCATTGAACGGGT VNNVNNVNN GTTGTACTCCAGCTTGTGCCCCA

As you correctly noted, the theoretical sequence space ($> 10^7$) vastly exceeds the number of variants we experimentally screened. While it is practically infeasible to achieve full coverage for a library of this magnitude, our empirical results demonstrate that screening a representative subset (typically hundreds to a few thousand variants) is highly effective for

identifying substantially improved biosensors.

To clearly illustrate the linker mutation strategy and the high-throughput screening workflow, we have included a process diagram in the revised manuscript (Supplementary Fig. 2d; also provided as **Fig. 3** below for your convenience).

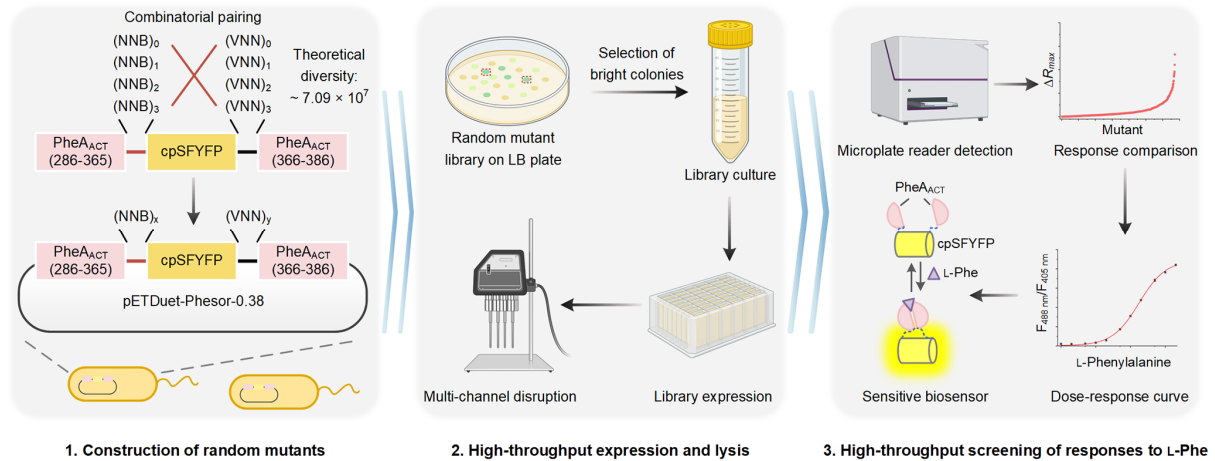


Figure 3 Schematic representation of the optimization workflow for the L-phenylalanine biosensor through random linker mutagenesis and high-throughput screening. The linker regions were randomized using NNB or VNN codons and combinatorially assembled at both termini (0, 1, 2, or 3 random amino acids), 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. (Supplementary Fig. 2d in the revised manuscript)

Related descriptions were added in the “Results” section of the revised manuscript as follows:

“Subsequently, we conducted random mutagenesis and high-throughput screening for linkers connecting the cpSFYFP and the PheA_{ACT} domain (Supplementary Fig. 2b, d). Among a total of 672 mutants, the biosensor with the N-terminal linker of “Ala-Arg-Leu” and the C-terminal linker of “Gln-His-Leu” possessed the highest response magnitude to L-phenylalanine (Fig. 2b and Supplementary Fig. 2b, c).”

Furthermore, we have added detailed descriptions regarding the linker randomization strategy, degenerate primer design, and theoretical library diversity in the “Methods” section of the revised manuscript, as follows:

“Construction of Phesor, Valsor, Ilesor, SAMsor, and SAMsor_L

Random linker mutagenesis combined with high-throughput screening. To construct the

randomized linker library of Phesor-0.38, the plasmid backbone of pETDuet-Phesor-0.38 was linearized by inverse PCR using primer pair Phesor-0.38 inverse PCR-F/Phesor-0.38 inverse PCR-R. The DNA fragment of cpSFYFP carrying random linkers was PCR-amplified using degenerate primers containing NNB or VNN codons (for example, primer pair Phesor-0.38-3N-mutation/Phesor-0.38-3C-mutation). A theoretical diversity of 7.09×10^7 distinct sequences was generated by combinatorially pairing the N-terminal and C-terminal linkers of varying lengths (0, 1, 2, or 3 random amino acids). Then, the obtained cpSFYFP fragment and linearized plasmid were ligated by TEDA method and transferred into *E. coli* BL21 (DE3) strain. The transformants were incubated on LB plates containing $100 \mu\text{g mL}^{-1}$ ampicillin at 37°C for 12 h, followed by incubation at 23°C for 24 h. Bright colonies were batch-picked and incubated in 5 mL LB medium containing $100 \mu\text{g mL}^{-1}$ ampicillin and 1 mM isopropyl- β -D-1-thiogalactopyranoside (IPTG) for 16 h at 23°C . The cultures were harvested by centrifugation at $4,000 \times g$ for 10 min at 4°C , resuspended in 2.5 mL of 50 mM Tris-HCl (pH 7.4) containing 1 mM phenylmethylsulfonyl fluoride (PMSF), transferred to 48-well deep well plates, and then lysed by a SCIENTZ-48TD multi-channel ultrasonic disrupter (Scientz Biotechnology, China) on ice. The supernatants were extracted by centrifugation at $12,000 \times g$ for 5 min at 4°C and mixed with 5 mM L-phenylalanine at a volume ratio of 3:1. The changes in fluorescence ratio were then high-throughput detected using an EnSight microplate reader (PerkinElmer, USA). Mutants with high response magnitude were then subjected for sequencing. The random linker mutagenesis and the high-throughput screening of Valsor-0.03, Ilesor-0.23, SAMsor-0.85, and SAMsor-0.34 were performed using the same procedure.”

2. The authors demonstrate that the ratiometric GEFB responses are independent of sensor concentration. To further support this point, I suggest including the corresponding single-channel fluorescence responses for comparison.

Response: We sincerely appreciate your constructive suggestion. In the revised manuscript, we have included the single-channel fluorescence responses alongside the ratiometric data for Phesor, Valsor, Ilesor, and SAMsor. As shown in **Fig. 4-Fig. 7**, the single-channel fluorescence intensities ($F_{488 \text{ nm}}$ and $F_{405 \text{ nm}}$) increase proportionally with biosensor

concentration, whereas the ratiometric readouts ($F_{488\text{ nm}}/F_{405\text{ nm}}$) remain stable and concentration-independent. This ratiometric property effectively eliminates potential interference from variable expression levels, enabling reliable measurements in living cells.

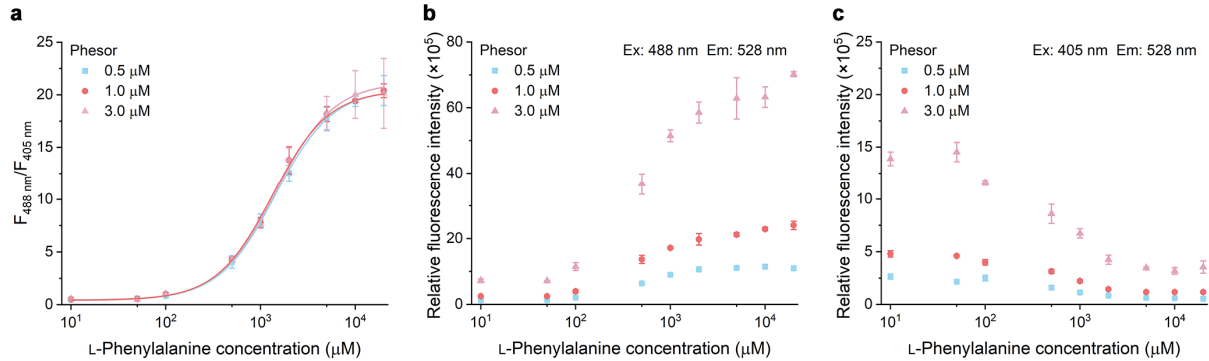


Figure 4 Analysis of the effect of Phesor concentration on its detection of L-phenylalanine. (a-c)

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 ($F_{488\text{ nm}}/F_{405\text{ nm}}$) (a), alongside the corresponding single-channel fluorescence intensities, $F_{488\text{ nm}}$ (b) and $F_{405\text{ nm}}$ (c). All data shown are means $\pm$ s.d. (n = 3 independent experiments). (Supplementary Fig. 5g-i in the revised manuscript)

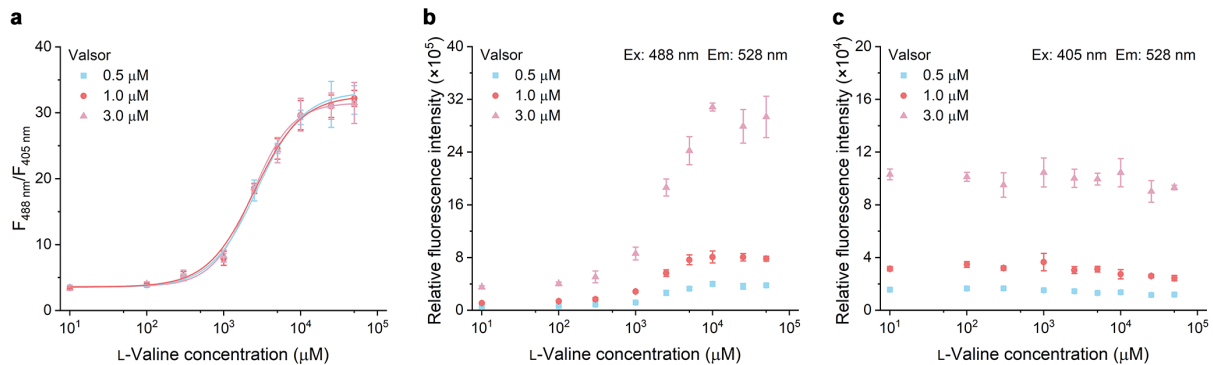


Figure 5 Analysis of the effect of Valsor concentration on its detection of L-valine. (a-c) Fluorescence responses of Valsor to increasing concentrations of L-valine at varying biosensor concentrations (0.5, 1, and 3 μM). The data show the ratiometric readout ($F_{488\text{ nm}}/F_{405\text{ nm}}$) (a), alongside the corresponding single-channel fluorescence intensities, $F_{488\text{ nm}}$ (b) and $F_{405\text{ nm}}$ (c). All data shown are means $\pm$ s.d. (n = 3 independent experiments). (Supplementary Fig. 18g-i in the revised manuscript)

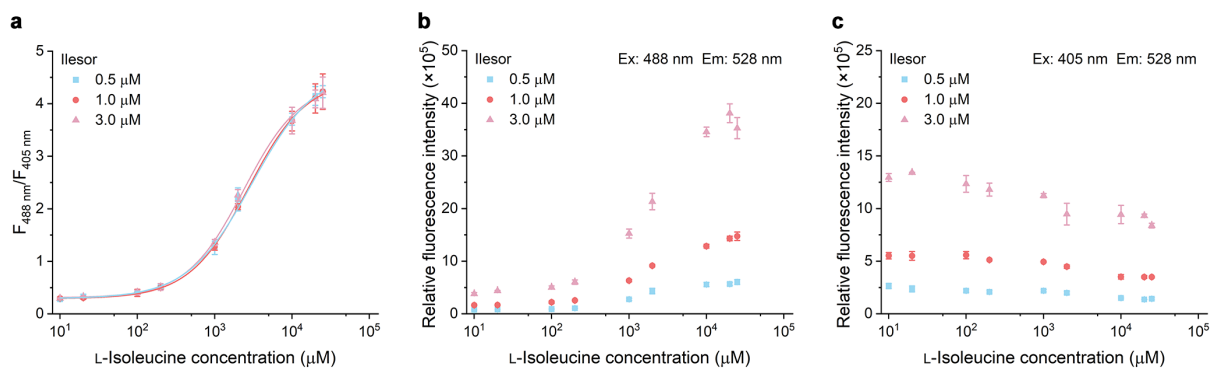


Figure 6 Analysis of the effect of Ilesor concentration on its detection of L-isoleucine. **(a-c)** Fluorescence responses of Ilesor to increasing concentrations of L-isoleucine at varying biosensor concentrations (0.5, 1, and 3 μM). The data show the ratiometric readout ($F_{488\text{ nm}}/F_{405\text{ nm}}$) **(a)**, alongside the corresponding single-channel fluorescence intensities, $F_{488\text{ nm}}$ **(b)** and $F_{405\text{ nm}}$ **(c)**. All data shown are means $\pm$ s.d. ($n = 3$ independent experiments). (Supplementary Fig. 30g-i in the revised manuscript)

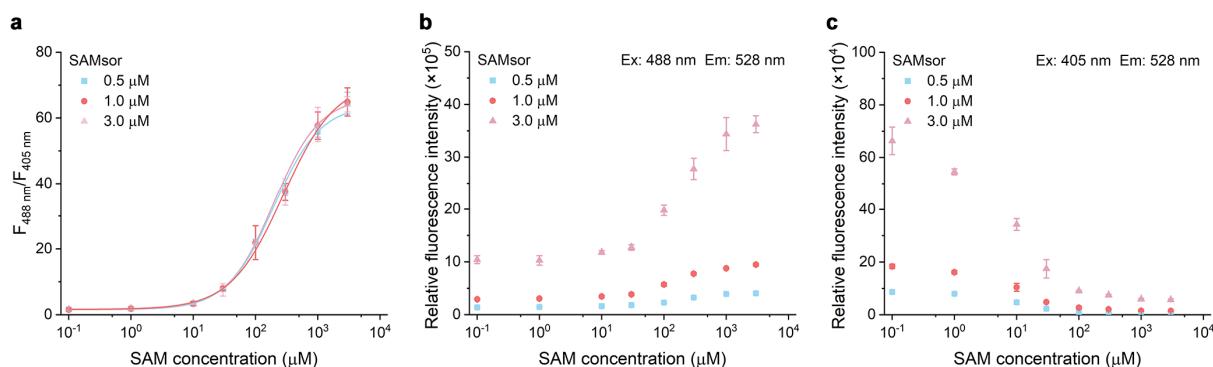


Figure 7 Analysis of the effect of SAMsor concentration on its detection of SAM. **(a-c)** Fluorescence responses of SAMsor to increasing concentrations of SAM at varying biosensor concentrations (0.5, 1, and 3 μM). The data show the ratiometric readout ($F_{488\text{ nm}}/F_{405\text{ nm}}$) **(a)**, alongside the corresponding single-channel fluorescence intensities, $F_{488\text{ nm}}$ **(b)** and $F_{405\text{ nm}}$ **(c)**. All data shown are means $\pm$ s.d. ($n = 3$ independent experiments). (Supplementary Fig. 40g-i in the revised manuscript)

Related data were presented in [Supplementary Fig. 5g-i](#), [Supplementary Fig. 18g-i](#), [Supplementary Fig. 30g-i](#), and [Supplementary Fig. 40g-i](#) in the revised manuscript. Related descriptions were provided in the “Results” section of the revised manuscript as follows:

For Phesor:

“As a ratiometric biosensor, Phesor displayed a robust response independent of its own concentration ([Supplementary Fig. 5g-i](#)).”

For Valsor:

“In addition, Valsor demonstrated a robust ratiometric response independent of its own concentration, high specificity, good reversibility, and correctable pH-stability from pH 6.6 to 8.2 for L-valine detection ([Supplementary Fig. 17d-f](#), [Supplementary Fig. 18g-m](#), and [Supplementary Fig. 19a](#)).”

For Ilesor:

“In addition, Ilesor demonstrated a robust ratiometric response independent of its own concentration, high specificity, good reversibility, and correctable pH-stability from pH 6.6 to

8.2 for L-isoleucine detection (Supplementary Fig. 19a, Supplementary Fig. 29d-f, and Supplementary Fig. 30g-m).”

For SAMsor:

“SAMsor displayed a robust ratiometric response independent of its own concentration (Supplementary Fig. 40g-i).”

3. What is the difference between ΔR_{max} and $\Delta R/R_{min}$, as both metrics are frequently used throughout the manuscript? I also notice that some sensor variants exhibit negatively-going responses, such as Phesor-0.38, Valsor-0.03, MTAsor-0.64 and SAMsorL. For such sensors, reporting ΔR_{max} and $\Delta R/R_{min}$ may be less appropriate, particularly for imaging in living cells or for quantifying exogenous ligands, since achieving R_{min} requires saturate ligand concentrations, which are usually hard to reach. I therefore suggest presenti $\Delta R/R_0$ (R without ligands) instead of ΔR_{max} in the dose-response curves like Fig. 7d and in high-throughput screening results like Fig. 7c.

Response: We sincerely appreciate your valuable and insightful comments. As you correctly noted, this study encompasses multiple biosensors with two distinct response modalities. Some exhibit a direct response with an upward dose-response curve (e.g., SAMsor-0.34 in Fig. 8a), while others demonstrate an inverse response with a downward curve (e.g., SAMsor_L in Fig. 8b).

In this context, the metric $\Delta R/R_{min}$ defines the amplitude of the fluorescence ratio ($F_{488\text{ nm}}/F_{405\text{ nm}}$) shift under assay conditions relative to the lowest fluorescence state, while ΔR_{max} denotes the maximum attainable $\Delta R/R_{min}$ achieved at ligand saturation. To precisely evaluate the detection sensitivity across both types of biosensors, the calculation formulas are inherently different. Specifically, for direct-response biosensors, $\Delta R/R_{min} = \frac{R - R_{apo}}{R_{apo}}$ and $\Delta R_{max} = \frac{R_{sat} - R_{apo}}{R_{apo}}$. For inverse-response biosensors, $\Delta R/R_{min} = \frac{R_{apo} - R}{R}$ and $\Delta R_{max} = \frac{R_{apo} - R_{sat}}{R_{sat}}$. Here, R , R_{sat} , and R_{apo} represent the experimental, ligand-saturated, and ligand-free $F_{488\text{ nm}}/F_{405\text{ nm}}$ ratios, respectively.

We deeply appreciate your insightful suggestion to utilize $\Delta R/R_0$ (R without ligands) to represent the signal changes. We carefully considered this approach; however, we opted to

retain ΔR_{max} for evaluating inverse-response biosensors. Our primary concern is that substituting ΔR_{max} with $\Delta R/R_0$ mathematically caps the theoretical maximum response of inverse-response biosensors at 100%, which might inadvertently mislead readers when evaluating them against direct-response biosensors.

For example, the direct-response biosensor SAMsor-0.34 exhibits a ~ 1.8 -fold increase in the $F_{488\text{ nm}}/F_{405\text{ nm}}$ ratio upon SAM saturation, resulting in a ΔR_{max} of $\sim 80.0\%$ (**Fig. 8a**). Its optimized variant, SAMsor_L, demonstrates a superior ~ 2.28 -fold $F_{488\text{ nm}}/F_{405\text{ nm}}$ reduction (decreasing from 4.29 in the apo state to 1.88 at saturation), yielding a ΔR_{max} of $\sim 128.5\%$ (**Fig. 8b**). However, calculating $\Delta R/R_0$ mathematically constrains this value to $(4.29 - 1.88) / 4.29 \approx 56.2\%$. We are concerned that this metric would inaccurately depict the optimized SAMsor_L as inferior to SAMsor-0.34, despite its intrinsically larger dynamic range.

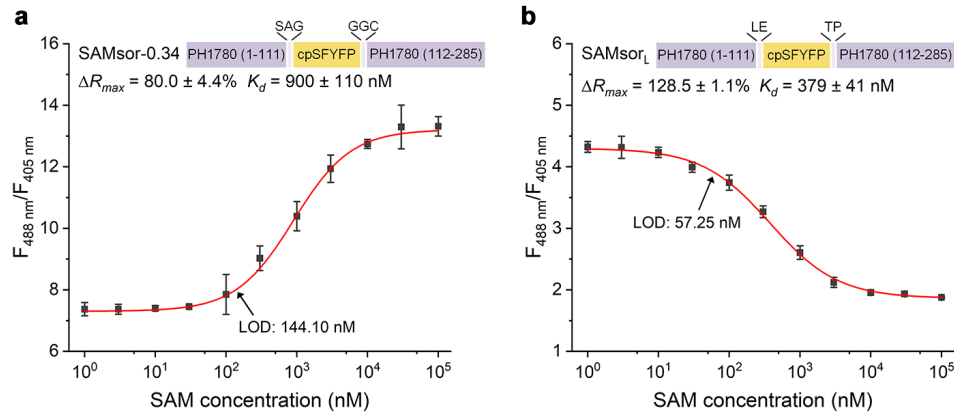


Figure 8 Optimization of the SAM biosensor SAMsor_L with low detection range. **(a, b)** Dose-response curve of SAMsor-0.34 **(a)** and SAMsor_L **(b)** for increasing concentrations of SAM. The structural compositions of SAMsor-0.34 and SAMsor_L were shown. The ΔR_{max} of SAMsor-0.34 was calculated by $(R_{sat} - R_{apo}) / R_{apo}$, and the ΔR_{max} of SAMsor_L was calculated by $(R_{apo} - R_{sat}) / R_{sat}$. All data shown are means $\pm$ s.d. (n = 3 independent experiments). (**Fig. 7b, d in the revised manuscript**)

To further elucidate the rationale of our approach, characterizing the response of SAMsor_L using the reciprocal ratio ($F_{405\text{ nm}}/F_{488\text{ nm}}$) converts it into an upward dose-response curve (**Fig. 9**). Under apo and SAM-saturated conditions, these reciprocal values are 0.23 and 0.53, respectively. Applying the standard direct-response formula $\frac{R_{sat} - R_{apo}}{R_{apo}}$ to these reciprocal values consistently yields a ΔR_{max} of $\sim 128.5\%$. This suggests that our ΔR_{max} calculation accurately reflects the true sensitivity across different response modalities.

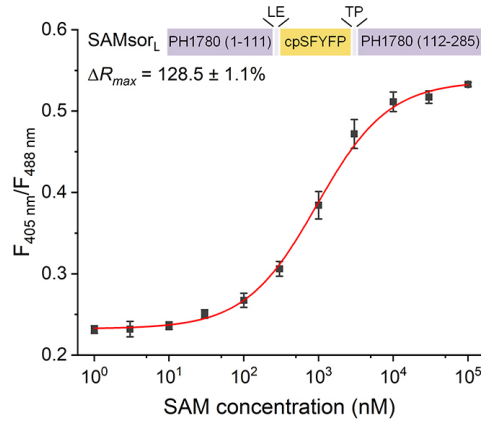


Figure 9 Dose-response curve of SAMsor_L for increasing concentrations of SAM. The fluorescence signal of SAMsor_L is represented as the ratio of F_{405nm} to F_{488nm}. The ΔR_{max} of SAMsor_L was calculated by $(R_{sat} - R_{apo}) / R_{apo}$. All data shown are means $\pm$ s.d. (n = 3 independent experiments). **(only for review)**

Furthermore, we would like to respectfully note that our analytical calculations align with established conventions in related biosensor studies, facilitating accurate performance comparisons among biosensors with distinct response trajectories. For example, the metric $\Delta F/F_{min}$ was previously utilized to benchmark the sensitivities of various NEMO Ca²⁺ biosensor variants (*Nat. Methods*, 2023, 20:918–924). Similarly, Zhao *et al.* adopted the same mathematical rationale to evaluate both direct-response (Citron1) and inverse-response (Citroff1) citrate biosensors (*ACS Cent. Sci.*, 2020, 6:1441–1450).

Nevertheless, we fully agree with your rigorous assessment that metrics such as ΔR_{max} and $\Delta R/R_{min}$ are less appropriate for complex application contexts, such as live-cell imaging. Therefore, to accurately reflect authentic signal dynamics, all data involving living bacteria, living human cells, and clinical samples are presented strictly as raw F_{488 nm}/F_{405 nm} ratios. The metrics ΔR_{max} and $\Delta R/R_{min}$ are exclusively reserved for *in vitro* comparative analyses (where ligand concentrations can be precisely controlled), such as dose-response profiling and high-throughput mutant screening.

We fully acknowledge that the absence of explicit definitions and formulas for ΔR_{max} and $\Delta R/R_{min}$ in the original manuscript led to this ambiguity, and we sincerely apologize for the oversight. In response, we have incorporated detailed clarifications in both the “Results” section and “Supplementary Methods” section of the revised manuscript, as follows:

“We constructed four biosensors by fusing a circularly permuted superfolder yellow fluorescent protein (cpSFYFP) to the C-terminus of full-length PheA (Phe_{ACM}-Phe_{APDT}-

PheA_{ACT}) or truncated PheA (PheA_{CM}-PheA_{PDT}, PheA_{PDT}-PheA_{ACT}, or PheA_{ACT})⁷.

Subsequently, we analyzed the fluorescence ratio ($F_{488\text{ nm}}/F_{405\text{ nm}}$, the ratio of fluorescence at 528 nm when excited at 488 and 405 nm) changes ($\Delta R/R_{min}$) of these biosensors before and after the addition of chorismate, prephenate, phenylpyruvate, or L-phenylalanine.”

“Firstly, we constructed 50 biosensor variants by inserting cpSFYFP between amino acid residues located in the loop regions of the PheA_{ACT} domain with an N-terminal linker of “Ser-Ala-Gly” and a C-terminal linker of “Gly-Gly-Cys” (**Supplementary Fig. 2 and Supplementary Fig. 4**). The 38th variant (denoted as Phesor-0.38, with cpSFYFP inserted between residues 365I/366T of the PheA_{ACT} domain) exhibited a higher maximum fluorescence ratio change (ΔR_{max} , $\Delta R/R_{min}$ at ligand saturation) of $67.2 \pm 2.9\%$ and an appropriate apparent dissociation constant (K_d) of $440 \pm 80\text{ }\mu\text{M}$, making it suitable for L-phenylalanine detection both in living cells and in human body fluids (**Fig. 2a and Supplementary Table 2**).”

“Characterization of Phesor, Valsor, Ilesor, and SAMsor *in vitro*

Dose-response curve determination. Purified biosensors were diluted to a final concentration of 1 μM with 50 mM Tris-HCl (pH 7.4) and mixed with increasing concentrations of L-phenylalanine (for Phesor), L-valine (for Valsor), L-isoleucine (for Ilesor), or SAM (for SAMsor) in a black 384-well plate at a volume ratio of 3:1 (biosensor to ligand, total 20 μL per well). After a 10-min incubation at room temperature, the fluorescence intensities of the biosensors at 528 nm with excitation at 405 or 488 nm were measured using an EnSight microplate reader (PerkinElmer, USA). Dose-response curves of different biosensors were then generated using OriginPro 2019 (OriginLab, USA) to fit the $F_{488\text{ nm}}/F_{405\text{ nm}}$ values against ligand concentrations according to the following equation:

$$R = R_{sat} + \frac{R_{apo} - R_{sat}}{1 + ([\text{Ligand}] / K_d)^p} \quad (1)$$

where R , R_{sat} , R_{apo} , $[\text{Ligand}]$, K_d , and p represent the experimental $F_{488\text{ nm}}/F_{405\text{ nm}}$, ligand-saturated $F_{488\text{ nm}}/F_{405\text{ nm}}$, ligand-free $F_{488\text{ nm}}/F_{405\text{ nm}}$, ligand concentration, apparent dissociation constant, and Hill slope, respectively.

To precisely evaluate the detection sensitivity across both direct-response ($F_{488\text{ nm}}/F_{405\text{ nm}}$ -increasing, e.g., Phesor) and inverse-response ($F_{488\text{ nm}}/F_{405\text{ nm}}$ -decreasing, e.g., Phesor-

0.38) biosensors, the response magnitude ($\Delta R/R_{min}$) is calculated as the absolute amplitude of the signal change divided by the lowest fluorescence state. Specifically, for direct-response biosensors, $\Delta R/R_{min} = (R - R_{apo}) / R_{apo} \times 100\%$; for inverse-response biosensors, $\Delta R/R_{min} = (R_{apo} - R) / R \times 100\%$. The maximum response magnitude (ΔR_{max}) is defined as the $\Delta R/R_{min}$ at saturating ligand concentrations. Specifically, for direct-response biosensors, $\Delta R_{max} = (R_{sat} - R_{apo}) / R_{apo} \times 100\%$; for inverse-response biosensors, $\Delta R_{max} = (R_{apo} - R_{sat}) / R_{sat} \times 100\%$.”

We sincerely hope you will agree with the rationale of our analytical choices. However, if you still recommend replacing ΔR_{max} with $\Delta R/R_0$ for the *in vitro* data, we will certainly implement the necessary revisions.

4. The authors have increased the utility of these tools by generating GEFB variants targeted to different subcellular compartments, which revealed lower amino acid/SAM levels in mitochondria compared with the cytosol and nucleus. To further highlight the advantage of high spatiotemporal resolution, I suggest using these compartment-localized sensors to monitor spatial and temporal differences in drug-induced amino acid fluctuations through time-lapse imaging. For example, the subcellularly targeted Ilesor and Valsor could be used to examine valine and isoleucine dynamics in response to gabapentin or octyl-D-2-HG, or different SAMsors variants could be applied to track SAHA- and AG-270-regulated SAM synthesis. Such experiments could potentially provide additional insight into the compartment-specific and kinetic features of valine/isoleucine/SAM metabolism.

Response: We sincerely appreciate your constructive suggestion. Following your recommendation, we utilized Valsor as a representative model to monitor the temporal dynamics of L-valine in different subcellular compartments upon treatment with 10 mM gabapentin, an inhibitor of cytosolic branched-chain amino acid transaminase 1 (BCAT1).

As shown in **Fig. 10f**, gabapentin addition induced a rapid and significant increase in the fluorescence ratios of cytosolic and nuclear Valsor, reaching a steady state by 10 h. The synchronous changes observed in these two compartments can be attributed to the rapid exchange of L-valine through the nuclear pore complex. By comparison, we observed a delayed increase in the fluorescence ratio of mitochondrial Valsor, which supports our previous speculation that mitochondrial L-valine accumulation relies on the influx of

cytosolic L-valine. Furthermore, the mitochondrial signal increased at a slower rate and plateaued earlier. This kinetic difference may result from the continuous degradation of L-valine by mitochondrial branched-chain amino acid transaminase 2 (BCAT2). Collectively, these results demonstrate the capability of Valsor for tracking intracellular L-valine dynamics with high spatiotemporal resolution.

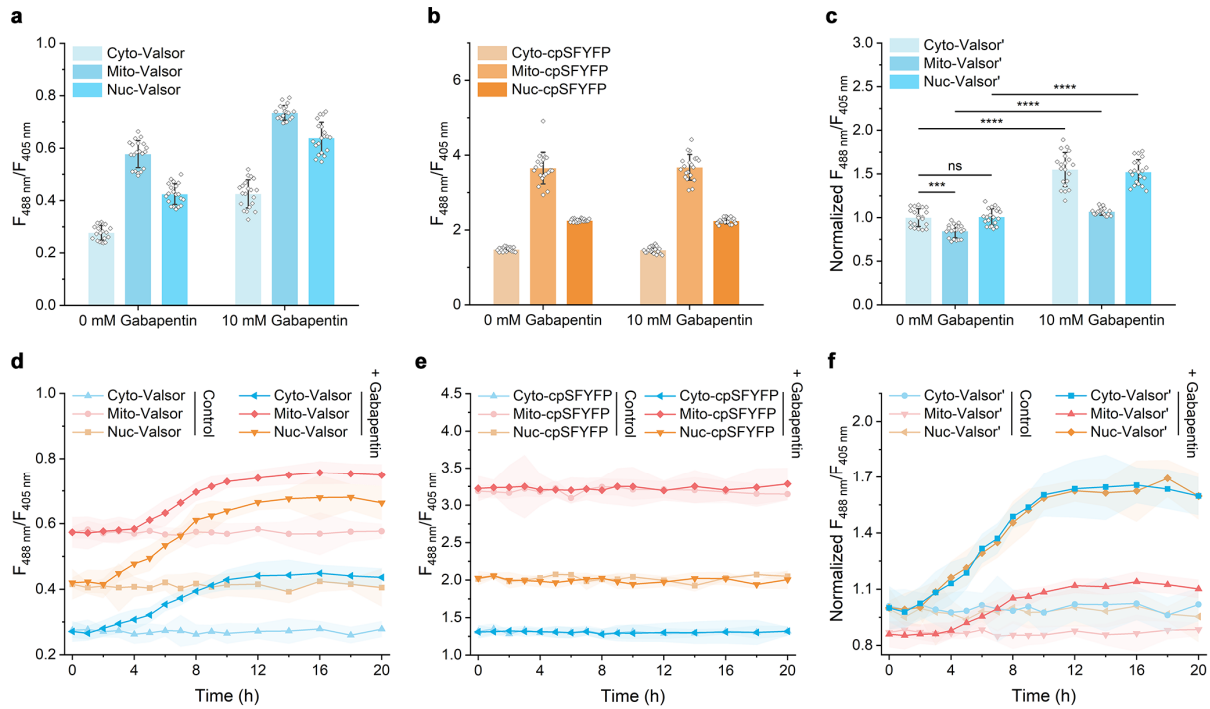


Figure 10 Analysis of subcellular distribution of L-valine using different subcellularly localized Valsor. **(a, b)** Fluorescence ratios of HEK293FT cells expressing different subcellularly localized Valsor **(a)** or cpSFYFP **(b)** in response to 10 mM gabapentin addition. Cells were treated with 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 test with Tukey's Multiple Comparison Test. (Supplementary Fig. 25 in the revised manuscript)

Related data were presented in **Supplementary Fig. 25** in the revised manuscript.

Related descriptions were added in the “Results” section of the revised manuscript as follows:

“Valsor could be correctly expressed in the cytosol, mitochondria, and nucleus, and responded to exogenous L-valine addition (**Supplementary Fig. 24**). Analysis of the subcellular distribution of L-valine demonstrated its lower abundance in the mitochondria (**Supplementary Fig. 25**). In addition, Valsor facilitated compartment-specific L-valine kinetic analysis. As shown in **Supplementary Fig. 25f**, gabapentin treatment induced a rapid and significant increase in the fluorescence ratios of cytosolic and nuclear Valsor. However, a delayed and slower signal increase of mitochondrial Valsor was also observed, which may be attributed to the influx of cytosolic L-valine.”

Within the limited revision timeframe, we focused on Valsor as a proof-of-concept to validate the applicability of these biosensors for spatiotemporally resolved imaging. However, we would be happy to perform the corresponding experiments for Ilesor and SAMsor if you consider them necessary.

5. In the table comparing detection performance between HPLC and biosensors, the authors say that HPLC needs > 100 μ L sample, which is unusual given the very high sensitivity of HPLC. I suggest revisiting this point for accuracy. Additionally, it may be more informative for readers to compare the concentration detection ranges (or limits of detection) of these approaches, rather than focusing on sample volume requirements.

Response: We sincerely appreciate your constructive comment. As you correctly noted, HPLC is a sensitive technique that typically requires small injection volumes (5–20 μ L). Our original statement regarding the “> 100 μ L” sample requirement was intended to encompass the total raw sample volume necessary for essential preparation steps (e.g., deproteinization, derivatization, centrifugation, and 0.22- μ m filtration), as well as to account for the dead volume of standard autosampler vials. In contrast, the biosensors developed in this study (Phesor, Valsor, Ilesor, and SAMsor_L) completely bypass these laborious pretreatments, requiring only 10 μ L of raw sample. Following your suggestion, we have refined the comparison table to explicitly distinguish between “injection volume” and “total sample volume” (as shown in **Table 3** below for your convenience).

Table 3 Comparison of detection performance between HPLC and the biosensors developed in this study. (**Supplementary Table 5 in the revised manuscript**)

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) 6 µM for L-Phe ^c	10 µL	10 µL	10 µL	/	10 µL
LOD ^b	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 [47].

^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.

Furthermore, we have now incorporated the limits of detection (LODs) for both approaches. As shown in **Table 3**, while the HPLC method exhibits lower LODs than the developed biosensors, the successful quantification of target metabolites in authentic clinical samples (from both healthy individuals and patients) and fermentation media clearly demonstrates the practical utility of these biosensors. More importantly, compared to the HPLC method, these biosensors are rapid, convenient, cost-effective, and compatible with high-throughput assays (**Table 3**), making them ideal tools for future clinical diagnostics and continuous bioprocess monitoring.

Related data were presented in **Supplementary Table 5** in the revised manuscript. Related descriptions were elaborated in the “Results” section of the revised manuscript as follows:

For Phesor:

“Compared with traditional HPLC- or mass spectrometry-based methods, Phesor provides a point-of-care, easy-to-handle, cost-effective, and high-throughput alternative for clinical serum and urine testing (**Supplementary Table 5**).”

For Valsor and Ilesor:

“In addition, both biosensors enabled convenient and precise bioprocess monitoring for fermentative production of L-valine or L-isoleucine (**Supplementary Table 5**). Such biosensor-based high-throughput screening may accelerate the engineering of the L-valine and L-isoleucine producing strains^{43,45}.”

For SAMsor_L:

“Importantly, SAMsor_L enables point-of-care quantification of SAM in human body fluids (**Supplementary Table 5**), offering a practical tool for the clinical diagnostics and therapeutic monitoring of SAM-related diseases.”

We hope these revisions adequately address your concerns.

Minor comments:

1. In Fig. 1b, do the colors represent different secondary structures? If so, this should be explained in the figure legend.

Response: Thanks for your good suggestion. The colors in Fig. 1b represent different

secondary structures. Specifically, cyan indicates α -helices, magenta indicates β -sheets, and wheat indicates random coils. We have updated the legend for Fig. 1b in the revised manuscript, as follows:

“(b) Schematic representation of the structure of the ACT dimer. The structure of the ACT dimer from D-3-phosphoglycerate dehydrogenase (PDB: 1PSD) was used as an example. Cyan: α -helix; magenta: β -sheet; wheat: random coil.”

2. In line 97, “feedback inhibits the activities of Phe_{ACM} and Phe_{APDT}” should be “act as a feedback inhibitor of Phe_{ACM} and Phe_{APDT} activities”.

Response: Thanks for your correction. The sentence has been revised to “act as a feedback inhibitor of Phe_{ACM} and Phe_{APDT} activities” in the revised manuscript.

3. For the high-throughput screening results (e.g., Fig. 2b, 3c, 4c, 6b and 7c), it would improve clarity to label the starting and final versions. For example, in Fig. 2b, the variants corresponding to Phesor-0.38 and the final Phesor could be explicitly marked.

Response: Thanks for your good suggestion. We fully agree that explicitly labeling both the starting and final versions in the high-throughput screening results is essential for clearly illustrating the optimization trajectory and the significant improvements of the biosensors. Accordingly, we have highlighted these key variants in the revised Figs. 2b, 3c, 4c, 6b, and 7c (summarized as **Fig. 11** below for your convenience).

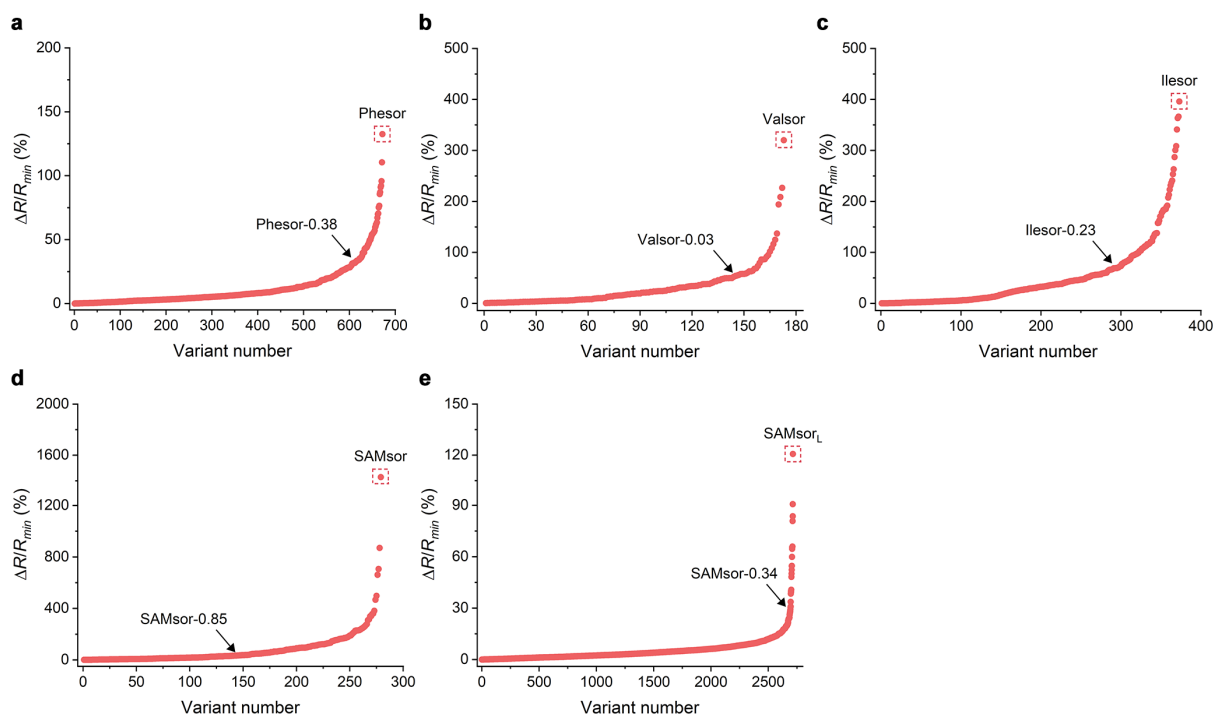


Figure 11 Optimization of biosensor sensitivity through random linker mutagenesis and high-throughput screening. **(a-e)** Comparison of response magnitudes of crude extracts from various linker mutant libraries to their respective ligands. The optimization of Phesor-0.38 were measured with 5 mM L-phenylalanine (672 mutants) **(a)**. The optimization of Valsor-0.03 was measured with 5 mM L-valine (173 mutants) **(b)**. The optimization of Ilesor-0.23 was measured with 25 mM L-isoleucine (375 mutants) **(c)**. The optimization of SAMsor-0.85 was measured with 500 μ M SAM (279 mutants) **(d)**. The optimization of SAMsor-0.34 was measured with 10 μ M SAM (2,715 mutants) **(e)**. (**Fig. 2b, Fig. 3c, Fig. 4c, Fig. 6b, and Fig. 7c in the revised manuscript**)

4. In line 376-379, the descriptions of “diminished” and “abolished” response appear to be reversed, as mutated MTAsor-0.64 still show some response to MTA. Besides, the two panels lack statistic comparison (at least no significance indicators are labeled), and therefore stating “significantly” is not appropriate.

Response: We sincerely appreciate your thorough review and corrections. The descriptions of “diminished” and “abolished” have been corrected in the updated manuscript. Furthermore, we have removed the term “significantly” and updated the corresponding figure panels to include explicit statistical comparisons and significance indicators (**Fig. 12**).

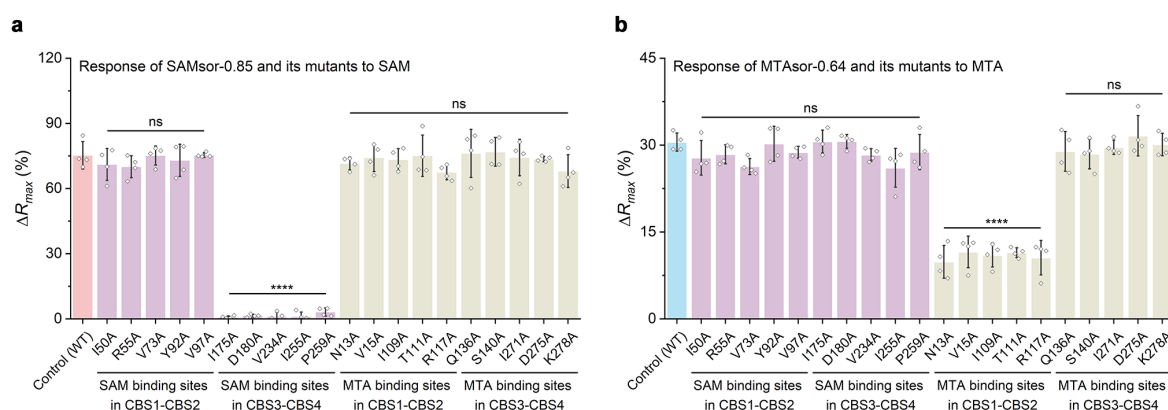


Figure 12 Utilization of PH1780 as a biorecognition element to derive an SAM biosensor and an MTA biosensor. **(a)** Effects of mutations in putative SAM or MTA binding sites in SAMsor-0.85 on SAM response. **(b)** Effects of mutations in putative SAM or MTA binding sites in MTAsor-0.64 on MTA response. All data shown are means $\pm$ s.d. ($n = 4$ independent experiments). ****, $P < 0.0001$; ns, no significant difference ($P \geq 0.05$); one-way ANOVA test with Tukey's Multiple Comparison Test. **(Fig. 5i, j in the revised manuscript)**

Related data were presented in **Fig. 5i, j** in the revised manuscript. Related descriptions were provided in the “Results” section of the revised manuscript as follows:

“Mutations at the SAM binding sites in CBS3-CBS4 of PH1780 abolished the response of SAMsor-0.85 to SAM (**Fig. 5i**). Mutations at the MTA binding sites in CBS1-CBS2 of PH1780 diminished the responsiveness of MTAsor-0.64 to MTA (**Fig. 5j**). These results demonstrate that PH1780 containing four CBS domains can be used as a biorecognition element for developing an SAM-specific GEFB and an MTA-specific GEFB.”

5. In Fig. 6e, the authors use 100 μ M MTA and 10 μ M SAH to demonstrate the selectivity of SAMor. However, even 10 μ M MTA induce a modest response. According to Supplementary Fig. 40, the physiological concentrations of MTA, adenosine and SAH fall within a similar range. Therefore, it will be more appropriate to use SAH at 100 μ M in Fig. 6e for a more balanced comparison.

Response: Thanks for your good comment. As suggested, we have adjusted the test concentration of SAH from 10 μ M to 100 μ M in the revised Fig. 6e. Correspondingly, we also updated the SAH concentration to 100 μ M in the parallel control experiment using cpSFYFP. As shown in **Fig. 13**, increasing concentrations of exogenous SAM induced a dose-dependent increase in the fluorescence ratio of SAMsor expressed in permeabilized *E. coli*

BL21(DE3), while 100 μ M MTA, 100 μ M SAH, 100 μ M adenosine, and 500 μ M L-methionine could not.

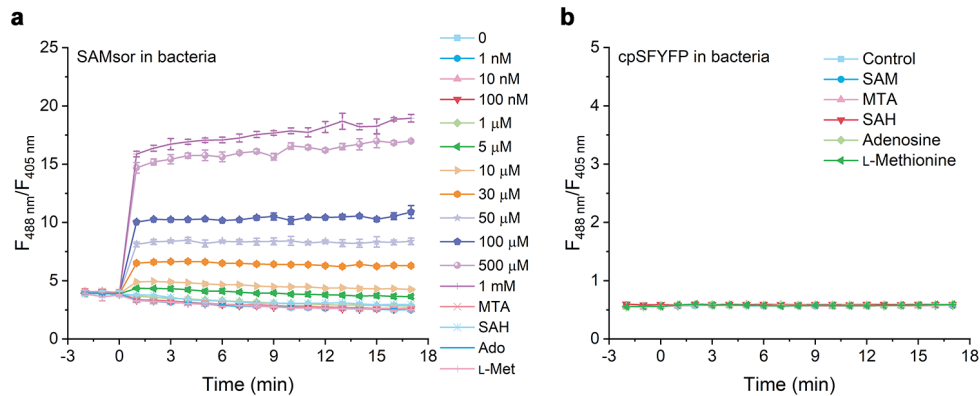


Figure 13 Characterization of SAMsor in bacteria. **(a)** Time courses of the fluorescence ratio changes of SAMsor expressed in permeabilized *E. coli* BL21(DE3) in response to exogenous gradient concentrations of SAM, 100 μ M MTA, 100 μ M SAH, 100 μ M adenosine, or 500 μ M L-methionine. **(b)** Time courses of the fluorescence ratio changes of cpSFYFP expressed in permeabilized *E. coli* BL21(DE3) in response to exogenous 100 μ M SAM, 100 μ M MTA, 100 μ M SAH, 100 μ M adenosine, or 500 μ M L-methionine. All data shown are means $\pm$ s.d. ($n = 3$ independent experiments). (Fig. 6e and Supplementary Fig. 41b in the revised manuscript)

Related data were presented in Fig. 6e and Supplementary Fig. 41b in the revised manuscript. Related descriptions were provided in the “Results” section of the revised manuscript as follows:

“Exogenous gradient concentrations of SAM induced a dose-dependent increase in the fluorescence ratio of SAMsor expressed in permeabilized *E. coli* BL21(DE3), while MTA, SAH, adenosine, and L-methionine could not (Fig. 6e and Supplementary Fig. 41b).”

6. In heatmaps like supplementary Fig. 4a, the legend of ΔR_{max} is missing a “%” symbol. In addition, I can not find such heatmaps for Valsor development.

Response: Thanks for your helpful reminder. We have added the missing “%” symbol to all heatmaps and their corresponding figure legends throughout the revised manuscript (Fig. 14, Fig. 15, and Fig. 16).

For the screening of cpSFYFP insertion sites within the biorecognition elements, we generated 51, 36, and 90 variants for the L-phenylalanine, L-isoleucine, and SAM biosensors, respectively (Fig. 14, Fig. 15, and Fig. 16). In contrast, only 7 variants were constructed for

the L-valine biosensor. Given this limited number of variants, we initially did not include the corresponding heatmap. However, for completeness, we have now generated and included this heatmap in the revised Supplementary Information (Fig. 17).

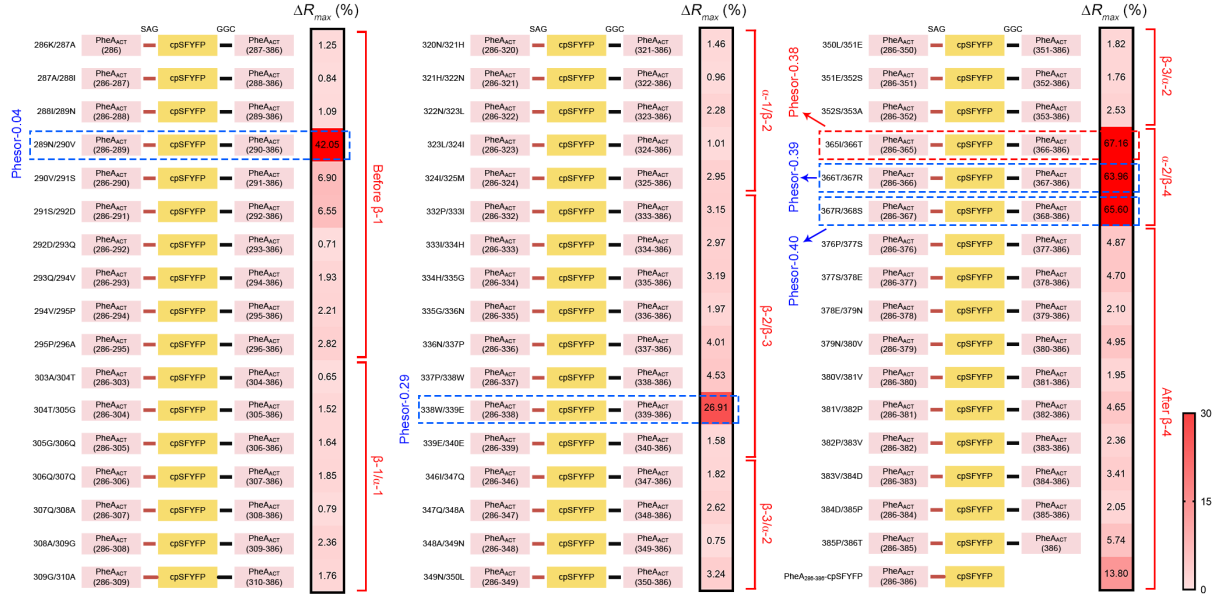


Figure 14 Schematic representation of the construction of the L-phenylalanine biosensor Phesor-0.38 based on insertion site screening. The ΔR_{max} of different biosensor variants in response to L-phenylalanine were presented in a heatmap, where the color depth represented the magnitude of ΔR_{max} (%). 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. (Supplementary Fig. 4a in the revised manuscript)

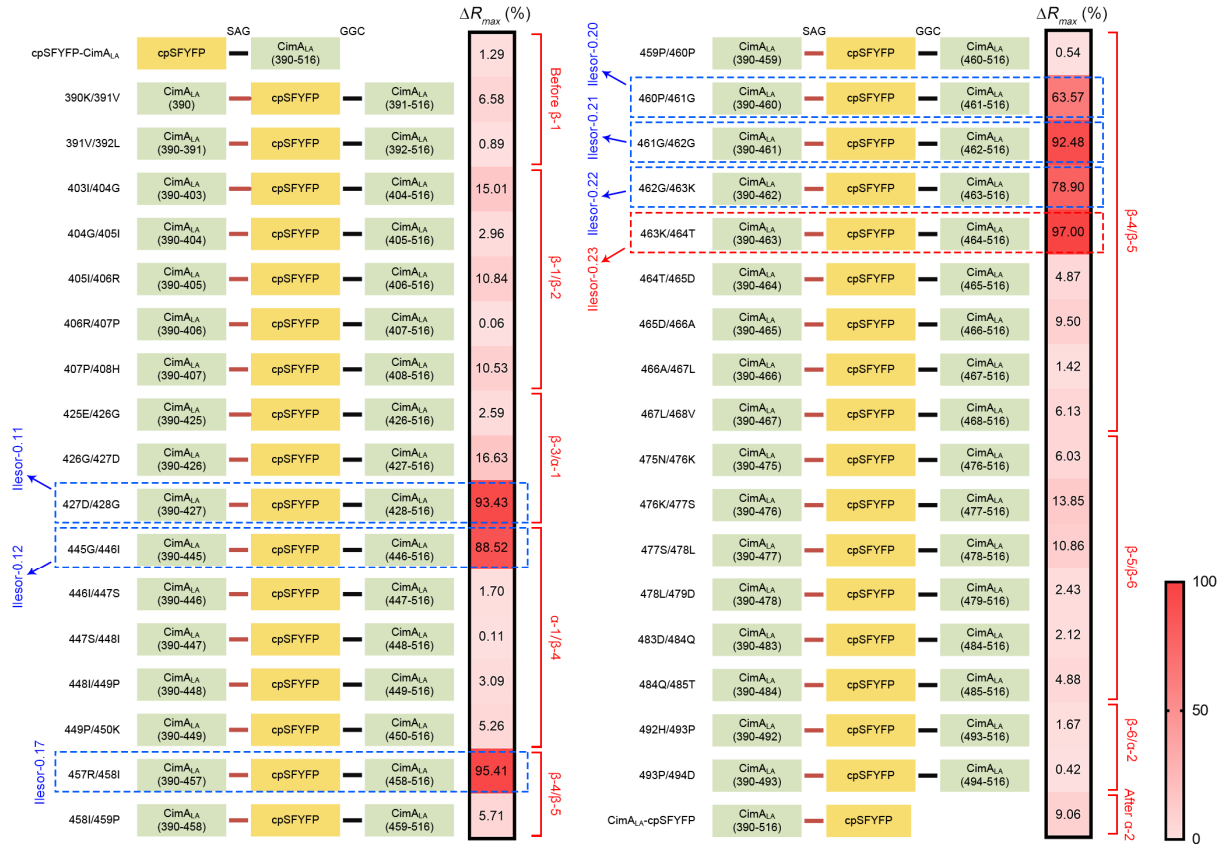


Figure 15 Schematic representation of the construction of the L-isoleucine biosensor Ilesor-0.23 based on insertion site screening. The ΔR_{max} of different biosensor variants in response to L-isoleucine were presented in a heatmap, where the color depth represented the magnitude of ΔR_{max} (%). The 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. (Supplementary Fig. 28a in the revised manuscript)

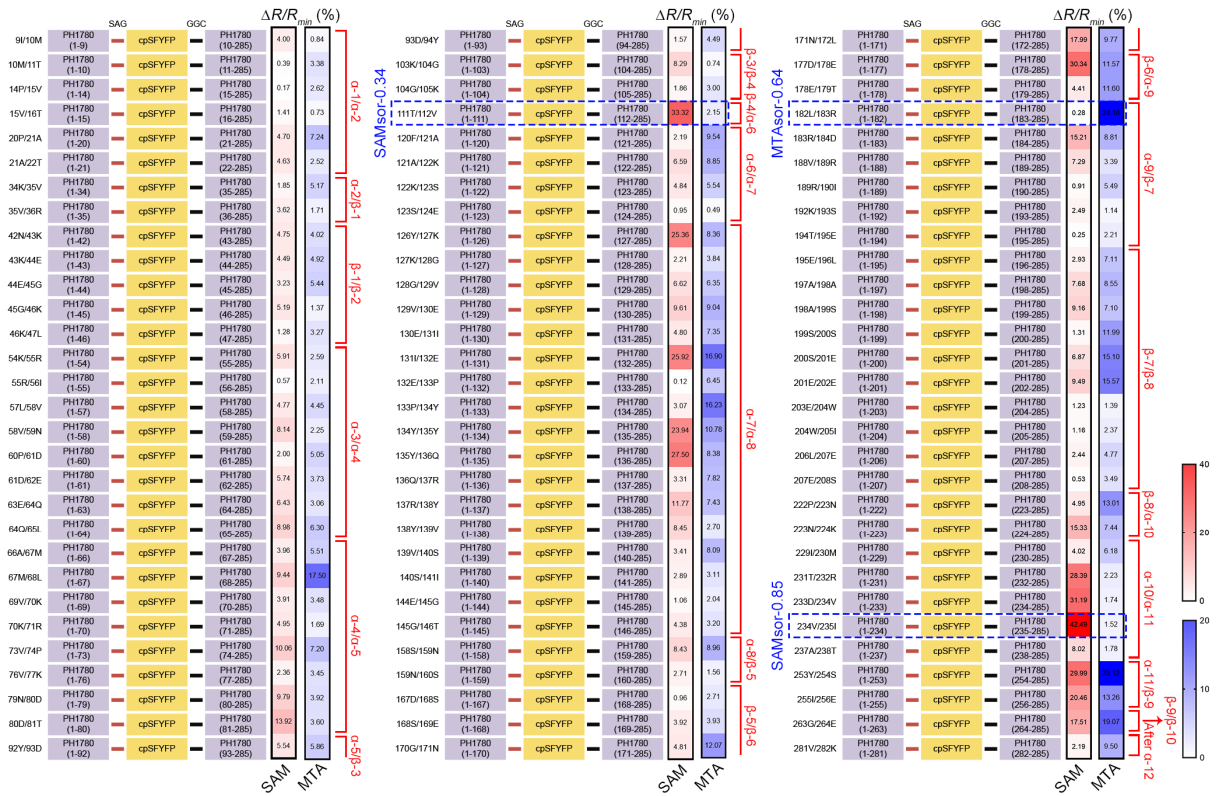


Figure 16 Construction of MTAsor-0.64, SAMsor-0.85, and SAMsor-0.34 based on insertion site screening. The response magnitudes ($\Delta R/R_{min}$) of different biosensor variants in response to 100 μM SAM or MTA were presented in a heatmap, where the color depth represented the $\Delta R/R_{min}$ value (%). 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. (**Supplementary Fig. 39 in the revised manuscript**)

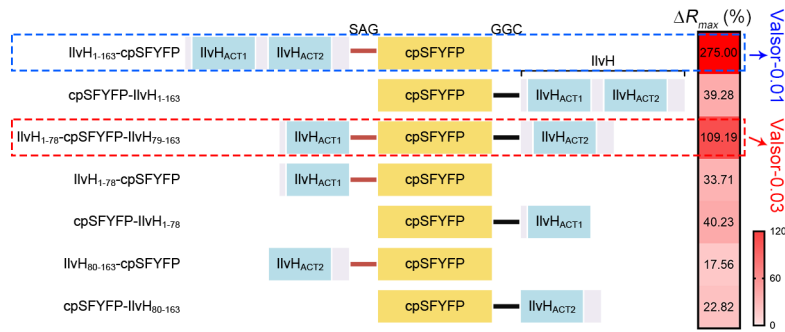


Figure 17 Schematic representation of the construction of the L-valine biosensor Valsor-0.03 based on insertion site screening. The ΔR_{max} of different biosensor variants in response to L-valine were presented in a heatmap, where the color depth represented the magnitude of ΔR_{max} (%). The structure 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. (**Supplementary Fig. 16b in the revised manuscript**)

7. The upper parts of some supplementary figures including Fig. 2, 4 and 37 appear to

be cropped or incomplete. For example, the label “ ΔR_{max} ” in supplementary Fig. 4 is partially obscured.

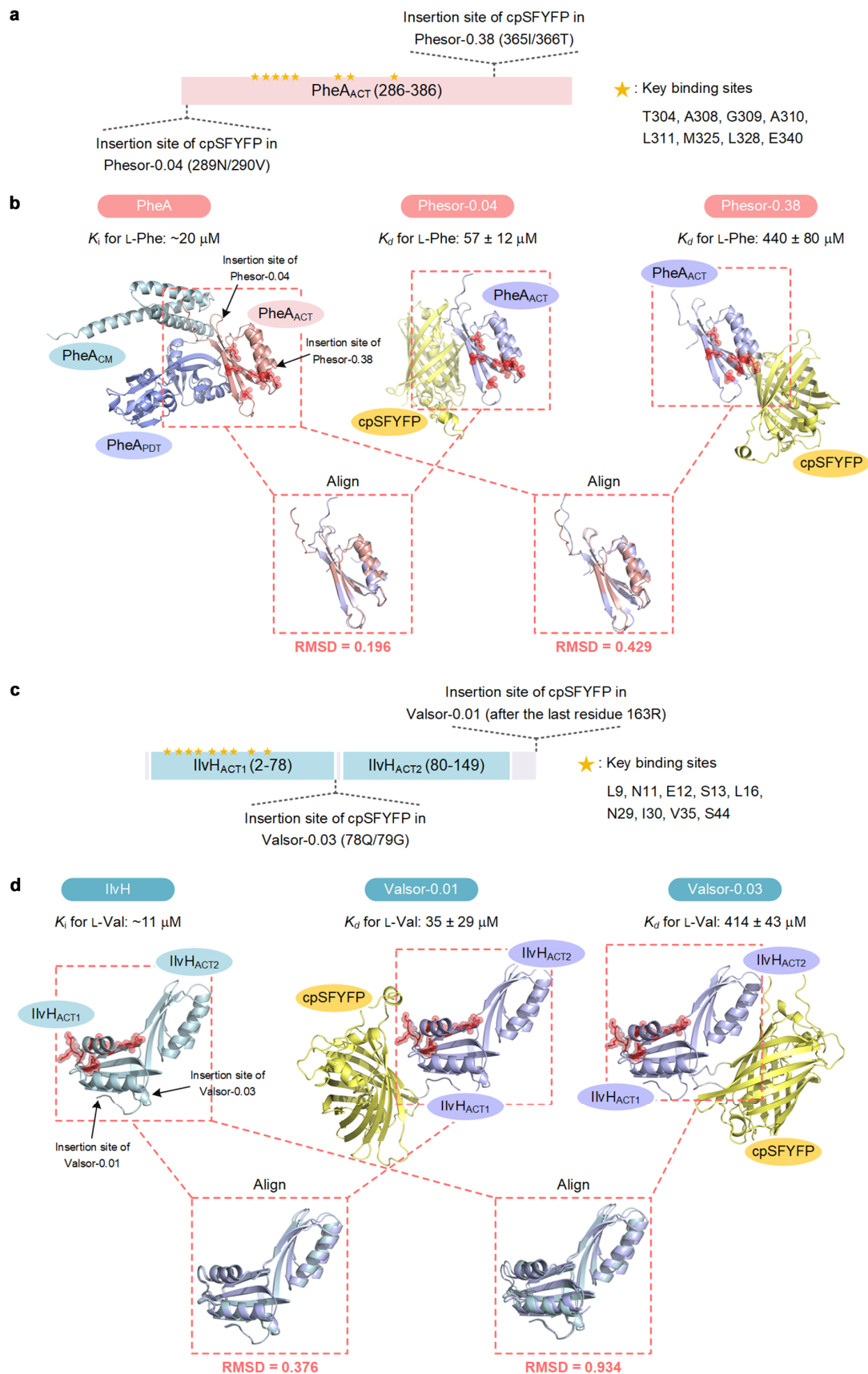
Response: We sincerely appreciate your careful review. We have corrected the cropping errors in Supplementary Figs. 2, 4, and 37 and thoroughly reviewed all other figures to ensure their proper formatting in the updated Supplementary Information.

8. Using structural modeling, the authors found that the low- K_d variants tend to have cpSFYFP insertion sites distal to the ligand-binding pockets. It will be clearer to highlight the ligand-binding sites in the structures. In addition, the figure legends should specify whether these structures were obtained from experimental PDB entries or predicted (e.g., by AF3).

Response: Thanks for your constructive suggestions. Following your recommendation, we have highlighted the ligand-binding sites and annotated the cpSFYFP insertion sites in the revised structural models. As shown in **Fig. 18**, the cpSFYFP insertion sites in the high-affinity (low- K_d) variants are consistently located distal to the ligand-binding pockets. This positioning minimizes structural disruption and maintains a higher architectural fidelity (lower RMSD) of the biorecognition element relative to its native conformation. Consequently, these variants exhibited K_d values closely approximating the native dissociation constant.

Furthermore, we have specified the sources of these structures in the figure legends of the revised manuscript as follows:

“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 [3].
[3] Kaplun, A. *et al.* Structure of the regulatory subunit of acetohydroxyacid synthase isozyme III from *Escherichia coli*. *J. Mol. Biol.* **357**, 951–963 (2006).”



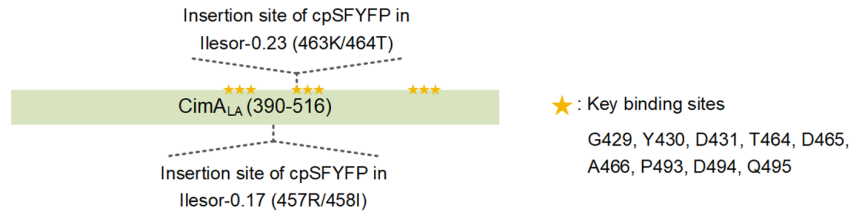
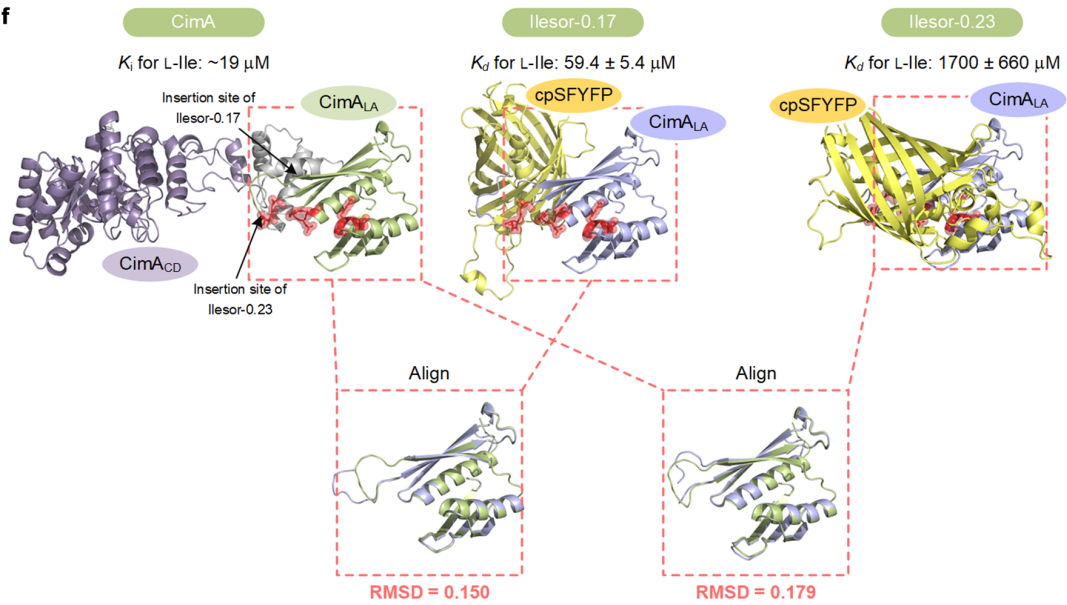
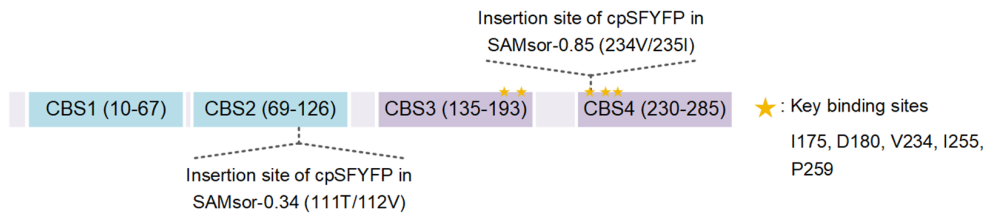
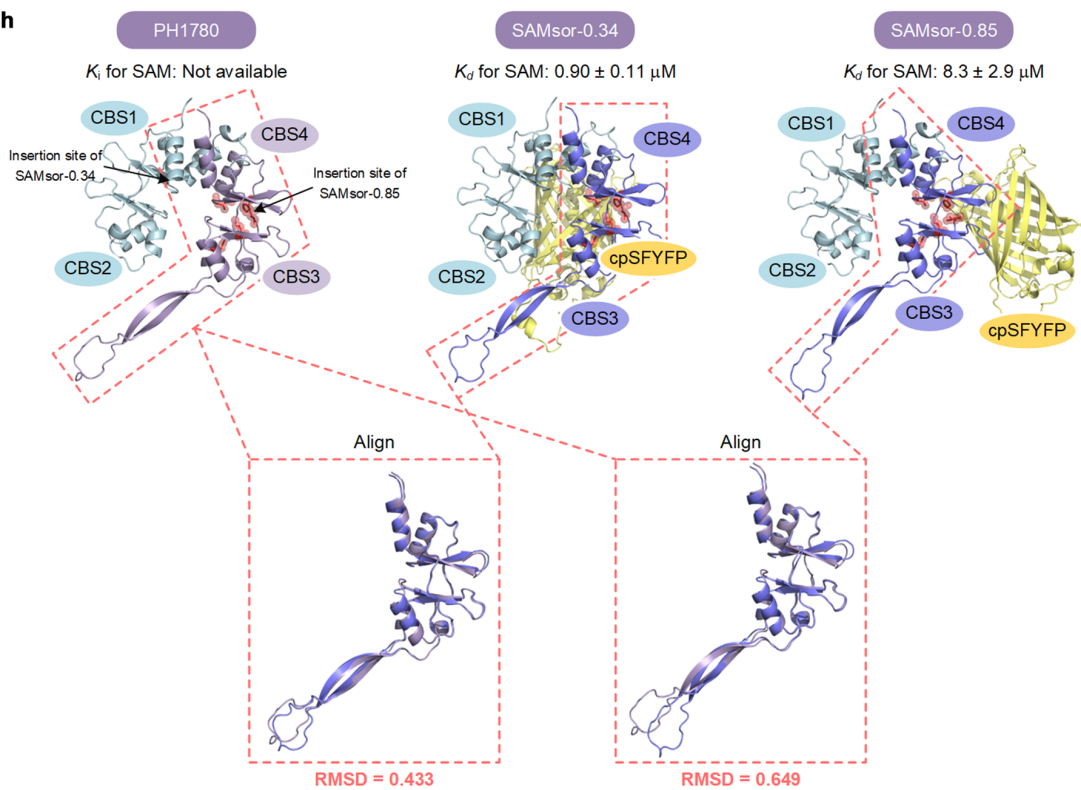
e**f****g****h**

Figure 18 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 [3]. 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 [3]; the L-isoleucine binding sites in CimA were derived from PDB entry 3F6G and reference [4]; and the SAM binding sites in PH1780 were identified through structural alignment with the regulatory domain of MJ0100 (Supplementary Fig. 38). **(Supplementary Fig. 49 in the revised manuscript)**

We hope these modifications satisfactorily address your concerns.

9. The authors state that PLIP website was used for binding site prediction for PheA. Was the same approach applied to the other sensors as well?

Response: Thanks for your question. The approaches for binding site analysis varied depending on the availability of existing experimental structures (**Fig. 18**). Specifically, 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 related literature (*J. Mol. Biol.*, 2006, 357:951–963); the L-isoleucine binding sites in CimA were derived from PDB entry 3F6G and related literature (*Biochem. J.*, 2009, 421:133–143); and the SAM binding sites in PH1780 were identified through structural alignment with the regulatory domain of MJ0100 (PDB entry 3KPC), a homolog of PH1780.

These detailed descriptions have been incorporated into the legend for Supplementary Fig. 49 in the revised manuscript, as follows (also noted in our response regarding **Fig. 18**):

“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 [3]; the L-isoleucine binding sites in CimA were derived from PDB entry 3F6G and reference [4]; and the SAM binding sites in PH1780 were identified through structural alignment with the regulatory domain of MJ0100 (**Supplementary Fig. 38**).

[3] Kaplun, A. *et al.* Structure of the regulatory subunit of acetohydroxyacid synthase isozyme III from *Escherichia coli*. *J. Mol. Biol.* **357**, 951–963 (2006).

[4] Zhang, P. *et al.* Molecular basis of the inhibitor selectivity and insights into the feedback inhibition mechanism of citramalate synthase from *Leptospira interrogans*. *Biochem. J.* **421**, 133–143 (2009).”

10. Compared with the 12-hour permeabilization shown in Fig. 4h of the original manuscript, the signal kinetics after 24-hour permeabilization in current Fig. 4e seems even slower. How to explain this unexpected result?

Response: We sincerely appreciate your careful observation. As you correctly noted, Ilesor exhibits slower on-kinetics after a 24-hour permeabilization compared to the 12-hour treatment (**Fig. 19a, b**). Chemical permeabilization with toluene and EDTA effectively disrupts the cell membrane to facilitate substrate entry. As demonstrated in our previous response, a 24-hour permeabilization is necessary to fully eliminate the membrane barrier, enabling the L-valine biosensor Valsor to achieve rapid kinetics (**Fig. 19c, d**).

However, prolonged exposure to toluene and EDTA can perturb protein conformation (*ACS Catal.*, 2013, 3:2823–2836). Considering the rapid on-kinetics of Ilesor in mammalian

cells (**Fig. 20**), we previously speculated that its delayed kinetics in bacterial environments might be attributed to impaired folding or maturation. This intrinsic structural instability likely makes Ilesor highly susceptible to extended chemical exposure. Therefore, the potential structural perturbation caused by the 24-hour treatment may counteract the benefits of enhanced membrane permeabilization, resulting in the observed slower kinetics. Taken together, these data further support our speculation that the delayed kinetics of Ilesor in bacterial environments likely result from its structural instability.

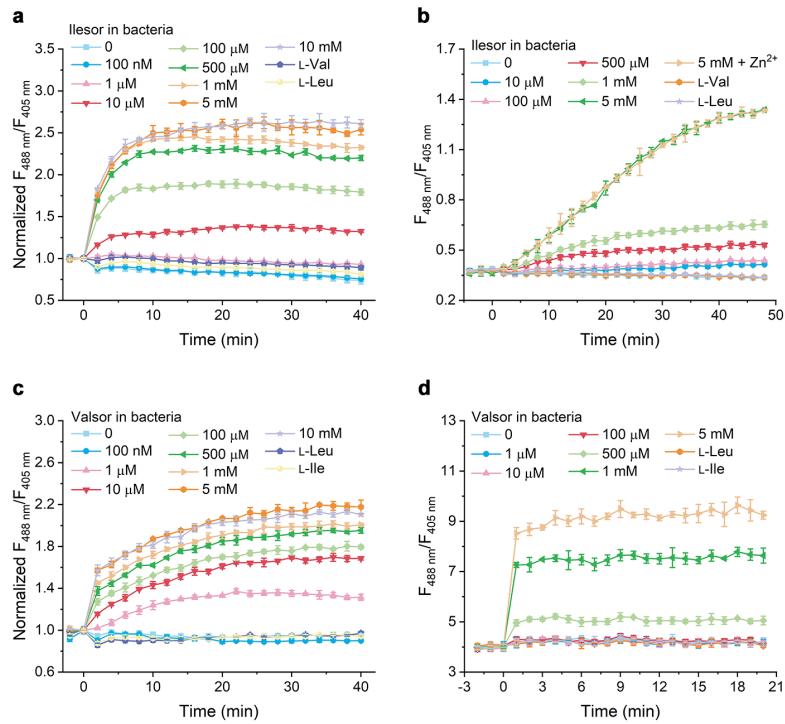


Figure 19 Analysis of the response kinetics of Ilesor and Valsor in *E. coli* BL21(DE3). **(a, b)** Time courses of the fluorescence ratio changes of Ilesor expressed in *E. coli* BL21(DE3) permeabilized for 12 h **(a)** or 24 h **(b)** in response to exogenous gradient concentrations of L-isoleucine and its structural analogs. 10 mM Zn^{2+} was used to facilitate the assembly of Ilesor tetramers. **(c, d)** Time courses of the fluorescence ratio changes of Valsor expressed in *E. coli* BL21(DE3) permeabilized for 12 h **(c)** or 24 h **(d)** in response to exogenous gradient concentrations of L-valine and its structural analogs. All data shown are means $\pm$ s.d. ($n \geq 3$ independent experiments). (**Fig. 3h and Fig. 4h in the original manuscript; Fig. 3e and Fig. 4e in the revised manuscript**)

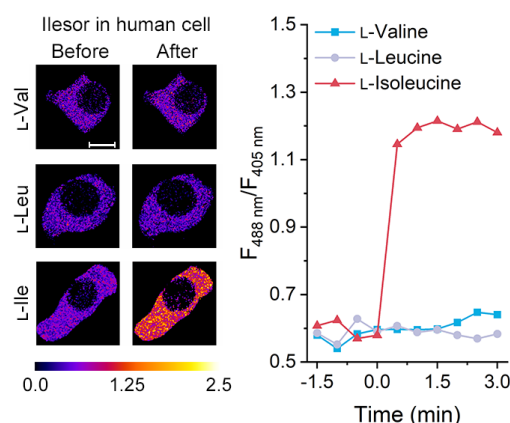


Figure 20 Ratiometric images and fluorescence ratios of permeabilized HEK293FT cells expressing Ilesor before and after the addition of 5 mM L-valine, L-leucine, or L-isoleucine. Scale bar, 10 μm . (**Fig. 4g in the revised manuscript**)

Accordingly, we have updated the manuscript to further discuss the potential impact of this intrinsic structural instability on the delayed kinetics, as follows:

“Ilesor expressed in permeabilized *E. coli* BL21 (DE3) specifically responded to the exogenous L-isoleucine addition in a dose-dependent manner (**Fig. 4e and Supplementary Fig. 19b**). However, Ilesor exhibited slow on-kinetics independent of its oligomeric state, potentially due to impaired folding or maturation, which compromises its structural stability in bacterial environments. Additional optimizations, such as introducing superfolder mutations or engineering binding sites⁴, may be required to improve the temporal resolution of Ilesor in bacteria.”

We hope these modifications satisfactorily address your concerns.

11. Alphafold3 is not a simple homology modeling tool. Please use the term “structural modeling” or “structural prediction” instead of “homology modeling”.

Response: Thanks for your correction. We have replaced the term “homology modeling” with “structural prediction” throughout the revised manuscript. For example:

“Next, we identified the L-phenylalanine binding sites of the PheA_{ACT} dimer through structural prediction and molecular docking analysis (Fig. 1j).”

“Structural prediction, molecular docking, and structural alignment

The structural prediction of PheA, Phesor-0.04, Phesor-0.38, IlvI, Valsor-0.01, Valsor-0.03, CimA, Ilesor-0.17, Ilesor-0.23, MTH1, PH1780, SAMsor-0.34, and SAMsor-0.85 was

performed using AlphaFold3 (<https://alphafoldserver.com/>).”

To Reviewer #4:

This manuscript has undergone a thorough revision in response to the reviewers comments. The manuscript has undergone considerable change addressing my primary concerns about the data that had been presented. I believe appropriate data is now included in both the main manuscript and the supplementary information to allow for publication.

Response: We appreciate your supportive comments and are encouraged that our revisions have addressed your initial concerns. Thank you for your time, your valuable critiques, and your support for the publication of this manuscript.

Reviewers' comments:

Reviewer #2 (Remarks to the Author):

The authors have carefully and comprehensively revised the manuscript in response to the reviewer's comments. The revised version is scientifically sound, clearly presented, and significantly improved in both rigor and clarity. I am fully convinced that this work will have a substantial impact on the field of biosensor engineering, and I strongly support its publication in Nature Communications.

Reviewer #3 (Remarks to the Author):

The authors have provided a thorough, point-by-point response to all my concerns and have incorporated the requested additional data. The revised manuscript is now robust and presents a complete narrative. The work is in an excellent state for publication, and I would like to thank the authors for their diligence and hard work.

Responses to the reviewers' comments point-by-point:

We sincerely thank the reviewers for their constructive comments and thoughtful suggestions, which have been very helpful in improving our manuscript.

To Reviewer #2:

The authors have carefully and comprehensively revised the manuscript in response to the reviewer's comments. The revised version is scientifically sound, clearly presented, and significantly improved in both rigor and clarity. I am fully convinced that this work will have a substantial impact on the field of biosensor engineering, and I strongly support its publication in Nature Communications.

Response: We sincerely thank you for the time and effort you have dedicated to reviewing our manuscript.

To Reviewer #3:

The authors have provided a thorough, point-by-point response to all my concerns and have incorporated the requested additional data. The revised manuscript is now robust and presents a complete narrative. The work is in an excellent state for publication, and I would like to thank the authors for their diligence and hard work.

Response: We sincerely thank you for the time and effort you have dedicated to reviewing our manuscript.