Cell Systems

Transparent Peer Review Record

Design, Mutate, Screen: Multiplexed creation and arrayed screening of synchronized genetic clocks

Andrew Lezia, Nicholas Csicsery, Jeff Hasty

Summary

Initial Submission: Received June 25, 2021
Preprint: https://doi.org/10.1101/2021.06.22.449269

Scientific editor: Ernesto Andrianantoandro, Ph.D.

First round of review: Number of reviewers: Two
Two confidential, Zero signed
Revision invited September 02, 2021
Major changes anticipated
Revision received November 15, 2021

Second round of review: Number of reviewers: Two
Two original, Zero new
Two confidential, Zero signed
Accepted February 17, 2021

Editorial decision letter with reviewers' comments, first round of review

Dear Dr. Hasty,

I'm enclosing the comments that reviewers made on your paper, which I hope you will find useful and constructive. As you'll see, they express interest in the study, but they also have a number of criticisms and suggestions. Based on these comments, it seems premature to proceed with the paper in its current form; however, if it's possible to address the concerns raised with additional experiments and/or analysis, we'd be interested in considering a revised version of the manuscript.

As a matter of principle, I usually only invite a revision when I'm reasonably certain that the authors' work will align with the reviewers' concerns and produce a publishable manuscript. In the case of this manuscript, the reviewers and I have make-or-break concerns that can be addressed by:

1. Appropriate context for your study in comparison to other approaches and clarifying the relationship to directed evolution.
2. More fulsome analysis of the results of your screen including quantitative characterization of oscillator properties and characterization of RBS strength and phenotype that compares predictions vs experimental data.
3. A clearer rationale for the approach (Reviewer #2 provides great guiding questions in this regard)

To help guide revision, I've highlighted portions of the reviews that strike me as particularly critical.

As you address these concerns, it's important that you and I stay on the same page. I'm always happy to talk, either over email or by Zoom, if you'd like feedback about whether your efforts are moving the manuscript in a productive direction. Do note that we generally consider papers through only one major round of revision, so the revised manuscript would be either accepted or rejected based on the next round of comments we receive from the reviewers. If you have any questions or concerns, please let me know. More technical information and advice about resubmission can be found below my signature. Please read it carefully, as it can save substantial time and effort later.

I look forward to seeing your revised manuscript.

All the best,

Ernesto Andrianantoandro, Ph.D.
Scientific Editor, Cell Systems

Reviewers' comments:

Reviewer #1: The ability to screen cells with interesting phenotypes has been tremendously useful in biology. Until recently, the methods for screening these cells were limited to static pictures of phenotypes.

Although many interesting phenotypes can be screened with snapshots, many properties of biological systems are dynamic and require temporal characterization. In this manuscript, the authors present a method for dynamic characterization of a small-medium scale library with suggested applications in directed evolution. As a proof-of-principle, they build and characterize two libraries. The first one is through modification of an existing oscillator circuit, the synchronized lysis circuit, and the second one is through the creation of a new synchronized oscillator. Although a handful of methods have been recently developed to screen dynamical properties, additional, complementary methods are very much needed. This manuscript and method will provide a useful tool to the community, but several improvements are needed to the manuscript.

First, a proper discussion and comparison to the recently developed techniques is necessary. The manuscript properly acknowledges the technique by Luro et al., but does not mention the recently developed barcoding techniques for screening complex phenotypes (references below). These other cutting-edge techniques do not necessarily remove from the novelty of the manuscript but should be compared. For example, the technique proposed by the authors is reminiscent of a high-throughput arrayed screening, where the genotype-phenotype relation can be characterized for each mutant. It also does not require genetic barcoding like these other techniques.

Emanuel, G., Moffitt, J. R. & Zhuang, X. High-throughput, image-based screening of pooled genetic-variant libraries. Nat. Methods 14, 1159-1162 (2017).

Lawson, M. J. et al. In situ genotyping of a pooled strain library after characterizing complex phenotypes. Mol. Syst. Biol. 13, 947 (2017).

Daniel Camsund, Michael J. Lawson, Jimmy Larsson, Daniel Jones, Spartak Zikrin, David Fange & Johan Elf (2019). Time-resolved imaging-based CRISPRi screening. Nature Methods

In addition, a bit more details about the microfluidic technique should be included in the manuscript. What is the innovation that increases the throughput and how does it work? Looking at the SI, the authors appear to use an acoustic liquid handler (Echo) to array their strains. More details are necessary both from an innovation perspective as well as a reproducibility perspective. The authors introduce the technique as being able to screen 48 strains in parallel. However, they show two experiments, one with 24 strains and the other with 8 strains. The throughput should be clarified, and only claimed as high as what was achieved in the paper. On a sidenote, 8 to 16 strains is what can be typically achieved with standard microfluidic devices.

Other comments:

- There is a lot of discussion about directed evolution, but in the end the manuscript does not really perform directed evolution, but rather a single round of screening (directed evolution would imply at least 2 rounds of mutations and screening). I do not think it is necessary to do a second round of screening, but some of the discussion about directed evolution could be replaced by a discussion on the different methods for screening dynamics (as mentioned above)
- The real-world application of population oscillator could be introduced the first such applications are

mentioned in the text
- Throughout the screening, I found it striking that the different mutants are not sequenced. A big advantage of an arrayed screening method like this is that it is simpler to get genotype-phenotype map. For example, the different RBS could be sequenced and compare the theoretical prediction for the RBS strength to the observed phenotype. A few sequences are presented in Fig S8B, but it would have been interesting to compare the theoretical predictions from e.g. RBS calculator https://salislab.net/software/predict_rbs_calculator and simulations to the experimental data)
- The y axis in figure 3CD should probably be the same to make comparison easier
- For all the oscillators discussed, their properties are only vaguely described ("the frequency increasing", "more regular", "robust oscillations", etc.) without being quantitively measured. Such properties can easily be measured using a microfluidics time-lapse microscopy platform with for example period histograms. The period and the variation in the period could then be used to support such statements. The period is indicated on some graphs (Fig. 2D and Fig S3B), but not the noise on the period and is not well referenced.
- The new synchronized gene oscillator should be compared to the other population-based oscillator (2 strains oscillator, Chen, Y., Kim, J. K., Hirning, A. J., Josić, K., & Bennett, M. R. (2015). Emergent genetic oscillations in a synthetic microbial consortium. Science, 349(6251), 986-989), in terms of design and properties
- I do not understand this sentence: "The SLC library presented in this study, as an example, demonstrated variance between the magnitude of lysis events and the expression of a reporter gene. "
- Minor detail, but the SI figures should be numbered in the order that they come up in the text

Reviewer #2: Directed evolution has been a powerful complement to rational design methods since the early days of synthetic biology. As synthetic biology continues to stretch towards more complex designs, methods for efficient characterization and screening of complex outputs are essential for capitalizing on the potential of directed evolution. The authors focus on this need, building on a series of important works on oscillator design and sophisticated characterization devices by the Hasty group. The authors examine two different oscillator gene circuits and alter a key ribosome binding site in each circuit. They show that a batch approach is ineffective for characterizing performance, while their microfluidic devices enable reliable quantification and comparison to modeling predictions. With the ability to monitor up to 48 variants, the presented device in its current state might not be practical for truly conducting directed evolution, particularly for cases where protein sequences or network architecture variants are to be explored. However, the approach enables precise, quantitative characterization of dynamic behaviors for which few, if any other options exist. In addition, the authors utilize devices for individual variants to characterize responses to different inducer concentrations and to study the effects of confinement volume. Thus, the presented work marks a significant advancement in capabilities for the design-build-test cycle, while also stepping towards directed evolution of circuits for complex functionality.

1. The authors present important advancements in approaches for testing complex gene circuit variants, given the requirements for environmental stability, long periods of observation, and control over scale. My one concern is that some readers may take issue with the efforts being labelled as directed evolution. Indeed, the presented efforts are a key step in that direction. However, only one round of selection is

performed, and the throughput is many orders of magnitude below what most people are used to through methods such as chemotaxis assays or flow cytometry. It is hard to imagine scaling to a point where protein sequences or even combinations of multiple smaller genetic elements could be characterized, given that the number of variants simultaneously handled here is on the order of 10. These considerations should at least be addressed in the discussion.

2. Although ribosome binding sites (RBSs) are often important to optimize, there is a well established tool for rational design from the Salis lab. It is understandable why the authors did not focus on things like gene sequences, which would require the screening of far more variants, but why weren't other elements like operators, promoters, or degradation tags considered instead? Why were very large libraries of RBSs used instead of designing a targeted small library, given that only a few variants were tested? It would be helpful for readers considering approaches like this to know the extent to which rational design approaches can be relied upon for certain components, so that screening throughput can be budgeted towards components for which rational design tools are currently underdeveloped. Along these lines, it may also be informative to use the Salis tool to predict translation rates for the studied RBS variants and examine the degree of correspondence between predictions and experiments.

3. The transition to the second oscillator circuit (p. 12) is a bit abrupt. Why was this particular design chosen out of many options? There's no lysis in the second design, but how is it expected to compare to previous oscillator designs in general? Is there any particular design objective other than simply the production of any form of oscillations? Why is tetR placed under control of a separate promoter — would tetR autoregulation be detrimental? The authors eventually make an interesting point in the discussion that the first circuit has an interesting telltale in batch mode (presence of a lysis event), while the second one doesn't. However, more explanation for the choice of the second design when it is initially presented would be helpful.

4. More description of why batch mode fails would be interesting. Is it primarily due to the limited time window of strong expression capacity? To what extent are other factors like loss of coherence and system scale important? Presumably, the weights of these factors would differ between the two constructs. Growth dynamics and a constitutive GFP control may be informative for interpreting the batch results.

5. The authors note insensitivity to aTc concentrations between 0 and 50 ng/mL. Is this fundamental to the design (e.g. predicted by the model), or is there something else going on? For instance, could aTc be binding to surfaces, meaning that a critical concentration must be crossed to affect the cells?

6. Does aTc degrade during the experiments, given for instance its photosensitivity (including to the blue light range used for GFP imaging)? While these are subtle details, it can be important to know what effects are due to observation vs. fundamental performance.

7. Why was a delay used in the model for the second circuit but not the first?

8. Why are two separate copies of LuxR used in the second circuit? How are problems with recombination avoided?

Cell Systems

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Authors' response to the reviewers' first round comments

Attached.

Editorial decision letter with reviewers' comments, second round of review

Dear Dr. Hasty,

I'm very pleased to let you know that the reviews of your revised manuscript are back, the peer-review process is complete, and only a few minor, editorially-guided changes are needed to move forward towards publication.

In addition to the final comments from the reviewers, I've made some suggestions about your manuscript within the "Editorial Notes" section, below. Please consider my editorial suggestions carefully, ask any questions of me that you need, make all warranted changes, and then upload your final files into Editorial Manager.

I'm looking forward to going through these last steps with you. Although we ask that our editorially-guided changes be your primary focus for the moment, you may wish to consult our FAQ (final formatting checks tab) to make the final steps to publication go more smoothly. More technical information can be found below my signature, and please let me know if you have any questions.

All the best,

Ernesto Andrianantoandro, Ph.D.
Scientific Editor, Cell Systems

Editorial Notes

Transparent Peer Review: Thank you for electing to make your manuscript's peer review process transparent. As part of our approach to Transparent Peer Review, we ask that you add the following sentence to the end of your abstract: "A record of this paper's Transparent Peer Review process is included in the Supplemental Information." Note that this ***doesn't*** count towards your 150 word total!

Also, if you've deposited your work on a preprint server, that's great! Please drop me a quick email with your preprint's DOI and I'll make sure it's properly credited within your Transparent Peer Review record.

Title: The term "high throughput" might be a bit misleading, since you are dealing with 24 and 8 samples here. I would recommend alternative verbiage, such as "…parallel creation of multiple genetic clocks…" or include the term "array" or "arrayed" as you have in the introduction.

As you re-consider your title, note that an effective title is easily found on Pubmed and Google. A trick for thinking about titles is this: ask yourself, "How would I structure a Pubmed search to find this paper?" Put that search together and see whether it comes up is good "sister literature" for this work. If it does, feature the search terms in your title. You also may wish to consider that PubMed is sensitive to small differences in search terms. For example, "NF-kappaB" returned ~84k hits as of March, 2018, whereas "NFkappaB" only returned ~8200. Please ensure that your title contains the most effective version of the search terms you feature.

Manuscript Text:

- Please remove the numbers from all the headings and subheadings.
- House style disallows editorializing within the text (e.g. strikingly, surprisingly, importantly, etc.), especially the Results section. These terms are a distraction and they aren't needed—your excellent observations are certainly impactful enough to stand on their own. Please remove these words and others like them. "Notably" is suitably neutral to use once or twice if absolutely necessary.
- We don't allow "priority claims" (e.g. new, novel, etc.). For a discussion of why, read: http://crosstalk.cell.com/blog/getting-priorities-right-with-novelty-claims, http://crosstalk.cell.com/blog/novel-insights-into-priority-claims.
- Please only use the word "significantly" in the statistical sense.

Figures and Legends:

Please look over your figures keeping the following in mind:

- When color scales are used, please define them, noting units or indicating "arbitrary units," and specify whether the scale is linear or log.
- Bar graphs are not acceptable because they obscure important information about the distributions of the underlying data. Please display individual points within your graphs unless their large number obscures the graph's interpretation. In that case, box-and-whisker plots are a good alternative.
- Please ensure that every time you have used a graph, you have defined "n's" specifically and listed statistical tests within your figure legend.
- When figures include micrographs, please ensure that scale bars are included and defined within the legend, montages are made obvious, and any digital adjustments (e.g. brightness) have been applied equally across the entire image in a manner that does not obscure characteristics of the original image (e.g. no "blown out" contrast). ***Note that all accepted papers are screened for image irregularities, and if this advice is not followed, your paper will be flagged.***
- Please ensure that if you include representative images within your figures, a "representative of XXX individual cells"-type statement is made in the legend.

STAR Methods:

Please make sure to list any software or algorithm you use (e.g. RBS calculator) in the Key Resources Table, even if you are not reporting original code.

Thank you!

Reviewer comments:

Reviewer #1: The authors have addressed my comments and improved the manuscript. My only concern is the sentence: "Our technique enables us to rapidly array up to 48 distinct strains on a microfluidic device directly from liquid culture." This claim is repeated multiple times throughout the manuscript. The authors clarified later in the text that they only screen 24 such that they can have more replicates and more data on each strain. This would perhaps suggest that using 48 strains would not give enough data on each strain to make reliable conclusions (there are only 4 traps per spotting region)? In any case, the two-fold factor between what could potentially be done and what is shown is slightly misrepresenting the work. Changing the sentence(s) to something like "Our technique enables us to rapidly array potentially up to 48 distinct strains on a microfluidic device directly from liquid culture." would solve the issue. While

the extension to 48 strains should be trivial "in theory", that is what should be written in the manuscript. I leave it up to the editor to decide the appropriate wording throughout the text.

Reviewer #2: The authors have done a satisfactory job of addressing my comments.

Summary:

Dear Dr. Andrianantoandro,

Thank you for consideration of our manuscript for publication. We have carefully gone over the reviewer critiques and have revised our original draft based on their feedback. The critiques can be broadly summarized as requesting (i) that we better contextualize our study through comparison with other approaches, (ii) inclusion of a more complete and quantitative analysis of oscillator properties and RBS strengths, respectively, and (iii) clarification of the motivation for our approach, especially regarding second part of the manuscript. We found the referee comments to be insightful and fair. They have helped us to elevate the quality of our manuscript and better delineate its place within the field. A detailed list of our changes is attached below. We look forward to a decision on our manuscript.

Sincerely,
Jeff Hasty

Reviewer 1:

1) Reviewer's Comment: *A proper discussion and comparison to the recently developed techniques is necessary. The manuscript properly acknowledges the technique by Luro et al., but does not mention the recently developed barcoding techniques for screening complex phenotypes (references below).*

Response: We have added a thorough discussion of other recently developed techniques for screening mutant libraries for unique phenotypes and connecting these phenotypes to the cell genotype. Specifically, we added a paragraph to the introduction section highlighting the suggested publications on barcoding techniques for screening complex phenotypes. We have also clarified that our technique is an arrayed screening approach as opposed to pooled screening, and included discussion regarding the difficulty of screening for population-level phenotypes with current pooled-library screening techniques.

2) Reviewer's Comment: *In addition, a bit more details about the microfluidic technique should be included in the manuscript. What is the innovation that increases the throughput and how does it work? Looking at the SI, the authors appear to use an acoustic liquid handler (Echo) to array their strains. More details are necessary both from an innovation perspective as well as a reproducibility perspective.*

Response: In response to this request for more details on our microfluidic technique, we've added additional text to the beginning of the Results section describing the technique in more detail. We have also added a supplementary figure (SI Figure 1) with a diagram explaining the

design and function of our platform and how it enables rapid arraying and growth of mutant libraries. Lastly, we have added information on the previous microfluidic work that this new device is based on and highlighted the changes that were made to make this new device compatible with arraying cells directly from liquid culture.

3) Reviewer's Comment: *The authors introduce the technique as being able to screen 48 strains in parallel. However, they show two experiments, one with 24 strains and the other with 8 strains. The throughput should be clarified, and only claimed as high as what was achieved in the paper.*

Response: We have altered the text to make it clear that the number of strains we screened on the multi-strain microfluidic device was 24 and 8 for each oscillator library respectively.

4) Reviewer's Comment: *There is a lot of discussion about directed evolution, but in the end the manuscript does not really perform directed evolution, but rather a single round of screening (directed evolution would imply at least 2 rounds of mutations and screening). I do not think it is necessary to do a second round of screening, but some of the discussion about directed evolution could be replaced by a discussion on the different methods for screening dynamics (as mentioned above)*

Response: We agree with the reviewer's critique that a single round of mutation and screening as performed here does not constitute directed evolution as typically defined in the field. We have modified the text to clarify that our work is only a step towards the directed evolution of synthetic gene circuits with complex phenotypes at this point in time. Furthermore, we have added text to the discussion mentioning that combining our screening workflow with more high-throughput approaches could allow our technique to play a meaningful role in the directed evolution of synthetic gene circuits. Lastly, we have changed our discussion to focus more on comparisons to other screening approaches (see point 1).

5) Reviewer's Comment: *The real-world application of population oscillator could be introduced the first such applications are mentioned in the text.*

Response: We have modified the text to state the real-world application of the synchronized lysis circuit (SLC) the first time it is mentioned in the text. Specifically, we added a sentence to the introduction citing this circuit that explains how it has been used as a population-control mechanism for bacterial-based therapy applications.

6) Reviewer's Comment: *I found it striking that the different mutants are not sequenced. A big advantage of an arrayed screening method like this is that it is simpler to get genotype-phenotype map. For example, the different RBS could be sequenced and compare the theoretical*

prediction for the RBS strength to the observed phenotype. A few sequences are presented in Fig S8B, but it would have been interesting to compare the theoretical predictions from e.g. RBS calculator https://salislab.net/software/predict_rbs_calculator and simulations to the experimental data)

Response:
SLC Library: We strongly agree with this point that a big advantage of a technique like ours is the ability to sequence variants to understand genotype-phenotype relationships. For the population oscillator library, we chose to only sequence a subset (5) of the library as these strains spanned the range of dynamics we saw in our library screen. We have added a detailed paragraph to the results section comparing predicted RBS strengths from the Salis Lab RBS calculator to our experimental characterization of RBS strength. To make this comparison, we did additional experiments characterizing these two RBS sequences in a simple circuit with constitutive GFP expression. This comparison is summarized in a bar plot in SI Figure 5.

TetR-GFP Synchronized Oscillator: We chose not to sequence many clones from this particular library as there seemed to be a relatively obvious trend of stronger RBS driving TetR expression leading to more sustained oscillations in bigger trap sizes. For the two oscillators that we investigated in more detail in the single strain variable-trap size device, we have included their RBS sequences now as well as the predicted strengths obtained with the Salis Lab RBS calculator. This comparison is summarized in a bar plot in SI Figure 5.

7) Reviewer's Comment: *The y axis in figure 3CD should probably be the same to make comparison easier*

Response: We have modified figure 3 so that the y axis scales in panels C and D are identical.

8) Reviewer's Comment: *For all the oscillators discussed, their properties are only vaguely described ("the frequency increasing", "more regular", "robust oscillations", etc.) without being quantitatively measured. Such properties can easily be measured using a microfluidics time-lapse microscopy platform with for example period histograms.*

Response: We performed additional, quantitative analysis for each oscillator story to address this critique and revised the text of the results section to use more precise, quantitative language. For the SLC library, we created period histograms for a subset of working oscillators (Figure 2E). For the TetR-GFP oscillator story, we added a quantitative comparison of the damping behavior of different oscillators in the multistrain microfluidic device in Figure 5B. We now discuss the improvements in the D1 oscillator compared to the original in terms of the percentage of cell traps where we observed oscillations (Figure 5D). Additionally, we created period histograms for the original oscillator and strain D1 (Figure 5E), that show reduced period variability for strain D1. Additional, quantitative analysis of strain D1 was added to SI Figure 4.

9) Reviewer's Comment: *The new synchronized gene oscillator should be compared to the other population-based oscillator (2 strains oscillator, Chen, Y., Kim, J. K., Hirning, A. J., Josić, K., & Bennett, M. R. (2015). Emergent genetic oscillations in a synthetic microbial consortium. Science, 349(6251), 986-989), in terms of design and properties.*

Response: We have added a detailed comparison of the network architecture of the TetR-GFP synchronized oscillator we developed here to the one referenced by the reviewer ((2015). Emergent genetic oscillations in a synthetic microbial consortium. Science, 349(6251), 986-989). Specifically, we discuss how they looked at different network motifs for their oscillator and compare their conclusions on the robustness of these different motifs with our oscillator results. We also provide a detailed analysis of the implications of the different forms of negative feedback they use in their two-strain oscillator compared to the negative feedback loop in our oscillator.

10) Reviewer's Comment: *I do not understand this sentence: "The SLC library presented in this study, as an example, demonstrated variance between the magnitude of lysis events and the expression of a reporter gene. "*

Response: We have deleted the following sentence from the discussion, "The SLC library presented in this study, as an example, demonstrated variance between the magnitude of lysis events and the expression of a reporter gene." While presenting the results surrounding Figure 3, we now more clearly delineate in what ways our library generates diversity among both lysis and GFP fluorescence phenotypes.

11) Reviewer's Comment: *Minor detail, but the SI figures should be numbered in the order that they come up in the text*

Response: We have revised the order of the SI Figures so that they appear in the same order that they were originally referenced in the text.

Reviewer 2:

1) Reviewer's Comment: *My one concern is that some readers may take issue with the efforts being labelled as directed evolution. Indeed, the presented efforts are a key step in that direction. However, only one round of selection is performed, and the throughput is many orders of magnitude below what most people are used to through methods such as chemotaxis assays or flow cytometry…. These considerations should at least be addressed in the discussion.*

Response: We agree with the reviewer's assessment that the current work is not truly directed evolution as only one cycle of mutation and screening is performed. We have modified text in

the introduction and discussion to reflect this. Specifically, we added detailed discussion of existing methods for screening dynamic phenotypes and clarified that our technique specifically advances screening for dynamic, population-level phenotypes. We also agree that the current throughput of the method would be insufficient to screen the very large libraries necessary for exploring the meaningful sequence space for proteins. While our platform does not come close to rivaling the throughput of techniques like flow cytometry, it enables dynamic, population-level circuit characterization that is not achievable with higher throughput methods. Ideally, our platform could serve as a tool for true directed evolution of **population-level** synthetic gene circuits (i.e. circuits with dynamic behavior that only emerges when the cell population is above some threshold size) in the future by adding it to an evolution pipeline that also includes significantly more high-throughput methods. For instance, we could envision orders of magnitude larger mutant libraries being initially screened using flow cytometry or a number of recently developed single cell dynamic platforms to identify promising library members to investigate in a smaller throughput characterization in our device. Additionally, the microfluidic device here could in theory be scaled up to accommodate on the order of ~1000 variants in future studies, as indicated by our group's previous work on multi-strain microfluidic platforms.

2) Reviewer's Comment: *Although ribosome binding sites (RBSs) are often important to optimize, there is a well established tool for rational design from the Salis lab. It is understandable why the authors did not focus on things like gene sequences, which would require the screening of far more variants, but why weren't other elements like operators, promoters, or degradation tags considered instead? Why were very large libraries of RBSs used instead of designing a targeted small library, given that only a few variants were tested? …. it may also be informative to use the Salis tool to predict translation rates for the studied RBS variants and examine the degree of correspondence between predictions and experiments.*

Response: We chose to modify ribosome binding sites (RBSs) in our two mutant libraries as we thought that these would be most likely to create significant differences among library members for proof-of-concept testing of our approach. We have updated our manuscript to compare our results to those that could be obtained from the Salis lab calculator (see point 6 in response to Reviewer 1). Additionally, while it is true that there has been great success in rationally tuning the relative strength of RBSs, it is difficult to predict what strength of RBS will lead to the desired dynamical behavior for complex circuits with multiple interacting parts. Thus, even for a part that can be rationally designed or tuned, we believe a mutant library screening approach is still important for dynamic gene circuits. The Salis Lab RBS calculator also acknowledges this point, as their tool includes a module for designing RBS libraries to vary protein translation initiation rates. In comparing our experimental RBS characterization with theoretical results from the RBS calculator, we found that the RBS calculator was not able to correctly predict the relative RBS strength. Lastly, we chose to create RBS libraries whose size significantly exceeded the screening capacity of our platform to greatly increase the probability that transformants selected after library creation would contain unique sequences. We have added a sentence explaining this point to the Results section of the manuscript.

3) Reviewer's Comment: *The transition to the second oscillator circuit (p. 12) is a bit abrupt. Why was this particular design chosen out of many options? There's no lysis in the second design, but how is it expected to compare to previous oscillator designs in general? Is there any particular design objective other than simply the production of any form of oscillations? Why is tetR placed under control of a separate promoter — would tetR autoregulation be detrimental? …. more explanation for the choice of the second design when it is initially presented would be helpful.*

Response: We have updated the text and figures in the results section for the second oscillator to better highlight our motivation. As shown in our overview in Figure 1, we wanted to demonstrate the potential for our mutagenesis and screening workflow to accomplish two goals: 1) To tune the behavior of previously realized dynamic gene circuits and 2) To facilitate the construction of new dynamic gene circuits. The second oscillator circuit was created primarily to demonstrate the power of our approach for goal 2. We had previously created and investigated a design with TetR negative autoregulation, but found that it did not lead to any form of oscillations likely due to the significantly lower cooperativity coefficient of TetR repression compared to other transcriptional repressors such as the LacI repressor protein. We have now added a new figure (Figure 4), comparing the design with TetR autoregulation to a design without autoregulation. We have also clarified that the relatively low cooperativity of TetR binding (compared to LacI for instance) can make it more challenging to find a circuit with properly tuned parameters to see oscillations, which makes it a great circuit to try and optimize via mutant library screening. We also highlight the oscillator design we investigated is the first, published quorum-sensing based oscillator that relies solely on transcriptional repression in the negative feedback loop.

4) Reviewer's Comment: *More description of why batch mode fails would be interesting. Is it primarily due to the limited time window of strong expression capacity? To what extent are other factors like loss of coherence and system scale important? Presumably, the weights of these factors would differ between the two constructs. Growth dynamics and a constitutive GFP control may be informative for interpreting the batch results.*

Response: It is likely that the TetR-GFP synchronized oscillator doesn't exhibit oscillations in batch culture because the time scale for oscillations (6+ hours) is longer than the time the population spends in the exponential phase during batch culture. For the synchronized lysis circuit (SLC), the accumulation of dead cell waste products could be one factor that impacts cell metabolism in a complex way leading to lack of oscillations. The population size in the batch culture screen is also orders of magnitude larger than in the microfluidic cell traps we used and the SLC creates an intensely strong selective pressure for cells that have either mutated the circuit itself or developed genomic mutations conferring resistance to the lysis protein. These mutations could also play a role in the lack of oscillations seen in batch culture for the SLC. It is

also worth noting that there are occasionally multiple lysis "peaks" in batch culture for the SLC (e.g. library strain 22 in Figure 2 B Top Left). Even in these cases where multiple peaks in OD are seen, they are insufficient for making conclusions about dynamical parameters such as oscillator period.

We have added discussion of these limitations of batch culture screening to our manuscript.

5) Reviewer's Comment: *The authors note insensitivity to aTc concentrations between 0 and 50 ng/mL. Is this fundamental to the design (e.g. predicted by the model), or is there something else going on? For instance, could aTc be binding to surfaces, meaning that a critical concentration must be crossed to affect the cells?*

Response: We believe that the insensitivity to aTc that we observed is most likely due to very high expression levels of TetR-GFP that effectively quench the effect of aTc on inhibiting repression by TetR. We also acknowledge that the extended length of the experiments as well as the frequent blue light excitation during GFP imaging could be further decreasing the actual aTc concentration that cells see. When investigating the oscillator design with TetR negative autoregulation, we found that aTc did have an effect on the steady-state GFP level. Moreover, for that design, aTc-mediated GFP differences were maintained for at least 20 hours in microfluidics, suggesting that aTc degradation is not hugely significant. We have added a discussion of all of these points to the results section of the paper, as well as including modeling data on the predicted effect of aTc on oscillatory behavior.

6) Reviewer's Comment: *Does aTc degrade during the experiments, given for instance its photosensitivity (including to the blue light range used for GFP imaging)? While these are subtle details, it can be important to know what effects are due to observation vs. fundamental performance.*

Response: See response to point 5.

7) **Reviewer's Comment:** *Why was a delay used in the model for the second circuit but not the first?*

Response: For this manuscript, we sought to use the simplest deterministic models that could reliably predict the oscillator properties we saw experimentally. For the synchronized lysis circuit (SLC), our view is that it is not important to account for delays in gene expression relative to the rapid binding of transcription factors to their respective operator sites and inducers. Essentially, the timescale of cellular growth and lysis significantly outweighs these smaller factors in determining population dynamics so we left them out for simplicity. On the other hand, we believe that adding a delay term to the TetR-GFP synchronized oscillator is crucial for explaining the circuit dynamics. In this simple model, we do not include individual equations to

fully describe the cascade of reactions leading to production of functional proteins and AHL. Thus, to account for these reactions, we decided to add a delay to the protein production differential equations of our model. Essentially if TetR expression occurred instantaneously, its production would immediately shut off LuxI mediated positive feedback which would not reflect what is actually happening in our experiments. We have now added more thorough analysis of the role of the delay parameter for different designs of the TetR oscillator circuit.

8) Reviewer's Comment: *Why are two separate copies of LuxR used in the second circuit? How are problems with recombination avoided?*

Response: In previous synchronized oscillator circuits developed by our lab, we have used multiple copies of the LuxR gene with great success. Specifically we have typically used the bidirectional pLux promoter from the native *V. fischeri* operon where the LuxR gene is transcribed in one direction from the promoter and a gene of interest is transcribed in the other direction in an AHL-inducible fashion. While the homology between the two plasmids in the TetR-GFP synchronized oscillator could lead to undesirable recombination, it is unlikely that this would occur with significant frequency in our microfluidic experiments due to the relatively small population size and time scale. If this circuit were to be deployed in an application where stability in larger-scale cultures was necessary, we agree with the reviewer that the sequence homology in the circuit should be minimized.

Other revisions:

1) We have updated our methods section to adhere to the STAR Methods guidelines outlined in the online Cell Press guide.