

Modular Scalable Synthetic Gene Circuits for Complex Functions Within Minimal Computational Layers in Human Cells

Corresponding Author: Dr Lior Nissim

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)

This paper presents an elegant and technically rich framework for constructing modular, scalable gene circuits in mammalian cells using RNA trans-splicing, synthetic microRNAs, and hybrid promoters. The authors demonstrate that this approach enables Boolean logic operations and complex computational behaviors (half adder, full adder, 3-to-1 multiplexer with SOS output) with reduced circuit layering. It's a conceptually significant and methodologically sound contribution that addresses a major challenge in mammalian synthetic biology: scalability and resource efficiency in multi-layer genetic computation.

However regarding the techniques and the comparison of the current work with the previous techniques I have some questions;

1- Can you quantify or at least discuss how much circuit complexity (plasmid number, base pairs, or layer depth) your framework reduces compared to CRISPR- or recombinase-based systems?

2-Did you observe any cytotoxicity, growth rate changes, or stress marker induction in high-plasmid conditions (especially in the full adder design)?

Some minor concerns :

- a. Please standardize "trans-splicing" vs. "transsplicing" (use consistently),
- b. in Materials and Methods, plasmid table can be transferred to the supporting information

Reviewer #2

(Remarks to the Author)

This authors of this paper introduce a new method for building sophisticated mammalian genetic circuits, using orthogonal genetic components that function together within a single processing layer. This approach employs RNA trans-splicing, which has been employed for bacterial genetic circuits but is here extended to mammalian genetic circuits. This study reports and characterizes a toolkit that will enable other researchers to build circuits employing these components. This technology holds promise for many use cases, including the diagnosis and treatment of combinatorial diseases that require processing multiple inputs, demonstrating its significance. In general, the figures are well crafted and the writing is clear, complete, and rigorous. This will make a valuable addition to the synthetic biology literature. There are a few concerns that should be addressed prior to publication.

There is a few major question that should be addressed:

- The double-stranded RNA used to drive trans-splicing is fairly long (50 bp, e.g. see Line 90). That should be long enough

to trigger antiviral innate immunity such as interferon production (>30 bp) and is potentially sufficient to trigger DICER processing and RISC-mediated RNA interference targeting the individual transcripts. Can the authors clarify whether this possibility was considered (or evaluated), and how that could affect any of the conclusions drawn or usages of this technology?

- The choice of negative input states introduced in Figure 4B (the full-adder circuit) requires some clarification. The authors explain that when characterizing this complex (10 plasmid) circuit design, definition of negative input states using plasmids with promoters that are largely inactive led to misinterpretation of results due to the impact of leakiness on cells that stochastically received some subset of the 10 required plasmids. This should probably be described as circuit failure (or fault) ascribable not necessarily to design but to the interaction of leakiness and random plasmid segregation. In any event, the current experimental design does not enable the reader to evaluate whether these circuits are actually more sensitive to leakiness of off states (in a manner that is independent of random plasmid segregation effects). Can the authors separate these contributions? If not, that limitation should be clarified and explained.

There are also a few specific items which should be addressed:

- In general, it seems that all “weighted mean (WM)” measures of reporter output require some units (either “arbitrary units” or some absolute unit).
- Lines 127-129: Is there a way to demonstrate that the canonical and AFP-based 3'SE are different enough to not interact?
- Line 186: It is unclear what is meant by “re-optimization”. Would the circuit modules need to be optimized, or would the splicing sites of the output protein need to be optimized? Please elaborate.
- Line 408 and 411: It is unclear how reactions (7) and (8) overcome inhibition of mKate2 production. Both transcripts M1.1 and M1.2 have an mKate2 module which should be inhibited by miRS2 since its binding sites are present in modules M2.1 and M3.1. Is there an interaction which renders these binding sites nonfunctional? Please clarify/explain.
- Lines 397-413 / Figure 4B: In state [1,1,1], there are four reactions that produce ECFP (compared to one reaction for inputs resulting in a “SUM” signal), but expression levels are comparable across these conditions. This seems surprising. Can the authors speculate as to why state [1,1,1] does not confer a much higher ECFP level?
- Figure 4B legend has a typo: “SUM Thrsehold”
- Lines 442-444: This demonstrates the benefit to using this type of circuit in an electrical computing system. Can you also explain the benefit of using this type of circuit in a biological system?
- Line 576 / Figure 5B: Threshold levels are referred to in the line of text but are not illustrated on the plot. Significance (either in the form of threshold lines or asterixis to denote statistical significance), should be indicated on the plot.
- Figure 1F: Arrow coming out of the AND gate is misaligned/extends through text
- Figure 3B: It is unclear how the threshold lines for Sum and Carry functions were calculated. If they were calculated in the same manner as the ones in figure 4B, this should be stated. Please also consider including descriptions of threshold calculations in the figure captions rather than text body.
- Figure 3B: Asterixis indicating statistical significance should be added to support conclusions drawn. If the criteria for signal is that it is higher than the threshold, that should be mentioned explicitly and the calculations for the threshold values should be explained.
- Figure S6B: Can you please clarify the conditions listed? For example, when L1 is deleted, are all other choices the same as indicated in panel A?
- Figure S6A/C: Can you please explain why linker 4, shown, was not investigated.

Reviewer #3

(Remarks to the Author)

In this manuscript, the authors developed a modular design framework for engineering scalable synthetic gene circuits in human cells, integrating orthogonal trans-splicing-based AND gates, tunable native-synthetic hybrid promoters, and synthetic microRNAs for inhibitory logic to execute complex functions (e.g., three-input combinatorial gate, half adder, full adder, 3-to-1 multiplexer with SOS output) within minimal computational layers, while systematically optimizing key elements like splicing sequences and binding domains to enhance efficiency and orthogonality. The study is highly innovative, addressing critical scalability barriers of traditional multi-layered circuits (e.g., excessive orthogonal components, high genetic payload). However, some concerns need to be addressed before the manuscript is suitable for publication:

Major comments :

1. While the flow cytometry data are quantitatively robust, the authors are encouraged to include representative fluorescence microscopy images for each transfection condition. Such images would provide visual validation of transfection efficiency, cellular morphology, and spatial distribution of the signal, thereby strengthening the credibility of the reported results.
2. To better demonstrate the system's generalizability, the authors should validate it with secreted proteins (e.g., Gaussia Luciferase). This would confirm its applicability to therapeutically relevant protein classes beyond intracellular fluorescent reporters.
3. The trans-splicing efficiency is currently inferred indirectly from protein-level outputs. Direct molecular evidence at the RNA level is required. The authors should perform junction-specific RT-PCR or Northern blot analysis to confirm the formation of the expected trans-spliced mRNA products and quantify splicing efficiency. This is critical for validating the core mechanism of the system.
4. The claim of improved scalability and reduced circuit complexity should be supported by a quantitative comparison with previously published mammalian gene circuits for constructed logic gate. A supplementary table summarizing the number of regulatory components, plasmids, or layers required to achieve similar computational functions would greatly strengthen the impact of this work.

5. The system relies on intermolecular RNA hybridization, which may lead to cross-reactions with endogenous non-coding RNAs (such as miRNAs and lncRNAs). It is recommended to analyze potential off-target binding using RNA-seq or RIP-seq, and to evaluate whether the key BD sequences share homology with known regulatory RNAs, in order to assess biosafety.

Minor comments:

1. The authors should discuss the potential sources of basal leakage observed in OFF states and propose strategies to further minimize it. This would enhance the practical utility of the system.
2. It is recommended to add a brief paragraph at the end of the Discussion section to explore potential applications of this system in cell therapy, smart drug delivery, or in vivo diagnostics, and to highlight key technical challenges for future development.
3. It is recommended that the BD design rules be summarized in a flow chart or table in the supplementary figures to facilitate readers' quick understanding of the key parameters.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors have nicely responded all of the concerns in the review letter. In its current form the manuscript is mature enough to publish. Publish as it is

Reviewer #2

(Remarks to the Author)

The authors of this paper adequately address most concerns from the original review. Explanations and additional experiments strengthen the claims made in the manuscript. This manuscript will be a valuable contribution to the literature.

There is one minor items which should be addressed:

1. Figure 4B / Lines 426-437:

Original comment: "Lines 397-413 / Figure 4B: In state [1,1,1], there are four reactions that produce ECFP (compared to one reaction for inputs resulting in a "SUM" signal), but expression levels are comparable across these conditions. This seems surprising. Can the authors speculate as to why state [1,1,1] does not confer a much higher ECFP level?"

The response does not quite address this concern. The original concern was intended to refer to the expression level of ECFP in state [1,1,1] relative to the expression levels of ECFP in states [1,1,0], [1,0,1], and [0,1,1]. For example, in State [1,1,0] there is one reaction that produces ECFP (reaction 3). In State [1,1,1] there are four reactions that produce ECFP (reactions 1, 2, 3, and 4). Thus, one would expect that State [1,1,1] would result in higher expression of ECFP than State [1,1,0], and yet the outputs are comparable in Figure 4B. Can you please explain this and/or provide interpretation for the reader?

Reviewer #3

(Remarks to the Author)

I appreciate the authors' thorough and thoughtful responses to my previous comments. The revised manuscript has been significantly improved with the addition of relevant data and clarifications that have strengthened the overall validity and credibility of the research. The authors have adequately addressed all my concerns, and the manuscript now presents a more comprehensive and well-supported study. I believe the paper has met the standards for publication and therefore recommend it for acceptance in its current form.

Reviewer #4

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Version 2:

Reviewer comments:

Reviewer #2

(Remarks to the Author)

The authors of this paper adequately address the concern from the last review. Explanations are clear and strengthen the claims made in the manuscript.

Reviewer #4

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

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Dear Reviewers,

We appreciate your insightful comments. Below is a point-by-point response addressing each concern. We have revised the manuscript accordingly, incorporating your suggestions. All edits are highlighted in yellow in the revised document. We hope our response sufficiently addresses your concerns and that the manuscript is now suitable for publication.

Sincerely,
Lior Nissim

Reviewer #1

This paper presents an elegant and technically rich framework for constructing modular, scalable gene circuits in mammalian cells using RNA trans-splicing, synthetic microRNAs, and hybrid promoters. The authors demonstrate that this approach enables Boolean logic operations and complex computational behaviors (half adder, full adder, 3-to-1 multiplexer with SOS output) with reduced circuit layering. It's a conceptually significant and methodologically sound contribution that addresses a major challenge in mammalian synthetic biology: scalability and resource efficiency in multi-layer genetic computation.

- We thank the reviewer for this very positive and supportive evaluation of this manuscript, and particularly for acknowledging the technical depth and methodological soundness of our work. We appreciate the assessment that this framework addresses major challenges in mammalian synthetic biology, including scalability and resource efficiency in multi-layer genetic computation. We have addressed the specific comments raised by the reviewer to further strengthen the manuscript, and hope that our revision resolves these concerns.

Major Concerns

1. Can you quantify or at least discuss how much circuit complexity (plasmid number, base pairs, or layer depth) your framework reduces compared to CRISPR- or recombinase-based systems?

- Indeed, this comparison is essential to establish our framework in terms of minimizing circuit complexity and computational layer depth. Accordingly, we added **Table S7**, which summarizes our work and representative studies that implemented genetic half-adders, full-adders, and multiplexers. This table is referred to in the discussion section **(Lines 664-667)**: “This circuit design strategy effectively addresses challenges associated with the limited availability of

orthogonal genetic elements while reducing the number of genetic components and computational layers required to execute complex functions in mammalian cells (Table S7)."

2. Did you observe any cytotoxicity, growth rate changes, or stress marker induction in high-plasmid conditions (especially in the full adder design)?

- This comment is important, since multi-plasmid transfection can induce cytotoxicity. To minimize cytotoxicity, we limited the total DNA amount to 2.5µg per sample. Accordingly, in full-adder samples comprising 10 plasmids, the quantity of each plasmid was 0.25µg. This approach maintained sufficient transfection efficiency without inducing excessive cytotoxicity.
- To support this observation, we added representative fluorescence microscopy images showing cell morphology and density (bright field), fluorescence output, and merged images, including:
 - i. All possible input states for a BD6-based AND gate, using a CMVp for the ON input and the transcriptionally inactive sequence for the OFF input (Figure S6D). We also added the following text to the manuscript (Lines 185-188): "Representative fluorescence microscopy images of the AND gate were acquired to qualitatively validate the spatial distribution of output expression and cellular morphology across all input states, demonstrating high transfection efficiency with minimal cytotoxicity (Figure S6D)."
 - ii. All possible input states of the full adder, in the same experimental conditions described in the manuscript (Figure S8). We also added the following text to the manuscript (Lines 444-448): "Inactive modules in previous designs, comprising ≤4 plasmids, were regulated by inactive promoters with minimal basal activity. To avoid transfection-mediated cytotoxicity in the 10-plasmid full adder circuit, we kept the total delivered DNA similar to that used in previous experimental setups. However, although this minimized cytotoxicity, partial plasmid delivery into cells during transfection became significantly more common (Figure S8)."

Minor Concerns

a. Please standardize "trans-splicing" vs. "transsplicing" (use consistently)

- Thanks for noticing. Done as requested. We now consistently use "trans-splicing" in the revised manuscript.

b. In Materials and Methods, plasmid table can be transferred to the supporting information

- This was also done as requested. The plasmid table was removed to the supporting information file “**Plasmid compositions.xlsx**”.

Reviewer #2

This authors of this paper introduce a new method for building sophisticated mammalian genetic circuits, using orthogonal genetic components that function together within a single processing layer. This approach employs RNA trans-splicing, which has been employed for bacterial genetic circuits but is here extended to mammalian genetic circuits. This study reports and characterizes a toolkit that will enable other researchers to build circuits employing these components. This technology holds promise for many use cases, including the diagnosis and treatment of combinatorial diseases that require processing multiple inputs, demonstrating its significance. In general, the figures are well crafted and the writing is clear, complete, and rigorous. This will make a valuable addition to the synthetic biology literature. There are a few concerns that should be addressed prior to publication.

- We thank the reviewer for the positive evaluation and for acknowledging the clarity and rigor of our work. We appreciate the assessment that this toolkit constitutes a valuable addition to the synthetic biology literature and holds promise for the diagnosis and treatment of combinatorial diseases. We have addressed the specific comments raised by the reviewer to strengthen the manuscript, and hope our revision resolves these concerns.

Major Concerns

The double-stranded RNA used to drive trans-splicing is fairly long (50 bp, e.g. see Line 90). That should be long enough to trigger antiviral innate immunity such as interferon production (>30 bp) and is potentially sufficient to trigger DICER processing and RISC-mediated RNA interference targeting the individual transcripts. Can the authors clarify whether this possibility was considered (or evaluated), and how that could affect any of the conclusions drawn or usages of this technology?

- Reviewer 2 raised an important question about the triggering of antiviral activity. This is indeed a critical point that we took into account in circuit design and should have been explicitly addressed in the manuscript. Indeed, 50bp dsRNA can activate antiviral innate immunity. Yet recognition by innate immune sensors are multifactorial, including RNA length, sequence, secondary structures, cellular location, post-transcriptional processing, and modifications,

which are often distinct from those of mammalian RNAs^{1,2,3}. Since our dsRNA is synthetic and nuclear rather than cytoplasmic, the risk for an innate immune response is reduced.

- DICER processing and RISC-mediated RNA interference are additional risks that could potentially reduce circuit efficiency and interfere with cellular machinery. However, as we and other experienced, efficient loading and processing by the miRNA machinery requires careful optimization of specific motifs in the dsRNA, including basal UG, Drosha, and Dicer motifs^{4,5}.
- Nevertheless, two safety measures can be introduced into the circuit design to mitigate these risks. First, BD lengths can be minimized to 30bp, as evident by BDs 7-10 (**Fig. 1J**). Second, we included mismatches within the BDs and their cognate BD-ASs to disrupt dsRNA continuity and thereby hinder recognition by cellular innate immunity sensors^{1,2,3}.
- We edited the manuscript accordingly to address these considerations, by adding the following text, references, and flow chart detailing the recommended BD design rules (**Figure S5B**) (**Lines 150-159**): “Nevertheless, additional considerations must be taken into account in BD design. 50bp dsRNA can activate antiviral innate immunity, although innate immune sensing depends on multiple factors, including RNA length, sequence, secondary structures, subcellular localization, post-transcriptional processing, and modifications^{1, 2, 3}. Therefore, it is recommended to limit BD length to 30bp, and introduce mismatches into longer BDs to disrupt dsRNA continuity and thereby hinder recognition by cellular innate immunity sensors^{1, 2, 3}. Furthermore, since long intronic dsRNA could be processed by the miRNA production machinery, it is critical to exclude basal UG, Drosha, and Dicer processing motifs^{4,5}. Finally, it is essential to avoid known regulatory sequences, such as binding sites for native miRNAs.”

The choice of negative input states introduced in Figure 4B (the full-adder circuit) requires some clarification. The authors explain that when characterizing this complex (10 plasmid) circuit design, definition of negative input states using plasmids with promoters that are largely inactive led to misinterpretation of results due to the impact of leakiness on cells that stochastically received some subset of the 10 required plasmids. This should probably be described as circuit failure (or fault) ascribable not necessarily to design but to the interaction of leakiness and random plasmid segregation. In any event, the current experimental design does not enable the reader to evaluate whether these circuits are actually more sensitive to leakiness of off states (in a manner that is independent of random plasmid segregation effects). Can the authors separate these contributions? If not, that limitation should be clarified and explained.

- We agree with the observation made by the reviewer that the observed background is likely a composite effect of intrinsic promoter leakiness and faults arising from stochastic plasmid segregation. We addressed this limitation in the text by adding the additional sentence: “Since proper circuit function requires the simultaneous presence of all 10 plasmids, the absence of essential modules prevents correct processing of the input state, and amplifies the impact of basal activity from leaky promoters in OFF-state modules, leading to misinterpreted outputs^{6, 7}. In contrast, empty plasmid backbones do not produce any transcript, thereby eliminating unintended expression from missing modules. (Lines 455-456): “Consequently, the ability to precisely estimate the contribution of input promoter leakiness to the generation of improper outputs is limited in this experimental setup.”

Specific items:

In general, it seems that all “weighted mean (WM)” measures of reporter output require some units (either “arbitrary units” or some absolute unit).

- Reviewer 2 is correct. The appropriate unit is indeed arbitrary units (a.u.), and we labeled the axes in the revised manuscript accordingly.

Lines 127-129: Is there a way to demonstrate that the canonical and AFP-based 3’SE are different enough to not interact?

- Indeed, both canonical and AFP-based 3’SE function as splicing enhancers, which could theoretically interact. However, the AFP-based 3’SE was employed exclusively in initial optimization constructs, while the canonical 3’SE V1 was utilized in all subsequent optimized constructs. Consequently, these elements are not mutually encoded within the same circuit. Therefore, we did not evaluate their physical interactions.
- Nevertheless, we did address the issue of cross-interactions of various 3’SE elements by RNA-Seq. In this analysis, we did not observe off-target interactions between the circuit 3’SEs and native 3’SEs, thereby supporting the hypothesis that there are minimal interactions among various 3’SE elements. This was emphasized by adding the following text. (Lines 233-238): “Finally, to assess potential off-target intermolecular RNA hybridization, we performed paired-end RNA seq (median depth ~66 million reads per sample) and STAR alignment with chimeric junction detection enabled. Across all BD constructs and mKate2 controls, off-target junctions (chrMKATE2) constituted only 0.06–0.75% of the total chimeric events and ≤revised MS this

0.013% of all mapped reads, comparable to the expected technical chimeric background of RNA seq libraries (Supplementary Table S8)."

Line 186: It is unclear what is meant by "re-optimization". Would the circuit modules need to be optimized, or would the splicing sites of the output protein need to be optimized? Please elaborate.

- We agree that this statement was unclear and requires clarification. Trans-splicing efficiency can be affected by multiple factors in the pre-mRNA encoding the output, such as RNA length and secondary structures. Therefore, similar quantities of pre-mRNAs encoding distinct output genes may yield different protein concentrations. Consequently, additional optimization of splicing motifs, linkers, and BDs may be required to yield specific output concentrations.
- This was clarified by adding the following text (Lines 199-203): "Trans-splicing efficiency can be affected by multiple factors in the output pre-mRNA, such as RNA length and secondary structures. Consequently, comparable quantities of pre-mRNAs encoding distinct output genes may produce different protein concentrations. Therefore, further optimization of splicing motifs, linkers, and BDs may be required to yield defined output concentrations."

Line 408 and 411: It is unclear how reactions (7) and (8) overcome inhibition of mKate2 production. Both transcripts M1.1 and M1.2 have an mKate2 module which should be inhibited by miRS2 since its binding sites are present in modules M2.1 and M3.1. Is there an interaction which renders these binding sites nonfunctional? Please clarify/explain.

- Indeed this point should have been better clarified in the text. The binding of GAD to the G3BSs in M1.1 and M1.2 increases mKate2 transcription from these modules. This additional expression increases mKate2 mRNA levels to a level sufficient to overcome inhibition by the miRs produced by M2.1 and M3.1, thereby enabling mKate2 protein production.
- This was emphasized by revising the text (Lines 435-438): "(7) GAD binds G3BSs in M1.1, and together with the upstream active enhancer sequence restores a full activation of the native-synthetic hybrid promoter, further enhancing mKate2 expression to overcome inhibition by mirS1 and mirS3, and therefore mKate2 is produced; and, in addition..."

Lines 397-413 / Figure 4B: In state [1,1,1], there are four reactions that produce ECFP (compared to one reaction for inputs resulting in a "SUM" signal), but expression levels are comparable across

these conditions. This seems surprising. Can the authors speculate as to why state [1,1,1] does not confer a much higher ECFP level?

- We agree that this point requires better explanation. The production of mKate2 (SUM) and ECFP (CARRY) is regulated by two independent splicing mechanisms with distinct efficiencies. The production of mKate2 is regulated by cis-splicing, which is more efficient than the trans-splicing regulating ECFP expression. This difference in protein production efficiencies determines the mKate2 and ECFP production ratios.
- This was emphasized by adding the following text **(Line 96): “Trans-splicing efficiency is inherently lower than that of cis-splicing.”**

Figure 4B legend has a typo: “SUM Thrsehold”

- Corrected. Thanks for noticing.

Lines 442-444: This demonstrates the benefit to using this type of circuit in an electrical computing system. Can you also explain the benefit of using this type of circuit in a biological system?

- This is an important point discussed later: “This architecture facilitates design optimization by enabling independent verification of each selector-input output and expands circuit functionality by allowing selection of output combinations for downstream iterations. For example, this design can be converted into a combinatorial gate by assigning specific outputs to predefined input combinations. Such functions are essential to address challenges in diseases of a combinatorial nature. For instance, treating autoimmune disorders requires selective inhibition of specific immune pathways only in response to disease-specific input combinations, which often overlap with normal inflammatory signals. This challenge can be addressed by assigning distinct therapeutic outputs to predefined combinations of inflammatory markers. Nonetheless, the modular architecture allows straightforward reconfiguration of the distinct outputs into a single output to execute MUX functionality.”

We also revised the paragraph at the end of the discussion to further discuss this point **(Lines 657-666): “In conclusion, this modular synthetic gene circuit engineering platform constitutes a significant advancement in synthetic biology, offering a robust, tunable, scalable, and orthogonal toolkit for executing complex computational operations in mammalian cells. This approach provides essential building blocks for precision diagnostics, targeted therapies, sophisticated biosensors, and cellular computing devices. For example, multi-input circuits**

enable the programming of immune cells for cell therapies^{8,9}, enhance the precision of gene therapies, and facilitate in vivo diagnostics of complex diseases¹⁰. Furthermore, combinatorial and MUX circuits are well-suited to address inherently heterogeneous diseases such as cancer¹¹ or immunological disorders that require targeting of specific inflammatory patterns¹². Therefore, this work supports next-generation biomedical and biotechnological applications.”

Line 576 / Figure 5B: Threshold levels are referred to in the line of text but are not illustrated on the plot. Significance (either in the form of threshold lines or asterixis to denote statistical significance), should be indicated on the plot.

- We agree with the reviewer’s comment. Threshold lines and calculations were added to the relevant graphs.

Figure 1F: Arrow coming out of the AND gate is misaligned/extends through text

- Corrected. Thanks for noticing (This is now **Figure 1G**).

Figure 3B: It is unclear how the threshold lines for Sum and Carry functions were calculated. If they were calculated in the same manner as the ones in figure 4B, this should be stated. Please also consider including descriptions of threshold calculations in the figure captions rather than text body.

- The importance of this aspect was not emphasized in the original manuscript. Therefore threshold lines and calculations were added to this Figure and Legend, as well as the main text, including for Figure 3B of the revised manuscript (**Lines 320-323**): “Specifically, the OFF/ON threshold was calculated as $\left(\frac{Max\ WM - Min\ WM}{2}\right) + Min\ WM^{13}$, yielding a threshold of ~464,666 WM for mKate2 and ~532,168 WM for ECFP.”

Figure 3B: Asterixis indicating statistical significance should be added to support conclusions drawn. If the criteria for signal is that it is higher than the threshold, that should be mentioned explicitly and the calculations for the threshold values should be explained.

- The criteria for the signal are indeed that it is higher than the threshold. We indicated this in the main text, added threshold lines, and included threshold calculations in all relevant figures and legends, as required by the reviewer’s comment.

Figure S6B: Can you please clarify the conditions listed? For example, when L1 is deleted, are all other choices the same as indicated in panel A?

- This observation is correct. This issue was clarified in the figure legend **(Lines 38-41): “(B) Splicing output with various linker configurations using AFP-BD. Excluding deleted linkers that are indicated with Δ , and modified linker size is indicated in bp, the configuration of all other linkers is as indicated in S6A.”**

Figure S6A/C: Can you please explain why linker 4, shown, was not investigated.

- Linker 4 was added as a 3'UTR. Therefore, we focused only on linkers located within exons and most likely involved in the trans-splicing process (Linkers 1-3). We better addressed this issue in the main **text (Lines 168-169): “Since linkers within exons are most likely to affect trans-splicing, we focused on optimizing L1-L3.”**

Reviewer #3

In this manuscript, the authors developed a modular design framework for engineering scalable synthetic gene circuits in human cells, integrating orthogonal trans-splicing-based AND gates, tunable native-synthetic hybrid promoters, and synthetic microRNAs for inhibitory logic to execute complex functions (e.g., three-input combinatorial gate, half adder, full adder, 3-to-1 multiplexer with SOS output) within minimal computational layers, while systematically optimizing key elements like splicing sequences and binding domains to enhance efficiency and orthogonality. The study is highly innovative, addressing critical scalability barriers of traditional multi-layered circuits (e.g., excessive orthogonal components, high genetic payload). However, some concerns need to be addressed before the manuscript is suitable for publication.

- We thank Reviewer 3 for the accurate summary of the work and the positive assessment of its innovation and potential to address scalability barriers in synthetic biology. This support is very encouraging. We have addressed the specific comments detailed below and hope our revision resolves his concerns.

Major Comments

While the flow cytometry data are quantitatively robust, the authors are encouraged to include representative fluorescence microscopy images for each transfection condition. Such images

would provide visual validation of transfection efficiency, cellular morphology, and spatial distribution of the signal, thereby strengthening the credibility of the reported results.

- We strongly agree with Reviewer 3. Therefore, we added representative fluorescence microscopy images showing cell morphology and density (bright field), fluorescence output, and merged images, including:
 - i. All possible input states for a BD6-based AND gate, using a CMVp for the ON input and the transcriptionally inactive sequence for the OFF input (**Figure S6D**). We also added the following text to the manuscript (**Lines 185-188**): “Representative fluorescence microscopy images of the AND gate were acquired to qualitatively validate the spatial distribution of output expression and cellular morphology across all input states, demonstrating high transfection efficiency with minimal cytotoxicity (**Figure S6D**).”
 - ii. All possible input states of the full adder, in the same experimental conditions described in the manuscript (**Figure S8**). We also added the following text to the manuscript (**Lines 444-448**): “Inactive modules in previous designs, comprising ≤ 4 plasmids, were regulated by inactive promoters with minimal basal activity. To avoid transfection-mediated cytotoxicity in the 10-plasmid full adder circuit, we kept the total delivered DNA similar to that used in previous experimental setups. However, although this minimized cytotoxicity, partial plasmid delivery into cells during transfection became significantly more common (**Figure S8**).”

To better demonstrate the system's generalizability, the authors should validate it with secreted proteins (e.g., Gaussia Luciferase). This would confirm its applicability to therapeutically relevant protein classes beyond intracellular fluorescent reporters.

- We strongly agree with this comment. Demonstrating secreted therapeutic output facilitates the generalizability and potential applicability of our system. We therefore decided to demonstrate this applicability with an actual secreted immunostimulator. For this purpose, we replaced the mKate2 output in the GAD-based AND gate with human IL-15, a key chemokine that regulates the activation and proliferation of T and natural killer (NK) cells. As expected, we observed significant IL-15 secretion in ELISA assay in the [1,1] state, but not in other states.
- These results, which constitute a major improvement to the manuscript, were added to the manuscript as (**Fig. 1I**), and are addressed in the main text (**Lines 219-224**): “To demonstrate the generalizability and potential applicability of this system using secreted effector protein

outputs, we replaced the mKate2 output in the GAD-based AND gate with human IL-15, a key cytokine that regulates the activation and proliferation of T and natural killer (NK) cells¹⁴. Consistent with AND gate logic, significant IL-15 secretion was detected with ELISA exclusively in the [1,1] state (**Figure 1I**)."

The trans-splicing efficiency is currently inferred indirectly from protein-level outputs. Direct molecular evidence at the RNA level is required. The authors should perform junction-specific RT-PCR or Northern blot analysis to confirm the formation of the expected trans-spliced mRNA products and quantify splicing efficiency. This is critical for validating the core mechanism of the system.

- We agree with this an important point to better establish our findings. Therefore, we performed the suggested experiment. These results were added to the manuscript and are addressed in the main text and **Figure 1E (Lines 188-191)**: "To quantify output mRNA levels, junction-specific qPCR was performed across all input states of the BD6-based AND gate. As expected, significant levels of trans-spliced mKate2 mRNA were detected exclusively in the [1,1] state (**Figure 1E**)."
- To further address this concern, we first sequenced the trans-spliced mRNA to confirm the fidelity of the trans-splicing process. These results were added to the revised manuscript and are addressed in the main text and supplementary **Figure S6E (Lines 191-195)**: "To provide direct molecular evidence for the formation of appropriate mKate2 mRNA, we reverse-transcribed from the trans-spliced product generated by this AND gate and sequenced the resulting cDNA (**Figure S6E**). Sanger sequencing results validated the formation of the expected mKate2 sequence, thereby confirming the fidelity of the trans-splicing process."

The claim of improved scalability and reduced circuit complexity should be supported by a quantitative comparison with previously published mammalian gene circuits for constructed logic gate. A supplementary table summarizing the number of regulatory components, plasmids, or layers required to achieve similar computational functions would greatly strengthen the impact of this work.

- We are happy that Reviewer 3 raised this issue, as this comparison is essential to establishing our framework in terms of minimizing circuit complexity and computational layer depth. Accordingly, we added **Table S7**, which summarizes our work and representative studies that

implemented genetic half-adders, full-adders, and multiplexers. This table is referred to in the discussion section **(Lines 664-667)**: “This circuit design strategy effectively addresses challenges associated with the limited availability of orthogonal genetic elements while reducing the number of genetic components and computational layers required to execute complex functions in mammalian cells **(Table S7)**.”

The system relies on intermolecular RNA hybridization, which may lead to cross-reactions with endogenous non-coding RNAs (such as miRNAs and lncRNAs). It is recommended to analyze potential off-target binding using RNA-seq or RIP-seq, and to evaluate whether the key BD sequences share homology with known regulatory RNAs, in order to assess biosafety.

- Indeed, the potential of off-target intermolecular RNA hybridization with endogenous non-coding RNAs is an important safety consideration in circuit design. To directly address this concern, we performed paired-end RNA-seq (median depth ~66 million reads per sample, min: ~43M reads, and max: ~698M reads) and aligned the data with STAR using chimeric read detection (e.g., --chimSegmentMin 20, --chimJunctionOverhangMin 15, --chimOutType WithinBAM HardClip Junctions) to sensitively that captures trans-splicing and other fusion-like events. Across all BD constructs and controls, mKate2-involving off-target chimeric junctions accounted for only 0.06–0.75% of total chimeric events and ≤0.013% of all mapped reads (i.e., below the expected technical chimeric background of RNA-seq libraries sequenced on NovSeqX). These results support the hypothesis that there are minimal cross-reactions with endogenous RNAs. This was emphasized by adding the following text **(Lines 233-238)**: “Finally, to assess potential off-target intermolecular RNA hybridization, we performed paired-end RNA seq (median depth ~66 million reads per sample) and STAR alignment with chimeric junction detection enabled. Across all BD constructs and mKate2 controls, off-target junctions (chrMKATE2) constituted only 0.06–0.75% of the total chimeric events and ≤0.013% of all mapped reads, comparable to the expected technical chimeric background of RNA seq libraries **(Supplementary Table S8)**.”
- In addition, all RNA-Seq data were uploaded to the public GEO database, and appropriate details of data analysis were added to the Methods section.

Minor Comments

The authors should discuss the potential sources of basal leakage observed in OFF states and propose strategies to further minimize it. This would enhance the practical utility of the system.

- The main source of basal leakage is the non-specific DNA sequence chosen to regulate the inactive modules. This sequence was deliberately chosen because it is not completely inactive but rather exhibits basal activity (**Fig. S1**). This basal activity is further increased when this sequence regulates output genes that contain intronic sequences, to a level that is 15-fold lower than the active SSX1p. Thereby, we simulated the leaky basal activity typical of native mammalian promoters, which must be taken into account in circuit design. We addressed this comment in the manuscript by adding the following text (**Lines 179-183**): “Unless otherwise indicated, active modules in all experiments were regulated by an active promoter, whereas inactive modules were regulated by a non-specific DNA sequence. This sequence was selected for its residual activity, which simulates the leaky basal activity typical of native mammalian promoters that must be taken into account in circuit design (**basal activity levels, enhanced in the presence of intronic miRNA, are shown in Figure S1**).”
- The most effective way to minimize basal input promoter activity is to use synthetic promoters as circuit inputs. Artificial promoters, such as Synthetic Promoters with Enhanced Cell-state Specificity, typically exhibit superior performance over native promoters, and particularly minimal basal activity, enhanced specificity, and potent activity in the ON state¹⁵. Alternatively, it is possible to carefully choose the native input promoter and flank the circuit modules with synthetic transcriptional insulators that block activation by nearby enhancers^{16, 17}. We added the following text to the Discussion section to elaborate (**Lines 650-654**): “Artificial promoters, including Synthetic Promoters with Enhanced Cell-state Specificity (SPECS), typically exhibit minimal basal activity, enhanced specificity, and potent ON-state activity, which outperform native promoters¹⁵. Therefore, SPECS can be particularly useful for enhancing circuit performance and mitigating leaky promoter activity in the OFF-states.”

It is recommended to add a brief paragraph at the end of the Discussion section to explore potential applications of this system in cell therapy, smart drug delivery, or in vivo diagnostics, and to highlight key technical challenges for future development.

- We thank the reviewer for acknowledging the broad applicability of our system. Accordingly, we added the following paragraph to the Discussion (**Lines 659-666**): “This approach provides essential building blocks for precision diagnostics, targeted therapies, sophisticated biosensors,

and cellular computing devices. For example, multi-input circuits enable the programming of immune cells for cell therapies^{8,9}, enhance the precision of gene therapies, and facilitate *in vivo* diagnostics of complex diseases¹⁰. Furthermore, combinatorial and MUX circuits are well-suited to address inherently heterogeneous diseases such as cancer¹¹ or immunological disorders that require targeting of specific inflammatory patterns¹². Therefore, this work supports next-generation biomedical and biotechnological applications.”

- In addition, technical challenges for future development are discussed in the following paragraphs **(Lines 631-656)**: “Future studies focusing on the comprehensive optimization of circuit components are expected to enhance performance and orthogonality^{18, 19}. The generation of BD sequences would benefit from a systematic evaluation of key parameters, including length, GC content, internal mismatches, nucleotide composition, secondary structures, and sequence complexity. In addition, the design of synthetic miRNAs must account for sequences present in native RNAs to avoid unintended off-target interactions with the cellular machinery. In parallel, circuit designs should avoid components that could become potential targets for endogenous regulators, such as sequences targeted by native miRNAs.

Moreover, integrating complex multi-vector circuits into mammalian genomes presents an additional layer of complexity. To address this challenge, future work should explore methods such as landing pad technologies or CRISPR/Cas-mediated integration to facilitate precise, stable, and efficient genomic insertion of these circuits^{10, 20, 21, 22, 23, 24, 25, 26, 27}. To maintain circuit functionality, appropriate insulator sequences could be incorporated to avoid cross-regulation with flanking regulatory elements or silencing by DNA methylation^{28, 29, 30}. Consequently, further optimization of circuit design that accounts for vector copy number and delivery platform may be required.

Finally, this approach could be expanded by incorporating additional synthetic components. Cell-state-specific promoters that are selectively activated in defined cellular contexts can be used as circuit inputs. Such promoters would provide precise regulation, reduce off-target effects, and enable rational programming of cellular activities essential for biomedical research and therapeutic interventions^{15, 31, 32, 33}. **Artificial promoters, including Synthetic Promoters with Enhanced Cell-state Specificity (SPECS), typically exhibit minimal basal activity, enhanced specificity, and potent ON-state activity, which outperform native promoters¹⁵. Therefore, SPECS can be particularly useful for enhancing circuit performance and mitigating leaky promoter activity in the OFF-states.** Furthermore, orthogonal synthetic

transcription factors based on CRISPR-Cas9 systems, TALEs, or zinc-fingers would provide additional scalability^{21, 34, 35, 36, 37}. Such genetic components could be easily integrated into our circuits, given the modular nature of these designs.”

It is recommended that the BD design rules be summarized in a flow chart or table in the supplementary figures to facilitate readers’ quick understanding of the key parameters.

- We agree with this comment. We edited the manuscript accordingly to address these considerations, by adding the following text, references, and flow chart detailing the recommended BD design rules (**Figure S5B**) (**Lines 150-159**): “Nevertheless, additional considerations must be taken into account in BD design. 50bp dsRNA can activate antiviral innate immunity, although innate immune sensing depends on multiple factors, including RNA length, sequence, secondary structures, subcellular localization, post-transcriptional processing, and modifications^{1, 2, 3}. Therefore, it is recommended to limit BD length to 30bp, and introduce mismatches into longer BDs to disrupt dsRNA continuity and thereby hinder recognition by cellular innate immunity sensors^{1, 2, 3}. Furthermore, since long intronic dsRNA could be processed by the miRNA production machinery, it is critical to exclude basal UG, Drosha, and Dicer processing motifs^{4, 5}. Finally, it is essential to avoid known regulatory sequences, such as binding sites for native miRNAs.”

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Dear Reviewers,

We appreciate your insights, which significantly improved this manuscript. Below is our response to the final concern raised by Reviewer #2. We have revised the manuscript accordingly and highlighted all edits in yellow. We hope our response sufficiently addresses this concern and that the manuscript is now suitable for publication.

In this version of the manuscript, we added a link to the RNA-Seq data, detailed under the 'data Availability' section.

Sincerely,
Lior Nissim

Reviewer #1

The authors have nicely responded all of the concerns in the review letter. In its current form the manuscript is mature enough to publish. Publish as it is.

- We thank Reviewer #1 for accepting the manuscript and appreciate the time and effort they spent reviewing it.

Reviewer #2

The authors of this paper adequately address most concerns from the original review. Explanations and additional experiments strengthen the claims made in the manuscript. This manuscript will be a valuable contribution to the literature.

- We thank the reviewer for their support, and hope that we adequately addressed their last concern.

There is one minor items which should be addressed:

1. Figure 4B / Lines 426-437:

Original comment: "Lines 397-413 / Figure 4B: In state [1,1,1], there are four reactions that produce ECFP (compared to one reaction for inputs resulting in a "SUM" signal), but expression levels are comparable across these conditions. This seems surprising. Can the authors speculate as to why state [1,1,1] does not confer a much higher ECFP level?" The response does not quite address this concern. The original concern was intended to refer to the expression level of ECFP in

state [1,1,1] relative to the expression levels of ECFP in states [1,1,0], [1,0,1], and [0,1,1]. For example, in State [1,1,0] there is one reaction that produces ECFP (reaction 3). In State [1,1,1] there are four reactions that produce ECFP (reactions 1, 2, 3, and 4). Thus, one would expect that State [1,1,1] would result in higher expression of ECFP than State [1,1,0], and yet the outputs are comparable in Figure 4B. Can you please explain this and/or provide interpretation for the reader?

- We apologize for misunderstanding this concern, and hope that our response clarifies this issue. Indeed, in State [1,1,1], there are four reactions that could produce ECFP: M1.3 + M3.3, M1.2 + M2.3, M1.2 + M3.3, and M2.3 + M3.4. Yet, the key limiting factor for ECFP production in this state is likely the concentration of pre-RNA encoding the second exon of ECFP (EC-Ex2), expressed from modules M2.3 and M3.3. As a result of this critical stoichiometric bottleneck, the maximal theoretical ECFP levels in this state can be produced from the total pre-RNA expressed from M2.3 + M3.3. In comparison, the limiting factor for ECFP production in States [1,1,0], [1,0,1], and [0,1,1] is the maximal pre-RNA expressed by a single module. Therefore, the maximal theoretical difference in ECFP levels produced in either States [1,1,0], [1,0,1], and [0,1,1] compared to State [1,1,1] is up to two-fold. However, doubling the number of possible reactions and pre-mRNAs that produce ECFP does not translate into a two-fold increase in ECFP levels. For example, ECFP levels depend on multiple factors, including BD-S and BD-AS binding efficiency and competition among EC-Ex1 donors for EC-Ex2 acceptors (*i.e.*, M1.2 and M3.4, which can both trans-splice with M2.3 via BD9-AS, and M1.2 and M1.3, which can both trans-splice with M3.3 via BD5-AS). Consequently, we observe a plateau in ECFP expression in all corresponding states.
- We added the following text to discuss this issue in the main text (lines 465-469): **“While State [1,1,1] enables more productive EC-Ex1/EC-Ex2 trans-splicing reactions compared to States [1,1,0], [1,0,1], and [0,1,1], ECFP levels were comparable across all four states. These results are consistent with constraints on ECFP production in State [1,1,1], including a stoichiometric bottleneck in the availability of EC-Ex2 pre-mRNAs expressed from plasmids M2.3 and M3.3.”**

Reviewer #3

I appreciate the authors' thorough and thoughtful responses to my previous comments. The revised manuscript has been significantly improved with the addition of relevant data and clarifications

that have strengthened the overall validity and credibility of the research. The authors have adequately addressed all my concerns, and the manuscript now presents a more comprehensive and well-supported study. I believe the paper has met the standards for publication and therefore recommend it for acceptance in its current form.

- We thank Reviewer #1 for accepting the manuscript and appreciate the time and effort they spent reviewing it.