nature portfolio

Peer Review File

A Framework for Complex Signal Processing via Synthetic Biological Operational Amplifiers

Corresponding Author: Dr Ye Chen

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

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
As a cornerstone of synthetic biology, genetic circuit engineering faces inherent challenges due to the non-orthogonal and nonlinear nature of biological systems, which hinders the direct application of electronics-inspired design paradigms. In this work, Wenjun Cao et al. address this limitation by introducing an innovative genetic operational amplifier (OA) framework. The authors establish a theoretical foundation by drawing parallels to matrix diagonalization principles and implement OAs using well-characterized σ/anti-σ pairs.
A key demonstration involves engineering a growth-state sensor that significantly enhances signal resolution, expanding the dynamic range from <10-fold to 150/680-fold. The framework's practical utility is further validated through its successful application in metabolic engineering, addressing the persistent challenge of growth-expression coupling, and re-ducing crosstalk in multiple quorum-sensing systems.
This work provides a systematically validated design framework combining theoretical modeling, component fine-tuning, and multi-scenario validation. While scaling challenges persist (as acknowledged), the OA concept represents a conceptual leap toward predictive genetic circuit engineering that actively accommodates biological complexity. The research demonstrates both theoretical and experimental robustness, meriting dissemination in high-impact journals such as Nature Communications. Its interdisciplinary implications - spanning engineering biology, synthetic biology, quantitative biology, and systems biology - promise to stimulate interdisciplinary dialogues and inspire new methodologies for addressing complex biological system challenges through engineering paradigms.
However, before formal acceptance, this manuscript requires further necessary refinements and revisions, including additional scrutiny of figure-related issues. Below are my detailed comments:
Major Comments:
1. Page 8, Lines 144–148: The authors state that N sets of OA modules are required to achieve signal orthogonalization for N-dimensional inputs. While this suggests linear complexity, a preemptive evaluation accounting for biological context and genetic manipulation challenges remains critical. For instance: What is the theoretical upper limit of N under biological signal-to-noise ratio constraints? How does the required number of genetic parts (promoters, RBSs, proteins, terminators) scale with N?
2. Page 9, Equations 1–3: The protein expression model relies on the quasi-steady-state assumption. This may hold for exponential-phase bacteria undergoing stable division but raises concerns about its validity for modeling protein expression in stationary-phase bacteria.
3. Page 11, Lines 215–217: The textual description appears inconsistent with Fig. 2f, which lacks the clear "functional area" demarcation shown in Fig. 2c. The authors must rigorously verify the alignment between text and figures. In Fig. 2c, the description "the functional area $Otarget \geq 0$ and $Onontarget \leq 0$ are shown in white" differs from the display in the image.
4. Fig. 3a: The highlighted green circles presumably emphasize critical data points, yet neither the figure legend nor the main text clarifies their purpose. Are these sequences used to build the sequence logo (Fig. 3a, right panel) or for testing growth-state promoters in Fig. 3b (their quantity appears matched)? Enhanced annotation and/or methodological clarification are essential for readability.
5. Fig. 3b: No direct description of this figure exists in the main text. The authors should either integrate relevant explanations or consider relocating it to supplementary ma-terials.
6. Fig. 3d: The parameters on the x- and y-axes of the SNR heatmap are different from those in Figure 2c. Why are they labeled as RBS- and RBS+? It seems that BCD18 and BCD2 has the same effect on the parameter β.
7. Page 13, Line 277: While the bicistronic designs (BCD) concept is central to the OA module design, its mechanistic details in the main text could benefit from further elaboration. The Supplementary Materials briefly characterize BCD as

RBS-mediated translational control; however, incorporating a schematic diagram or referencing foundational studies (if applicable) would enhance accessibility for readers and clarify its structural or operational novelty.
8. Page 14, Line 298: Since your synthetic promoter is stage-specific, is there any signal crosstalk in the synthetic operational amplifier application shown in Figure 3?
9. Fig. 4: Why does the circuit exhibit varying performance across different strains? This may be due to differences in stage-specific promoter activity or cell burden caused by the expression levels of OA-related effector factors.
10. Fig. 5b: Multiple promoters are reused across cassettes (e.g., PlasO-cC/bE/bD, PcinO-bE/bC), yet Supp. Table 12 lists only one sequence per promoter. What do the suffix letters denote? Based on Page 18, Line 383, is this repetition necessary for promoter scaling? Clarification is needed. Additionally, could polycistronic structures achieve similar outcomes while avoiding part reuse—a critical consideration in genetic engineering? Given that the circuit employs multiple σ/anti-σ pairs, does the paper validate their mutual orthogonality? Would it be possible to incorporate additional experimental data or supporting literature references in this section?

Minor Comments:
1. Page 10, Line 195: The stated correlation coefficient conflicts with Fig. 2b. Please confirm if the described correlation coefficient is R2.
2. Page 10, Line 205: The symbol β' is inconsistent with its representation in Fig. 2d, and the description of the parameter XA here contradicts Supplementary Note 2.
3. Page 14, Line 286: Only the 688-fold dynamic range is emphasized, but the secondary value (153-fold) is equally notable. Both should be explicitly reported.
4. Fig. 4d: The protein bands of different samples are not singular. It would be better to add arrows in the figure to indicate the target protein location.
5. Fig. 5d: How many data points are shown here? Is the high correlation coefficient due to the points clustering around the zero point?

Reviewer #3

(Remarks to the Author)
This article presents a novel framework for complex signal processing using synthetic biological operational amplifiers (OAs). The key innovation of this study is the integration of orthogonal OAs into genetic circuits to enable efficient decomposition and amplification of multidimensional biological signals. By leveraging σ/anti-σ pairs, fine-tuning ribosome binding sites, and employing both open-loop and closed-loop configurations, the authors successfully engineered scalable OAs that enhance the precision and robustness of genetic circuits. Furthermore, the proposed framework demonstrates its versatility in applications such as growth-phase-responsive regulation, metabolic engineering, and resolving crosstalk in quorum sensing systems. Overall, the article is well-structured and provides a thorough theoretical foundation supported by experimental validation. The authors effectively demonstrate the advantages of OA-based signal processing over traditional binary genetic circuits. However, several aspects require further clarification or improvement:
1. To design more efficient OA circuits, the authors first conducted differential transcriptomic analysis of Escherichia coli in both the exponential and stationary phases, identifying promoters with significant expression differences between these growth stages. This approach effectively collected promoter data and provided a solid foundation for subsequent artificial promoter design. However, the data analysis methods used in this study appear relatively simplistic. For instance, the authors primarily selected promoters with the most pronounced differential expression and analyzed only the -10 and -35 regions before constructing artificial promoters. While this method is efficient and the experimental results confirmed the successful application of these synthetic promoters in dynamic regulation, its applicability to other microorganisms may be limited. Specifically, artificial promoters designed using this approach may not function effectively in different microbial species.
If the authors aim to establish a broadly applicable strategy for OA circuits construction rather than one limited to E. coli, further standardization of promoter design is necessary. One potential improvement is to leverage the large-scale transcriptomic data collected in this study and employ machine learning models, particularly language models, to analyze promoter design principles and optimize promoter sequences. This approach offers two key advantages. First, it allows for comprehensive utilization of transcriptomic data, minimizing the risk of information loss that may occur when focusing only on highly differentiated promoters. Second, using machine learning models to automate promoter design reduces manual intervention, improving the generalizability and scalability of the method. It is recommended that the authors explore this direction to further refine their study and enhance the applicability and automation of OA component design.

2. It is recommended that the authors further optimize the structure of the article from a writing perspective to improve readability and logical coherence. For example, in lines 294 to 299, the authors discuss the advantages of using OA circuits for dynamic regulation and compare them with other dynamic regulation methods. However, this section would be more appropriately placed in the Introduction to allow readers to grasp the innovations and advantages of this study over existing methods early on. Additionally, the current Introduction appears somewhat lengthy. The authors are advised to streamline it to more clearly highlight the core role of OA circuits in orthogonal regulation. Consider restructuring the content to focus more directly on the limitations of existing methods and introduce the novelty of OA components concisely, rather than providing excessive background information. This adjustment would not only enhance readability but also make the article more accessible to non-specialist readers.

3. Please ensure the proper usage of abbreviations. For example, in line 69, when "ECF" appears for the first time, its full name should be provided.

Reviewer #4

(Remarks to the Author)
Cao et al. describe a strategy to use OA architecture to process biological signals and provide a design strategy to obtain orthogonal outputs from signals with crosstalks. The manuscript provides a number of interesting aspects in synthetic biological designs with practical application examples. However, there are some parts that need to be improved for accessibility to readers in general.

1. The authors demonstrated a closed-loop circuit (Figure 2f) which significantly expanded the functional area and improved robustness. However, they did not show any applications using the closed-loop architecture. They need to provide at least one example experimental demonstration with the closed loop architecture. Otherwise, it only indicates that building a closed-loop architecture is impractical.
2. The authors provide nice examples with STA-ON and EXP-ON circuits. However, there was only a single output protein, LacI, for the STA-ON circuit. They need to provide at least three distinct protein outputs to show that the system can work with output proteins in general. Also, it is quite strange that BL21wt lane is very clear in Figure 4d, as if the there was less of a loading material. They need to provide an improved image for Figure 4d and provide clear quantification, for instance, by using other control bands, or using western blots, or His-tag purification.

There are also other minor points.
1. There are many instances where the parts of figures are not explicitly mentioned in the text. For instance, Figure 3b is not mentioned or clearly explained in the text. Figure 3e is mentioned before Figure 3d. Also, Figure 4a is not discussed in the text, and same with Figure 4c.
2. Line 293: cellss -> cells

Overall, the manuscript is well-written, but the authors need to address some critical points to improve the work.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have answered all my questions and commends. This paper should be published as is.

Reviewer #3

(Remarks to the Author)
The author has addressed my questions and concerns.

Reviewer #4

(Remarks to the Author)
The authors addressed the concerns raised by the reviewer with additional experiments. The authors performed additional experiments to build closed-loop circuits and demonstrated multiple protein outputs linked to regulatory circuits. They also used His-tag purification and additional PAGE experiments to confirm the protein outputs. The manuscript is largely improved in this aspect.
There is a minor question I have.
In Figure S16d and S16e, somehow GFP bands are quite strong even for OD_0-aTc condition, while those bands are usually mostly absent in all the other experiments. Also, in Figure S16e, lysozome expression from STA-ON2 circuits look very low. It would be good if the authors could comment on these briefly in the manuscript.

Version 2:

Reviewer comments:

Reviewer #4

(Remarks to the Author)
The authors addressed the comments by the reviewer adequately. I would like to support the manuscript for publication.

Reviewer #1

As a cornerstone of synthetic biology, genetic circuit engineering faces inherent challenges due to the non-orthogonal and nonlinear nature of biological systems, which hinders the direct application of electronics-inspired design paradigms. In this work, Wenjun Cao et al. address this limitation by introducing an innovative genetic operational amplifier (OA) framework. The authors establish a theoretical foundation by drawing parallels to matrix diagonalization principles and implement OAs using well-characterized σ/anti-σ pairs.

A key demonstration involves engineering a growth-state sensor that significantly enhances signal resolution, expanding the dynamic range from <10-fold to 150/680-fold. The framework's practical utility is further validated through its successful application in metabolic engineering, addressing the persistent challenge of growth-expression coupling, and reducing crosstalk in multiple quorum-sensing systems.

This work provides a systematically validated design framework combining theoretical modeling, component fine-tuning, and multi-scenario validation. While scaling challenges persist (as acknowledged), the OA concept represents a conceptual leap toward predictive genetic circuit engineering that actively accommodates biological complexity. The research demonstrates both theoretical and experimental robustness, meriting dissemination in high-impact journals such as Nature Communications. Its interdisciplinary implications - spanning engineering biology, synthetic biology, quantitative biology, and systems biology - promise to stimulate interdisciplinary dialogues and inspire new methodologies for addressing complex biological system challenges through engineering paradigms.

However, before formal acceptance, this manuscript requires further necessary refinements and revisions, including additional scrutiny of figure-related issues.

Response: We thank the reviewer for the valuable comments and for appreciating our work. We have carefully revised the manuscript following your suggestions. We are willing to address any further questions that you may have.

Below are my detailed comments:

Major Comments:

1. Page 8, Lines 144–148: The authors state that N sets of OA modules are required to achieve signal orthogonalization for N-dimensional inputs. While this suggests linear complexity, a preemptive evaluation accounting for biological context and genetic manipulation challenges remains critical. For instance: What is the theoretical upper limit of N under biological signal-to-noise ratio constraints? How does the required number of genetic parts (promoters, RBSs, proteins, terminators) scale with N?

Response: We thank reviewer for this important and insightful question regarding the scalability of our *N*-dimensional OA framework and the associated biological constraints. We have addressed these points in the revised manuscript.

1. Regarding the theoretical upper limit of *N* and signal-to-noise ratio (SNR) constraints:

As noted, our theoretical framework requires N orthogonal regulatory pairs to achieve complete decomposition of N-dimensional signals. Each regulatory pair consists of a σ/anti-σ factor set that functions independently and avoids interference with the host cell's native regulatory systems. As N increases, the metabolic burden on the host cell also increases. Each additional OA module requires resources for the expression of its components. This increased demand can strain cellular resources, potentially leading to altered global physiology.

To address your question more directly, we have revised the relevant section of the manuscript. The text now reads (Lines 146–150):

"This required N orthogonal regulatory pairs that functioned independently and avoided interference with the host cell's native regulatory systems. Theoretically, N is limited by available, biologically compatible, and truly orthogonal regulatory pairs. Practically, increasing N is constrained by cumulative host cell effects like metabolic burden.**"**

2. Regarding the scaling of genetic parts with N:

Regarding the scaling of genetic components:

- **Operons:** In the current architecture, where both scaling (input weights) and computation are handled genetically, a full-rank system may require up to N^2 operons (one per coefficient). However, if a tandem-promoter strategy is used—where all scaling is encoded via promoters—a minimal implementation can be achieved with just $2N$ operons: N for OA modules and N for scaling promoters. We have now clarified this trade-off in the revised text.

We have added this discussion to the manuscript. The text now reads (Lines 475–481):

"Applying this N-dimensional OA framework to higher N values increases genetic part numbers, impacting construct complexity and cellular burden. The genetic encoding presents a key design trade-off: systems with up to N^2 operons offer fine-grained coefficient control but are genetically intensive. Alternatively, strategies like tandem-promoter designs could reduce operon requirements to around $2N$, enhancing genetic economy, though potential issues like transcriptional roadblocking would then need careful management in future developments of complex biological signal processors[13, 77]."

- **Proteins (σ/anti-σ)** scale linearly with N, as each OA module requires one such orthogonal pair.
- **Promoters** scale with the input matrix; sparse matrices may reuse promoters, while dense ones may require more unique sequences.
- **RBSs** only need to be diversified when finer tuning of α/β ratios is required—otherwise, standardized RBSs suffice.
- **Terminators** are standardized and reused, as they do not participate in parameter tuning in the current design.

2. Page 9, Equations 1–3: The protein expression model relies on the quasi-steady-state assumption. This may hold for exponential-phase bacteria undergoing stable division but raises concerns about its validity for modeling protein expression in stationary-phase bacteria.

Response: We appreciate the reviewer's observation regarding the quasi-steady-state assumption in deep stationary phase. Our measurements were specifically conducted during an early steady phase where, importantly, while net population growth (cell division) slowed, the culture's OD continued to increase (as shown, for example, by the red circle in the following figure). This continuing growth, despite slowed division, signifies ongoing cellular metabolic activity and capacity for robust gene expression. Coupled with our data (e.g., Fig. 3f for STA-ON1) showing fluorescence signals evolving to or maintaining stable plateaus, it indicates that cells remain transcriptionally and translationally active. Characterizing our system in this physiologically active state, as expression levels of our constructs stabilize, therefore validates the application of the quasi-steady-state approximation for our modeling objectives.

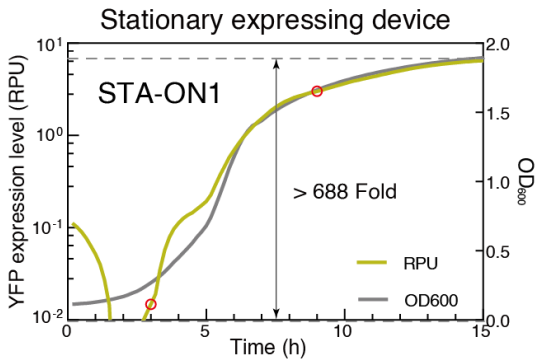


Figure 3f STA-ON1 device

3. Page 11, Lines 215–217: The textual description appears inconsistent with Fig. 2f, which lacks the clear "functional area" demarcation shown in Fig. 2c. The authors must rigorously verify the alignment between text and figures. In Fig. 2c, the description "the functional area $O_{target} \geq 0$ and $O_{nontarget} \leq 0$ are shown in white" differs from the display in the image.

Response: Thanks for spotting the inconsistency. We have corrected the description to:"The non-functional area $O_{target} \geq 0$ and $O_{off-target} \leq 0$ are shown in white."The figure (**Fig. 2c**) and caption have been checked and are now aligned with the corrected text.

4. Fig. 3a: The highlighted green circles presumably emphasize critical data points, yet neither the figure legend nor the main text clarifies their purpose. Are these sequences used to build the sequence logo (Fig. 3a, right panel) or for testing growth-state promoters in Fig. 3b (their quantity appears matched)? Enhanced annotation and/or methodological clarification are essential for readability.

Response: Thanks for the comment. The green-circled promoters in **Fig. 3a** were selected for experimental validation of growth-phase-specific expression, and their performance are presented in **Supplementary Fig. 9**. These promoters were chosen based on two criteria: (i) large SE ratio differences and (ii) consistency with the

identified sequence motifs from the logo plots in sequence logo (Fig. 3a, right panel). The experimental results confirmed their phase-specific behavior and supported the subsequent design of synthetic promoters.

This was briefly mentioned in the original text ("Based on these findings, we selected promoters from Fig. 3a…"), but we agree it was not sufficiently clear. We have revised both the figure legend and the main text to explicitly explain the purpose of the green circles.

The text now reads (Lines 262–269): "We further analyzed the sequence motifs of these phase-specific promoters from native RNAseq and identified distinct features consistent with prior studies[36, 37, 50, 51] (Supplementary Fig. 5-7): stationary-phase promoters favored AT-rich discriminator regions, cytosine enrichment at –13 nt and –36 nt, and a "TATACT" –10 motif; exponential-phase promoters displayed GC-rich discriminators and the canonical "TATAAT". To validate these features, we selected a subset of promoters from Fig. 3a (highlighted with green circles) that showed large SE ratio differences and matched the identified motif patterns. Their experimentally measured phase-specific expression strengths are shown in Supplementary Fig. 9, confirming their predicted behavior."

5. Fig. 3b: No direct description of this figure exists in the main text. The authors should either integrate relevant explanations or consider relocating it to supplementary materials.

Response: Thanks for your comment. In the revised manuscript, we now explicitly introduce **Fig. 3b** and its function. This panel illustrates the OA performance score calculated by screening all possible native promoter pairs across OA circuits. It serves to identify the best achievable OA performance when only native promoters are used as inputs. This analysis helps define the native benchmark for each OA circuit.

The same strategy was later applied to synthetic promoters, and the distribution of maximum OA scores is compared in **Fig. 3c**. We have now added this explanation to the Results section to clarify the role of **Fig. 3b**.

The text now reads (Lines 271–279): "Based on these results, we engineered a synthetic promoter library incorporating these phase-specific elements. Experimental tests (Supplementary Fig. 10) confirmed that synthetic promoters achieved significantly higher SE ratios and broader angular separation between input vectors than native promoters. To further quantify the performance ceiling of each OA configuration, we systematically screened all possible native promoter pairs as inputs to each OA, computing the best achievable OA score per combination (Fig. 3b). We repeated this analysis for synthetic promoters, allowing direct comparison between the native and synthetic promoter performance across OAs. This comparison, shown in Fig. 3c, demonstrated that synthetic inputs consistently outperformed native ones.

6. Fig. 3d: The parameters on the x- and y-axes of the SNR heatmap are different from those in Figure 2c. Why are they labeled as RBS- and RBS+? It seems that BCD18 and BCD2 has the same effect on the parameter β.

Response: Thanks for this detailed observation. While **Fig. 3d** and **Fig. 2c** both show how performance varies under parameter changes, their purposes and metrics differ:

- **Fig. 2c** uses **SNR** to represent general OA performance across signal space.
- **Fig. 3d** uses a **more application-driven "score"** metric that combines relative SNR and absolute signal output—better reflecting practical circuit usability.

The "RBS+" and "RBS−" axes in **Fig. 3d** denote the relative fold change in expression strength when swapping RBSs for the activator and repressor, respectively. For example, replacing BCD2 with BCD7 or BCD18 changes α or β. These changes shift circuit performance from the non-functional region (orange star, BCD2/BCD2) to the functional region (green star, BCD7/BCD18), as shown. Expressing axes in terms of RBS fold change makes the figure more intuitive for readers focused on component design. This distinction and rationale have been clarified in the revised figure legend and main text.

7. Page 13, Line 277: While the bicistronic designs (BCD) concept is central to the OA module design, its mechanistic details in the main text could benefit from further elaboration. The Supplementary Materials briefly characterize BCD as RBS-mediated translational control; however, incorporating a schematic diagram or referencing foundational studies (if applicable) would enhance accessibility for readers and clarify its structural or operational novelty.

Response: Thanks for the comment. The bicistronic design (BCD) is used in our work as a reliable method for tuning translational strength of RBSs, primarily to fine-tune the α/β ratio in OA circuits. While not conceptually central to the OA framework itself, BCD offers a practical and widely validated strategy for predictable expression tuning. As shown in Mutalik *et al.*(Mutalik, Guimaraes *et al*. 2013), BCDs mitigate context-dependent variability by reducing RBS–CDS interaction, enabling robust translational control without the need for iterative sequence optimization. This is particularly useful for modular circuit designs such as OAs, where precise control over component ratios is essential. We now cite this foundational study and have added a brief clarification in the revised manuscript to improve clarity for readers unfamiliar with BCD. Future developments in predictive RBS modeling may further enhance this approach.

The text now reads (Lines 284–290): "Some OA configurations were initially non-functional due to suboptimal α/β ratios (e.g., Fig. 3d, orange star). To resolve this, we tuned RBS strengths via bicistronic designs[41], a robust strategy for improving translation predictability and minimizing RBS–CDS context dependence. Axes in Fig. 3d are labeled "RBS+" and "RBS−" to represent activator/repressor translation strength changes. This figure illustrates how tuning RBSs (e.g., BCD2→BCD7, BCD2→BCD18) shifted the circuit into the functional zone (green star) without altering the underlying promoter or coding sequence."

8. Page 14, Line 298: Since your synthetic promoter is stage-specific, is there any signal crosstalk in the synthetic operational amplifier application shown in Figure 3?

Response: We appreciate this insightful question. While our synthetic promoters are designed for growth-stage specificity in the operational amplifier (OA) circuits, achieving complete signal orthogonality can be challenging. There might still be some background expression or "leakiness," meaning the input signal matrix may not be perfectly orthogonal.
However, our synthetic promoters demonstrate substantially improved performance over native promoters in this regard. As shown in Figure 3c, they exhibit significantly larger vector angles and reduced expression overlap between growth stages. This enhanced separation improves the overall performance and accuracy of the OA applications. Furthermore, by minimizing unknown native regulatory elements, our synthetic promoters are designed to reduce undesired interactions and potential crosstalk within the OA system. The application of these synthetic promoters in our OA circuits is demonstrated in Figures 3e and 3f.

9. Fig. 4: Why does the circuit exhibit varying performance across different strains? This may be due to differences in stage-specific promoter activity or cell burden caused by the expression levels of OA-related effector factors.
Response: Thanks for the comment. As noted, the observed variation in circuit performance across strains is primarily due to inherent differences in stage-specific promoter activity and protein expression capacity. Previous studies (Cardinale, Joachimiak *et al.* 2013) have shown that *E. coli* K-12 and *E. coli* B strains (such as BL21) differ significantly in promoter strengths, regulatory architecture (e.g., σ^{70}/σ^{38} distribution), and overall resource allocation. These strain-specific regulatory differences impact both the baseline transcriptional activity and the behavior of synthetic OA circuits. We have clarified this in the revised text and added appropriate references.

10. Fig. 5b: Multiple promoters are reused across cassettes (e.g., PlasO-cC/bE/bD, PcinO-bE/bC), yet Supp. Table 12 lists only one sequence per promoter. What do the suffix letters denote? Based on Page 18, Line 383, is this repetition necessary for promoter scaling? Clarification is needed. Additionally, could polycistronic structures achieve similar outcomes while avoiding part reuse—a critical consideration in genetic engineering? Given that the circuit employs multiple σ/anti-σ pairs, does the paper validate their mutual orthogonality? Would it be possible to incorporate additional experimental data or supporting literature references in this section?
Response: Thank you for your comment. We have provided detailed clarifications and made revisions to the manuscript as outlined below.
1. Promoter Variants (Suffixes), Scaling, and Supplementary Table 13 (Figure 5b):
The suffix letters (e.g., PlasO-cC, PlasO-bD) represent variants of the same core promoter that differ only in their -35 and -10 motifs, as described in (Chen, Ho *et al.*

2018). This promoter scaling method enables fine-tuning of promoter strength while preserving specificity and regulatory function. In our system, this scaling is necessary to match the output range required by each OA configuration without altering the encoded signal identity. As such, only one representative sequence is listed in Supplementary Table 13, since all variants share the same scaffold except for motif substitutions. We have clarified this in the revised figure legend and methods section.

2. Consideration of Polycistronic Structures:

We appreciate the reviewer highlighting the potential of polycistronic structures for reducing the overall number of genetic parts, a point discussed in our response to Major Comment 1 regarding N-scaling.

Our current modular design, utilizing independent transcriptional units, was chosen for its predictability and the precise, independent control it offers over each signal processing unit. This approach aligns with strategies proven effective in previous large-scale genetic circuit engineering(Chen, Zhang *et al*. 2020).

While polycistronic designs can indeed reduce part count, they present specific challenges for our analog OA circuits. Accurate quantitative control of each component is critical for OA function, and expression levels often attenuate along a polycistron(Qi, Haurwitz *et al*. 2012). This would necessitate highly precise calculations and fine-tuning of RBS strengths to achieve the target coefficients (β values) for each OA, complicating robust design.

Another common strategy for part economy, tandem promoters, has been successfully applied in digital logic circuits(Tamsir, Tabor *et al*. 2011). However, for our analog OAs, where operational errors can accumulate linearly (unlike threshold-based digital gates), the "roadblocking" phenomenon associated with tandem promoters would introduce significant design complexities(Shin, Zhang *et al*. 2020). We will briefly incorporate a discussion of these architectural trade-offs and their implications for analog circuit design in the revised manuscript.

3. Orthogonality of σ/anti-σ Pairs:

Finally, all σ/anti-σ pairs used in this work were selected from previously validated orthogonal sets (see Supplementary Table 2 and reference: Rhodius *et al.*(Rhodius, Segall-Shapiro *et al.* 2013). No significant cross-activation was observed across our tested OA circuits.

Minor Comments:

1. Page 10, Line 195: The stated correlation coefficient conflicts with Fig. 2b. Please confirm if the described correlation coefficient is R2.

Response: Thank you for pointing this out. The correlation coefficient ($R > 0.9$) originally mentioned in the text was a rounded interpretation of the actual R^2 value shown in Fig. 2b ($R^2 = 0.887$). We agree this could be misleading and have revised the text to explicitly report the R^2 value to maintain consistency with the figure and improve

accuracy.

2. Page 10, Line 205: The symbol β' is inconsistent with its representation in Fig. 2d, and the description of the parameter XA here contradicts Supplementary Note 2.
Response: Thank you for highlighting this point. We have revised the text and figure with corrected representations and description.

3. Page 14, Line 286: Only the 688-fold dynamic range is emphasized, but the secondary value (153-fold) is equally notable. Both should be explicitly reported.
Response: Thank you for your comment. We agree that both amplification values are meaningful. In the revised text, we now explicitly report both the 153-fold and 688-fold dynamic ranges to fully reflect the performance improvement achieved by our system.

4. Fig. 4d: The protein bands of different samples are not singular. It would be better to add arrows in the figure to indicate the target protein location.
Response: Thank you for your suggestion. We have revised Fig. 4d by adding arrows to indicate the position of the target protein bands for clarity.

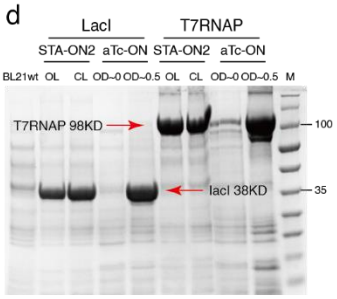
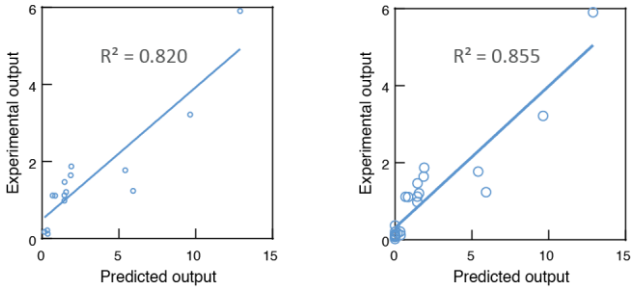


Fig. 4d, SDS-PAGE analysis of His-tagged protein expression. The STA-ON2 circuit was used to express His-tagged LacI (38 kDa) and T7RNAP (98 kDa) in both open-loop (OL) and closed-loop (CL) configurations. Arrows indicate the expected location of the target protein band. Protein levels were compared to those from aTc-inducible systems induced either at $t = 0$ or $OD_{600} \approx 0.5$. Both STA-ON2 configurations yielded expression comparable to the induced systems, confirming effective stationary-phase-specific activation without external inducers. BL21wt) showed no detectable expression. Additional His-tagged protein expression results are shown in Supplementary Fig. 16.

5. Fig. 5d: How many data points are shown here? Is the high correlation coefficient due to the points clustering around the zero point?
Response: Thank you for comment. A total of 24 data points are presented in Source data file of Fig. 5d. We agree that the high correlation coefficient partly arises from a cluster of low-expression values near the origin. However, these points are **biologically meaningful**—they represent conditions where the decoder predicted minimal or no gene expression, and the experimental results confirmed this. As such, these low-level outputs validate the decoder's performance in suppressing unintended crosstalk.

To further assess the model's accuracy across a broader dynamic range, we re-analyzed the dataset by excluding the 9 data points where both predicted and measured values were close to zero. The updated plot shows a correlation coefficient of $R^2 = 0.8201$, confirming that the decoder maintains high predictive fidelity even in non-zero regimes.



Left figure is the plot after removing the zero-value data points, while the right figure displays the original version used in main Fig. 5d. The corresponding numerical data are listed in the updated Source Data for Fig. 5, with the removed values highlighted in red.

Reviewer #3

This article presents a novel framework for complex signal processing using synthetic biological operational amplifiers (OAs). The key innovation of this study is the integration of orthogonal OAs into genetic circuits to enable efficient decomposition and amplification of multidimensional biological signals. By leveraging σ/anti-σ pairs, fine-tuning ribosome binding sites, and employing both open-loop and closed-loop configurations, the authors successfully engineered scalable OAs that enhance the precision and robustness of genetic circuits. Furthermore, the proposed framework demonstrates its versatility in applications such as growth-phase-responsive regulation, metabolic engineering, and resolving crosstalk in quorum sensing systems. Overall, the article is well-structured and provides a thorough theoretical foundation supported by experimental validation. The authors effectively demonstrate the advantages of OA-based signal processing over traditional binary genetic circuits.

Response: Thank you for reviewing our manuscript and for the helpful feedback. We have revised the manuscript focusing on your suggestions. We hope that our revisions have addressed your comments and improved understanding and presentation of the manuscript. We are willing to address any further questions from you.

However, several aspects require further clarification or improvement:

1. To design more efficient OA circuits, the authors first conducted differential transcriptomic analysis of Escherichia coli in both the exponential and stationary phases, identifying promoters with significant expression differences between these growth stages. This approach effectively collected promoter data and provided a solid foundation for subsequent artificial promoter design. However, the data analysis methods used in this study appear relatively simplistic. For instance, the authors primarily selected promoters with the most pronounced differential expression and analyzed only the -10 and -35 regions before constructing artificial promoters. While this method is efficient and the experimental results confirmed the successful application of these synthetic promoters in dynamic regulation, its applicability to other microorganisms may be limited. Specifically, artificial promoters designed using this approach may not function effectively in different microbial species.
If the authors aim to establish a broadly applicable strategy for OA circuits construction rather than one limited to E. coli, further standardization of promoter design is necessary. One potential improvement is to leverage the large-scale transcriptomic data collected in this study and employ machine learning models, particularly language models, to analyze promoter design principles and optimize promoter sequences. This approach offers two key advantages. First, it allows for comprehensive utilization of transcriptomic data, minimizing the risk of information loss that may occur when focusing only on highly differentiated promoters. Second, using machine learning models to automate promoter design reduces manual intervention, improving the generalizability and scalability of the method. It is recommended that the authors

explore this direction to further refine their study and enhance the applicability and automation of OA component design.

Response: Thank you for your suggestions. We agree that the transcriptomic analysis and synthetic promoter design strategy presented in this study are relatively streamlined and focused primarily on *E. coli*. This was a deliberate design choice, as the core objective of our current work was to validate the OA framework using a well-characterized model organism. As such, we prioritized establishing the general feasibility and effectiveness of growth-phase-responsive regulation using both native and rationally engineered promoters.

That said, as the reviewer correctly pointed out, our promoter analysis extended beyond the canonical -10 and -35 elements to include the extended -10 motif, the discriminator region, and the UP element. These features were selected based on their known relevance in *E. coli* transcriptional regulation and were cross-referenced with previous large-scale promoter architecture studies.

We fully recognize that extending the OA framework to other microbial species will require a more generalized and systematic promoter design approach. We appreciate the reviewer's suggestion to employ transcriptome-wide analysis coupled with machine learning or large language models for this purpose. We find this direction highly promising, particularly for expanding to non-model organisms or enabling promoter engineering without deep manual curation. In fact, we are currently pursuing such strategies in a follow-up study, which leverages large-scale transcriptomic datasets and transformer-based architectures to learn species-specific regulatory grammar and optimize promoter sequences in a data-driven manner. We will be sure to cite and credit this insightful recommendation in that future work.

To maintain focus and clarity, we have added a brief discussion of this limitation and future direction in the revised manuscript (Discussion section, Lines 459–467).

"Our synthetic promoter design strategy, while effective in E. coli, is currently based on transcriptional features such as SE ratio, -10， extended -10 and -35 motifs, and discriminator regions. Although these elements are well-supported in E. coli, their transferability to other organisms remains limited. To address this and expand the applicability of our OA framework, future work will explore more generalized promoter design strategies. In particular, leveraging large-scale transcriptomic datasets and integrating language models or transformer-based architectures may enable species-specific promoter optimization in a data-driven and automated manner. This would improve scalability, reduce manual intervention, and facilitate application across a wider range of microbial hosts."

2. It is recommended that the authors further optimize the structure of the article from a writing perspective to improve readability and logical coherence. For example, in lines 294 to 299, the authors discuss the advantages of using OA circuits for dynamic regulation and compare them with other dynamic regulation methods. However, this section would be more appropriately placed in the Introduction to allow readers to grasp the innovations and advantages of this study over existing methods early on.

Additionally, the current Introduction appears somewhat lengthy. The authors are advised to streamline it to more clearly highlight the core role of OA circuits in orthogonal regulation. Consider restructuring the content to focus more directly on the limitations of existing methods and introduce the novelty of OA components concisely, rather than providing excessive background information. This adjustment would not only enhance readability but also make the article more accessible to non-specialist readers.

Response: Thank you for your suggestions. We agree that relocating the discussion of OA-based dynamic regulation advantages (Lines 294–299) to the *Introduction* would help readers more quickly grasp the innovation and contextual relevance of our approach. We will revise this section accordingly.

Additionally, we acknowledge that the current *Introduction* contains background elements that could be condensed. We will streamline this section to more directly emphasize the core challenges in synthetic biology—particularly non-orthogonal signal processing and crosstalk—and introduce OA circuits as a novel solution earlier and more concisely. These adjustments will improve readability and accessibility, especially for non-specialist readers.

The text now reads (Lines 369–381): "Building upon the established challenge of crosstalk in multi-signal systems like quorum sensing (QS), we specifically investigated the LuxR (3-OC6HSL), LasR (3-OC12HSL), and CinR (3-OHC14HSL) QS pathways[58, 72]. These systems are frequently used in synthetic biology but are well-documented for exhibiting severe crosstalk and overlapping transcriptional responses[17]. Ideally, their cognate promoters—Plux, Plas, and Pcin—should operate orthogonally, responding exclusively to their respective N-acyl-homoserine lactone (AHL) signals. However, as illustrated in experimental characterizations (e.g., Fig. 5a), the natural encoding mechanisms within these systems lead to significant signal overlap, resulting in complex, non-orthogonal outputs.

To address this specific instance of three-dimensional signal interference without altering the native QS components, we applied our 3-dimensional Orthogonal Signal Transformation (3D-OST) framework. Our approach focused on computationally decoding the mixed transcriptional responses from these interacting pathways to reconstruct clear, orthogonal output signals."

3. Please ensure the proper usage of abbreviations. For example, in line 69, when "ECF" appears for the first time, its full name should be provided.

Response: Thank you for your suggestions. Corrected.

Reviewer **#4**

Cao et al. describe a strategy to use OA architecture to process biological signals and provide a design strategy to obtain orthogonal outputs from signals with crosstalks. The manuscript provides a number of interesting aspects in synthetic biological designs with practical application examples. However, there are some parts that need to be improved for accessibility to readers in general.

Response: Thank you for reviewing our manuscript and for the helpful feedback. We have revised the manuscript focusing on your suggestions. We hope that our revisions have addressed your comments and improved understanding and presentation of the manuscript. We are willing to address any further questions from you.

major

1. The authors demonstrated a closed-loop circuit (Figure 2f) which significantly expanded the functional area and improved robustness. However, they did not show any applications using the closed-loop architecture. They need to provide at least one example experimental demonstration with the closed loop architecture. Otherwise, it only indicates that building a closed-loop architecture is impractical.

Response: Thank you for your suggestions. We agree that demonstrating a practical application of the closed-loop architecture is important to support its utility beyond theoretical modeling.

In response, we have added new experimental data using closed-loop OA circuits constructed with **ECF11** and **ECF22** pairs. These circuits were applied to drive **protein expression**, demonstrating the feasibility of the closed-loop design in real applications. We further analyzed the system's **noise attenuation capabilities** and compared the **temporal expression profiles** under continuous culture conditions between open-loop and closed-loop designs.

These results confirm that the closed-loop architecture is not only feasible but also provide enhanced robustness and timing precision, supporting its practical use in dynamic gene regulation.

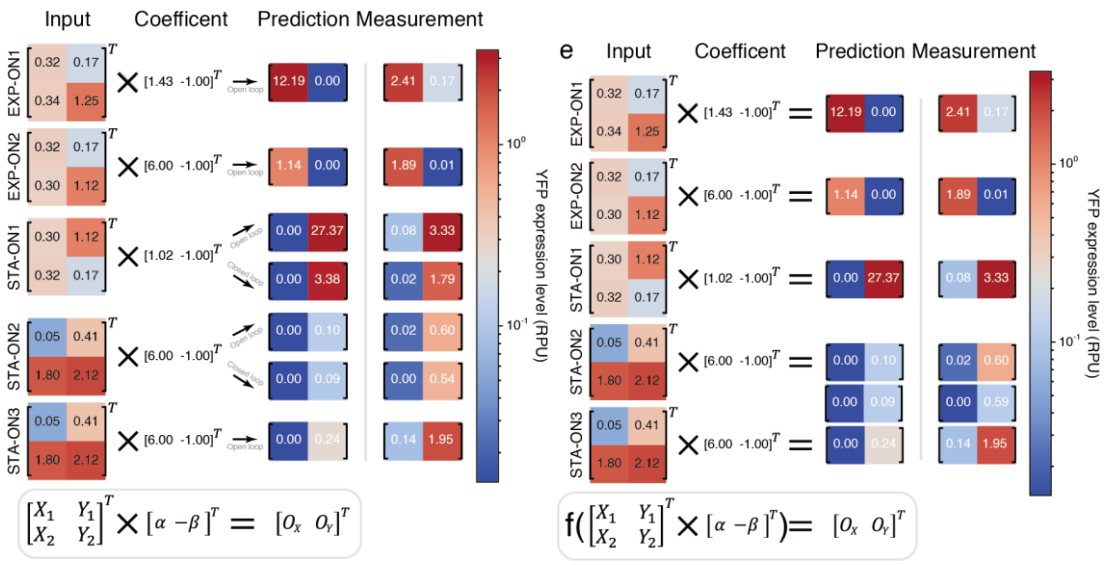
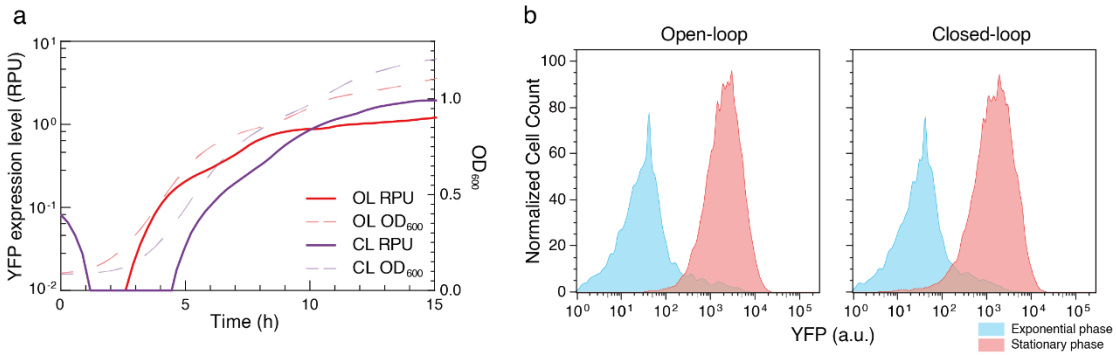


Fig. 3 | Functional circuit amplifier implementation. e, Functional circuits for EXP-ONs and STA-ONs. Input: Promoter activity under exponential (X) and stationary (Y) phases. Coefficient: OA parameters for signal processing. Prediction vs. Measurement: Modeled vs. experimental outputs (in RPU). STA-ON1 and STA-ON2 were constructed in both open-loop and closed-loop forms using the same input and coefficients. All EXP-ON reporters are tagged by ssrA to capture the real time expression(Andersen, Sternberg *et al*. 1998).

Note: Left is new main Fig. 3e. Right is previous main Fig.3e.



<u>Supplementary Figure S13:</u> Comparison of open-loop and closed-loop implementations of STA-ON2. a, Time-course fluorescence profiles of STA-ON2 in open-loop (OL, red) and closed-loop (CL, purple) configurations. Both circuits exhibit similar final expression levels during the stationary phase, indicating comparable performance in activating gene expression. However, the closed-loop variant shows a delayed activation onset, consistent with feedback-mediated regulation. This delay may help suppress premature expression during early growth, offering more precise temporal control. **b, Flow cytometry fluorescence distributions of STA-ON2 OL and CL circuits.** Both architectures demonstrate sharp OFF-to-ON transitions, with low background expression in the exponential phase (blue) and strong activation in the stationary phase (red). The narrow distributions suggest minimal signal noise, confirming that both open- and closed-loop designs reliably achieve growth-phase-specific expression with high fidelity.

2. The authors provide nice examples with STA-ON and EXP-ON circuits. However, there was only a single output protein, LacI, for the STA-ON circuit. They need to provide at least three distinct protein outputs to show that the system can work with output proteins in general. Also, it is quite strange that BL21wt lane is very clear in Figure 4d, as if the there was less of a loading material. They need to provide an improved image for Figure 4d and provide clear quantification, for instance, by using other control bands, or using western blots, or His-tag purification.

Response: We thank the reviewer for this insightful comment and fully agree that validating the STA-ON system with multiple output proteins is crucial for demonstrating its general applicability. In response, we have substantially expanded our experimental validation in the revised manuscript:

1. **Multiple protein outputs and circuits:** We tested three independent OA controllers based on ECF22, ECF11, and ECF38, in open-loop and closed-loop STA-ON configurations. These were used to express four distinct output proteins: LacI, GFP, T7 RNA polymerase, and T7 lysozyme. The resulting expression patterns were consistent and growth-phase-specific across all tested designs, confirming the generalizability and robustness of the STA-ON circuits. These data are now included in Fig. 4c–d, and Supplementary Figures S15–S16.
2. **Quantification and gel image improvement:** We acknowledge the reviewer's concern about the clarity of the BL21wt lane in the original Fig. 4d. In the revised version, we have provided an improved SDS-PAGE image (now Fig. 4d) with balanced sample loading. Additionally, we performed His-tag purification followed by densitometric quantification using a nucleic acid/protein analyzer to objectively compare expression levels across conditions. These quantified values are provided in Supplementary Table S12.

Together, these results provide strong evidence that the STA-ON architecture supports reliable and scalable protein expression across a range of protein types and circuit configurations. We appreciate the reviewer's suggestion, which helped us strengthen the demonstration of our system's versatility.

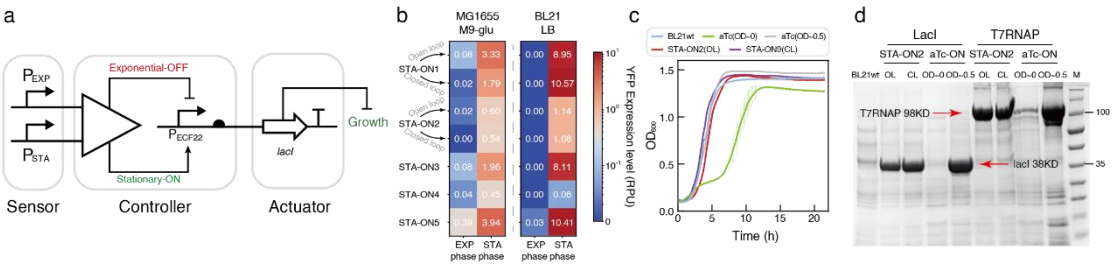
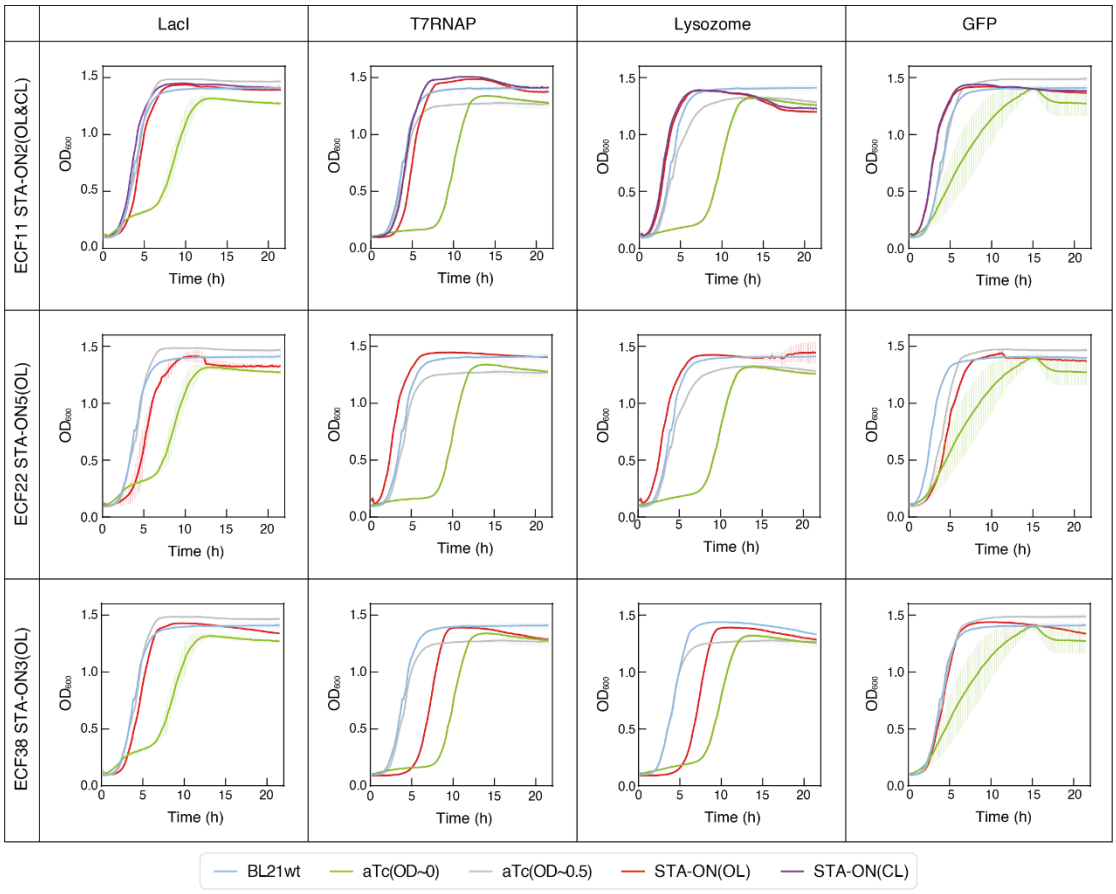
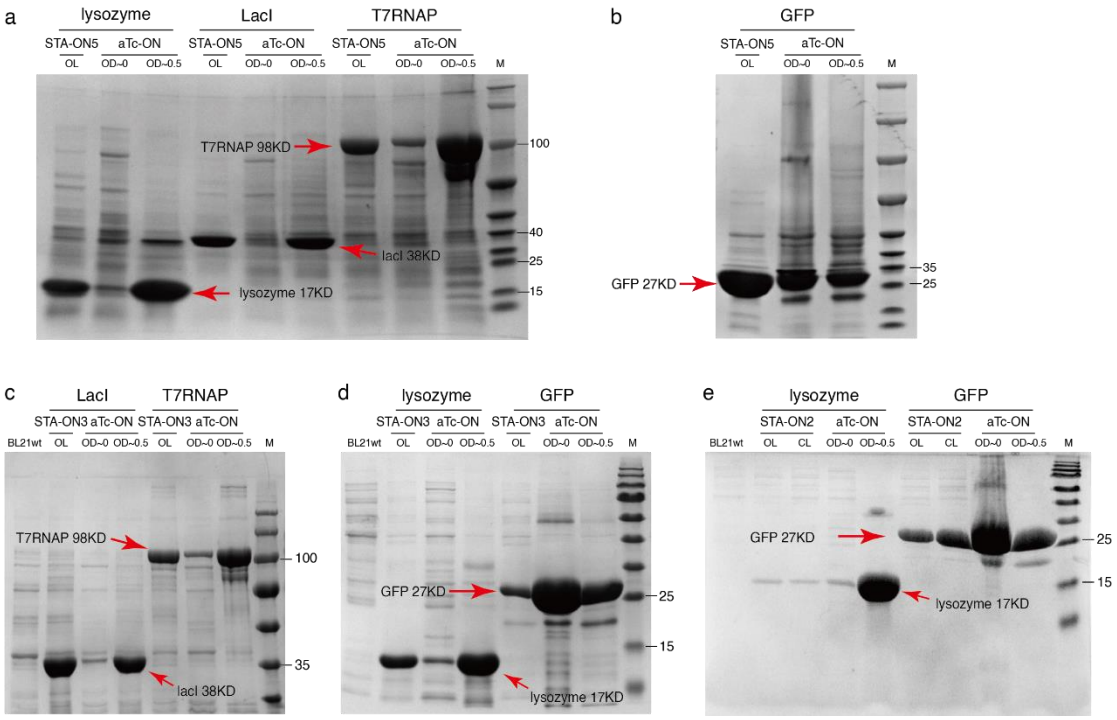


Fig.4 | Applications of functional amplifiers. a, Protein expression in BL21. The protein expression system uses the STA-ON5 circuit, comprising three modules: a sensor (a stationary-phase promoter as input$_1$ and an exponential-phase promoter as input$_2$), a controller [OA$_O$(E22.7.21.P$_{ECF22}$)], and an actuator (protein expression

module). The STA-ON circuit alternates between exponential-phase OFF and stationary-phase ON states, suppressing protein expression during the exponential phase to avoid growth inhibition and activating production during the stationary phase. **b, Cross-strain performance comparison of STA-ON circuits.** The expression profiles of several STA-ON circuits were evaluated in two E. coli strains (MG1655 and BL21) under their respective media (M9-glucose and LB). Most circuits showed consistent phase-specific behavior across strains, with low expression during the exponential phase and activation in the stationary phase. Both open-loop and closed-loop designs maintained functionality across hosts. Notably, a few circuits displayed reversed phase preference (e.g., higher expression in the exponential phase of MG1655), as detailed in Supplementary Fig. 14**. c, Growth curve analysis of STA-ON device in BL21.** The STA-ON device demonstrates growth comparable to wild-type BL21 and the induced system, where aTc was added at an OD_{600} of 0.5. In contrast, samples with circuits induced by aTc from the beginning (t=0) exhibited significant growth inhibition, likely due to metabolic stress. Shaded regions represent error bars from biological replicates. Additional growth curve results are shown in Supplementary Fig. 15. **d, SDS-PAGE analysis of His-tagged protein expression.** The STA-ON2 circuit was used to express His-tagged LacI (38 kDa) and T7RNAP (98 kDa) in both open-loop (OL) and closed-loop (CL) configurations. Arrows indicate the expected location of the target protein band. Protein levels were compared to those from aTc-inducible systems induced either at t = 0 or $OD_{600} \approx 0.5$. Both STA-ON2 configurations yielded expression comparable to the induced systems, confirming effective stationary-phase-specific activation without external inducers. BL21wt) showed no detectable expression. Additional His-tagged protein expression results are shown in Supplementary Fig. 16.



Supplementary Figure S15: Growth analysis of *E. coli* BL21 expressing four different proteins under various regulatory strategies. Growth curves of *E. coli* BL21 strains expressing four different heterologous proteins—LacI, T7 RNA polymerase (T7 RNAP), T7 lysozyme, and sfGFP—under five regulatory conditions: wild-type BL21 without expression (blue), constitutive expression induced with aTc at inoculation ($OD_{600} \approx 0$, green), delayed induction with aTc at $OD_{600} \approx 0.5$ (gray), STA-ON circuit with an open-loop design (OL, red), and STA-ON circuit with a closed-loop design (CL, purple). Each column corresponds to a different expressed protein. In all cases, STA-ON(OL) and STA-ON(CL) enabled robust growth comparable to wild-type and delayed induction, indicating minimal expression burden. In contrast, aTc induction at inoculation caused significant growth inhibition (especially for toxic proteins like LacI and T7 lysozyme), validating the need for growth-phase-specific control. The CL circuits exhibited slightly delayed activation compared to OL circuits, consistent with their feedback-regulated dynamics, yet both achieved similar final growth outcomes. These results demonstrate that the STA-ON strategy enables effective, inducer-free dynamic expression while minimizing cellular toxicity and preserving growth, with both OL and CL designs offering reliable and tunable expression control. Shaded areas represent standard deviations from biological triplicates (n = 3).



Supplementary Figure S16: SDS-PAGE analysis of heterologous protein expression driven by different STA-ON circuits. Coomassie-stained SDS-PAGE gels showing expression of target proteins—T7 RNA polymerase (98 kDa), LacI (38 kDa), T7 lysozyme (17 kDa), and GFP (27 kDa)—under growth-phase-specific regulation by STA-ON circuits in *E. coli* BL21. **a, Protein expression under control of STA-ON5.** Clear bands corresponding to T7 RNAP, LacI, and lysozyme indicate robust induction during the stationary phase, with minimal background expression in uninduced controls. **b, Expression of GFP driven by STA-ON5.** The distinct GFP band confirms successful dynamic regulation and effective phase-specific activation. **c, STA-ON3-driven expression of LacI and T7 RNAP.** Expression is visible and consistent with growth-phase-responsive activation. **d, Co-expression of lysozyme and GFP under STA-ON3.** Protein bands appear at the expected molecular weights, verifying reliable dual-protein output. **e, Comparison of lysozyme and GFP expression between STA-ON2 open-loop (OL) and closed-loop (CL) designs.** Both configurations produce similar expression levels, with the CL construct showing slightly delayed but strong induction, reflecting feedback stabilization. These results collectively demonstrate that STA-ON circuits can drive high-level, growth-phase-specific protein expression across diverse targets, achieving performance comparable to conventional induction systems without the need for external inducers.

There are also other minor points.

1. There are many instances where the parts of figures are not explicitly mentioned in the text. For instance, Figure 3b is not mentioned or clearly explained in the text. Figure 3e is mentioned before Figure 3d. Also, Figure 4a is not discussed in the text, and same with Figure 4c.

Response: Thank you for your suggestions. We have carefully reviewed the manuscript and revised the text to ensure that **all figure panels (including Figures 3b, 3d, 4a, and 4c)** are explicitly referenced and clearly explained in the correct order. Specifically, we added descriptions for **Figure 3b** to explain the native promoter performance benchmarking, corrected the sequence of **Figures 3d and 3e**, and included discussion of **Figures 4a and 4c** in the main text where relevant. These changes improve the clarity and logical flow of figure interpretation throughout the manuscript.

Description for Fig. 3b, Lines 271–279: "Based on these results, we engineered a synthetic promoter library incorporating these phase-specific elements. Experimental tests (Supplementary Fig. 10) confirmed that synthetic promoters achieved significantly higher SE ratios and broader angular separation between input vectors than native promoters. To further quantify the performance ceiling of each OA configuration, we systematically screened all possible native promoter pairs as inputs to each OA, computing the best achievable OA score per combination (Fig. 3b). We repeated this analysis for synthetic promoters, allowing direct comparison between the native and synthetic promoter performance across OAs. This comparison, shown in Fig. 3c, demonstrated that synthetic inputs consistently outperformed native ones."

Description for Fig. 3d, Lines 281–290: "Functional OAs were then constructed for applications requiring phase-specific outputs, such as exponential-phase-ON (EXP-ON) and stationary-phase-ON (STA-ON) circuits (Fig. 3e). Measured outputs closely matched theoretical predictions. The framework used for input-output matching is detailed in Supplementary Note 4. Some OA configurations were initially non-functional due to suboptimal α/β ratios (e.g., Fig. 3d, orange star). To resolve this, we tuned RBS strengths via bicistronic designs[32], a robust strategy for improving translation predictability and minimizing RBS–CDS context dependence. Axes in Fig. 3d are labeled "RBS+" and "RBS−" to represent activator/repressor translation strength changes. This figure illustrates how tuning RBSs (e.g., BCD2→BCD7, BCD2→BCD18) shifted the circuit into the functional zone (green star) without altering the underlying promoter or coding sequence."

Description for Fig. 4a, Lines 322–325: "As illustrated in Fig. 4a, the OA-based controller plays a key role by suppressing expression during the exponential phase—when cells are most sensitive to stress—and enabling high-level expression in the stationary phase. This strategy minimizes toxicity while maximizing protein output."

Description for Fig. 4c, Lines 339–344: "The growth profiles in Fig. 4c further demonstrate this effect: when aTc was added at the start of cultivation (t = 0), the induced system showed severely inhibited growth, consistent with toxic burden from premature expression. In contrast, both the STA-ON2 OL and CL circuit and late aTc induction (at OD~0.5) maintained growth curves comparable to the BL21 wild-type control, confirming the effectiveness of OA-based dynamic regulation."

2. Line 293: cellss -> cells
Response: Corrected as suggested.

Overall, the manuscript is well-written, but the authors need to address some critical points to improve the work.

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Reviewer #1

The authors have answered all my questions and commends. This paper should be published as is.

Response: We sincerely thank the reviewer for their positive evaluation and recommendation for publication.

Reviewer #3

The author has addressed my questions and concerns.

Response: We appreciate the reviewer's careful assessment and supportive feedback.

Reviewer #4

The authors addressed the concerns raised by the reviewer with additional experiments. The authors performed additional experiments to build closed-loop circuits and demonstrated multiple protein outputs linked to regulatory circuits. They also used His-tag purification and additional PAGE experiments to confirm the protein outputs. The manuscript is largely improved in this aspect.

There is a minor question I have.

In Figure S16d and S16e, somehow GFP bands are quite strong even for OD_0-aTc condition, while those bands are usually mostly absent in all the other experiments. Also, in Figure S16e, lysozome expression from STA-ON2 circuits look very low. It would be good if the authors could comment on these briefly in the manuscript.

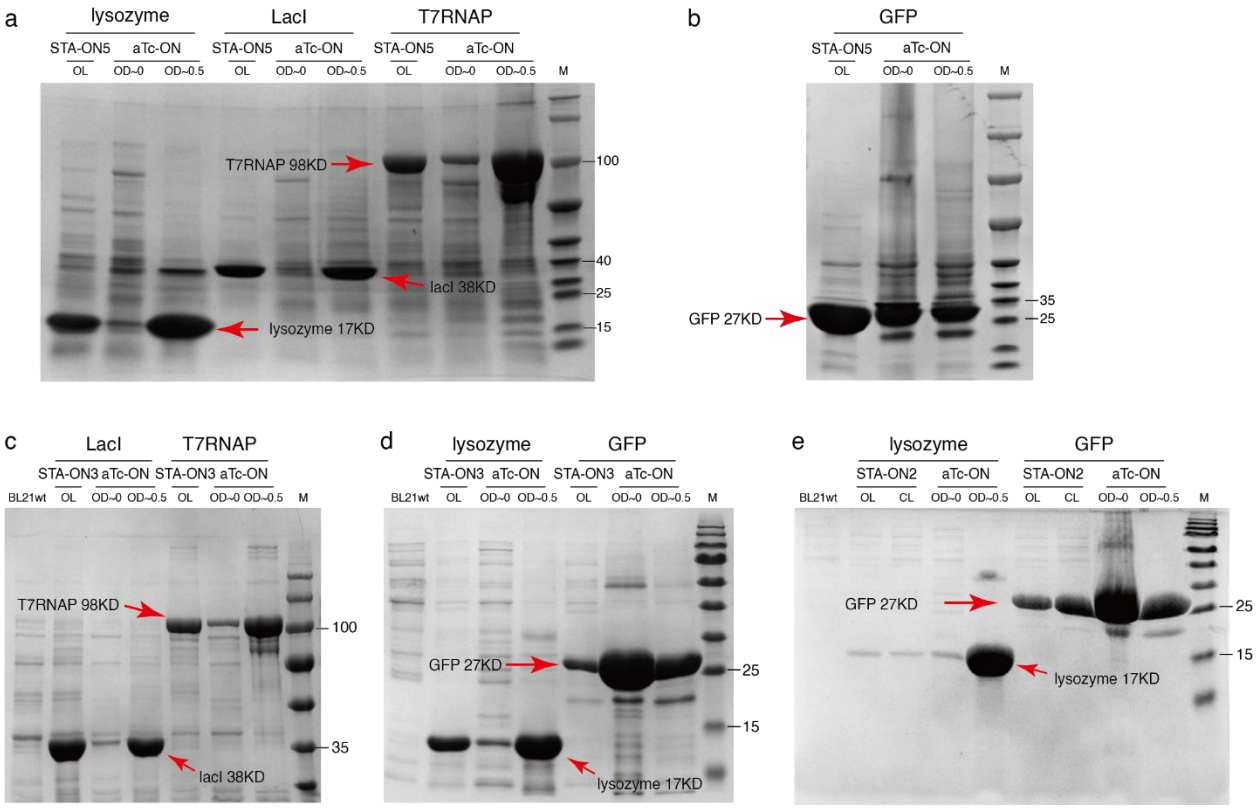
Response: We appreciate the reviewer's close inspection and thoughtful question.

The relatively strong GFP signal in the aTc-ON(OD~0) condition is likely due to the low toxicity of GFP. Unlike other proteins (e.g., lysozyme, T7RNAP), GFP imposes minimal metabolic burden on cells, which allows early induction (t=0) without triggering strong growth inhibition. This is consistent with Supplementary Fig. 15, where the OD_{600} growth curve for GFP under aTc(OD~0) showed less lag phase time than other proteins.

Regarding the STA-ON2 lysozyme expression in Fig. S16e, we agree that the bands appear weaker than expected. This likely reflects both the moderate expression strength of the STA-ON2 construct and gel loading variations. As shown in Fig. 3e and Fig. 4b, STA-ON2 exhibits slightly lower output compared to STA-ON1/3. This is further supported by quantitative data in Supplementary Table 12, where lysozyme expression levels for STA-ON2 circuits are slightly lower (52 – 54 mg/L) than STA-ON3 (91.2 mg/L), though the difference is not as pronounced as seen on gel. We have added a brief comment in the revised Supplementary text to clarify this point.

We revised main manuscript as follows:

"Among them, sfGFP exhibited strong expression even under early induction (aTc-ON(OD~0)), likely due to its minimal cytotoxicity, as supported by the corresponding growth profiles in Supplementary Fig. 15. While lysozyme expression of STA-ON2 appeared relatively weaker in SDS-PAGE, this is consistent with its lower quantified expression levels (Fig. 3e, Fig. 4b), as shown in Supplementary Table 12."



Supplementary Figure S16: SDS-PAGE analysis of heterologous protein expression driven by different STA-ON circuits. Coomassie-stained SDS-PAGE gels showing expression of target proteins—T7 RNA polymerase (98 kDa), LacI (38 kDa), T7 lysozyme (17 kDa), and GFP (27 kDa)—under growth-phase-specific regulation by STA-ON circuits in *E. coli* BL21. **a, Protein expression under control of STA-ON5.** Clear bands corresponding to T7 RNAP, LacI, and lysozyme indicate robust induction during the stationary phase, with minimal background expression in uninduced controls. **b, Expression of GFP driven by STA-ON5.** The distinct GFP band confirms successful dynamic regulation and effective phase-specific activation. **c, STA-ON3-driven expression of LacI and T7 RNAP.** Expression is visible and consistent with growth-phase-responsive activation. **d, Co-expression of lysozyme and GFP under STA-ON3.** Protein bands appear at the expected molecular weights, verifying reliable dual-protein output. **e, Comparison of lysozyme and GFP expression between STA-ON2 open-loop (OL) and closed-loop (CL) designs.** Both configurations produce similar expression levels, with the CL construct showing slightly delayed but strong induction, reflecting feedback stabilization. The relatively lower lysozyme band intensity likely reflects moderate expression output from STA-ON2, consistent with quantitative measurements in Supplementary Table 12. Notably, the relatively strong GFP signal in the aTc-ON(OD~0) condition of Fig. d&e is likely due to the low toxicity of GFP, which allows

early induction (t=0) without triggering growth inhibition. This is consistent with Supplementary Fig. 15, where the OD_{600} growth curve for GFP under aTc(OD~0) does not show significant lag phase.

These results collectively demonstrate that STA-ON circuits can drive high-level, growth-phase-specific protein expression across diverse targets, achieving performance comparable to conventional induction systems without the need for external inducers.