

The master regulator OxyR orchestrates bacterial oxidative stress response genes in space and time

Divya Choudhary, Kevin R. Foster, Stephan Uphoff

Summary

Initial Submission: Received March 07, 2024
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First round of review: Number of reviewers: Two
Two confidential, Zero signed
Revision invited May 08, 2024
Major changes anticipated
Revision received July 10, 2024

Second round of review: Number of reviewers: Two
Two original, Zero new
Two confidential, Zero signed
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This Transparent Peer Review Record is not systematically proofread, type-set, or edited. Special characters, formatting, and equations may fail to render properly. Standard procedural text within the editor's letters has been deleted for the sake of brevity, but all official correspondence specific to the manuscript has been preserved.

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

Dear Dr. Uphoff,

I hope this email finds you well. The reviews are back on your manuscript and I've appended them below. You'll see that the reviewers find the manuscript compelling and their comments are intended to strengthen an already strong piece of work. We're happy to invite a revision.

The reviewer concerns are straightforward to address, but if you have any questions or concerns about the revision, I'd be happy to talk about them, either over email or by Zoom/Teams. 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: In this interesting article, authors described diverse gene expression profiles to oxidative stress. The expression of 31 genes were followed, which showed distinct patterns (down-regulation, pulsate and graduate upregulation), which have distinct roles (sudden and prolonged stress). I am not an expert in oxidative stress, but was able to follow the manuscript. However, I believe the improvements can help general readers.

1. The title is too general: it does not properly summarize what the paper is about.
2. The effects of growth rate on gene expression can vary depending on how growth was inhibited. Therefore, testing a constitutive promoter is critical. It was nice that authors did that using Prna1. However, I don't think many people would have a priori knowledge about the nature of this promoter. The fact that this promoter is outside of the OxyR regulon does not prove it is constitutive. There could be other indirect effects. Please provide a reference or evidence that shows that this promoter is indeed constitutive.
3. Authors tracked 31 genes. How large is oxidative stress regulon? How was this set of 31 genes

chosen? Are they representative genes? Do they have important functions?

4. Details of the model were alluded to the previous publication. As it is written now, the model feels like a black box. It reads as if something happens in the model and what comes out explains the data. There are many models of gene expression out there. How is the model used here different from these models? What are the unique features of this model that are critical to explaining the data ?

Reviewer #2: In this study, Choudhary et al. use single-cell time-lapse imaging to understand the *Escherichia coli* response to oxidative stress. Their results reveal diversity in the expression dynamics of genes in the oxidative stress response network, with differences in timing, magnitude, and direction of expression exhibited between 31 genes in the network. However, responses follow a consistent pattern where all genes show a transient increase in protein levels due to slowed cell growth under oxidative stress. After controlling for this effect, the authors focus on three classes of gene regulation—pulsatile upregulation, gradual upregulation, and downregulation—primarily emphasizing the first two and the tradeoffs they offer in speed versus precision of the response. This study builds upon previous work from the authors, which characterized the effect of cell-to-cell interactions on the concentration of H₂O₂ across cells growing within mother machine chambers and how this relates to the induction of oxidative stress response via OxyR. In this manuscript, Choudhary et al. determine how the dynamics of genes regulated by OxyR can differ. They explain these differences using a mathematical model that describes the effects of OxyR-dependent gene regulation via changes in two key parameters. Their model is able to explain the differences in expression dynamics and predict the response of cells to gradual versus sudden addition of H₂O₂. Further testing using dual reporters and a gene knock out also support the model. The authors then use data from expression levels versus number of barrier cells to suggest how spatial positioning of cells can affect the oxidative stress response.

Overall, the authors have carried out a thorough examination of the single-cell dynamics associated with the expression of genes involved in the oxidative stress response. Their model provides insights into how different expression patterns can result from promoters acted upon by the same transcription factor. The experimental data are high quality and these are challenging measurements to conduct. However, there are various places where the manuscript requires improvement which I describe in greater detail below.

Major

1. The manuscript switches between analysis of frontier cells and mother cells for reasons that are not clearly justified. Fig. 3 and 6 are focused on mother cells while other figures are focused on frontier cells. This switch is not explained, and more importantly the frontier cells would be expected to have a stronger response so it seems the proper set of cells to focus on. I understand that the authors cannot show multi-hour lineages of the same frontier cell since they are flushed out of the chamber so there may be places where it is necessary to focus on the mother cell, but this should be clearly explained and justified.
2. The model presents an interesting and simple explanation on how K_d and K_{ind} for OxyR can result in differential expression from the promoters examined, but many of these genes are regulated by other transcription factors. Could some results be explained by other transcription factors interacting with these promoters differentially during oxidative stress? This likely possibility is not discussed in the text.

3. There are several places where the results described in the text do not line up with the data in the figures.

a. Line 88-89 indicates that all 31 genes showed an initial expression pulse within 10 min and references Fig. 1C, but Fig. 1C starts at 30 min, and this pulse is not clear in all the genes.

b. Please include the fluorescence data showing the expression pulse for the control Prna1 to help make the comparison mentioned in lines 122-123. Movie S3 does not show a pulse in fluorescence of Prna1 due to reduced growth that is described in the manuscript text.

c. Are the data in Fig. S1 fluorescence levels or promoter activity? The y axis label suggests fluorescence levels. However, the text says that cells with no change in promoter activity (black lines) still show a pulse in fluorescence due to decreased cell growth, and this is not visible in these plots.

d. Line 132 references Fig. 3D as a plot of basal versus peak expression, but Fig. 3D is a kymograph. Perhaps this is a typo and should be Fig. 3A?

e. Lines 192-193 discusses KD versus H₂O₂ sensitivity in relation to Fig. 5A, but the plots in 5A show no information about KD. This should be clarified in the plot legend or caption.

4. Although the manuscript purports to provide insight into dynamics across cell populations, the mother machine is limited to ~10 cells in a chamber in a single line. While the relationship between mother cells, barrier cells, and frontier cells provides interesting insights, it is a bit of a stretch to call this population or community level structure. I recommend revising the language throughout the manuscript to accurately reflect the experiments.

Minor

1. Although the authors strive to simplify the different categories of dynamic responses into clean groups, this is sometimes an oversimplification. For example, in Fig. 2C the Pflu and PiscS cases exhibit downregulation, but then end up with steady state values that are higher than the baseline. I appreciate that it is more straightforward to discuss distinct groups, but it could be more balanced to acknowledge that these distinctions are not always clear cut.

2. It would be helpful to include more x-axis tick marks in Figs. 1D and 2C since much of the focus is on the initial pulse period.

3. Fig. 1D: how is darker shaded region determined? It may be best to remove this.

4. Line 117-121: when discussing how there is a positive pulse in protein levels of negatively regulated genes, is the referenced protein the fluorescent reporter from Fig. 1C or is it the inferred protein level for the gene of interest?

5. Fig. 2C: It took me a little while to understand what the statistical significance bars were comparing. It would be helpful to describe in the caption what timepoints were actually used for the comparisons.

6. Fig. 3A and C are not referenced in the text.

7. It would be useful to show promoter activity and fluorescence levels together for each reporter (for example, in the supplement).

8. In Fig. 3A's caption there are three levels of H₂O₂ (25, 50, and 100 μ M), however it is not clear which points these different values correspond to on the plot.

9. The phrase "gradually induced" is a bit confusing in that it could be interpreted as a difference in the introduction rate of H₂O₂. A potential alternative would be "gradual response."

10. Fig. 4E: It would be helpful to plot actual values of K_{ind} and K_d from the model and show the lines they fall along as a supplementary figure.

11. Line 182-184: "experiments showed that the dissociation constant of oxidised OxyR from the pulsatile

gene promoter PkatG is an order of magnitude higher than for the gradually induced PahpC promoter" and references citation 72 and Fig. 4F. I see that reference 72 measures dissociation constants for the two promoters, but as written the text suggests that the values are measured and presented in Fig. 4F. Please clarify this wording.

12. There is a typo in the caption for Fig. 5A that labels both the peak and steady state plots as (bottom).

13. The fluorescence levels of PgrxA in delta_katG cells with gradual versus step increase in H₂O₂ look opposite in Movie S4 and Fig. 5E. The figure shows brighter response of the fluorescent reporter to a step in H₂O₂ in delta_katG cells, but the legend for Movie S4 says the step in H₂O₂ is the bottom set of cells, which show growth defects and dimmer fluorescence than the cells that received gradual H₂O₂ addition in the top of the video.

14. Line 208: The text describes delta_katG cells as being more sensitive to the step of H₂O₂. Does this refer to the induction of the PgrxA promoter or to growth rate/survival?

15. Line 243: My understanding is that Fig. 6F uses fluorescence. If promoter activity is used instead (which would be preferred), how much of the correlation remains?

16. Fig. 7D is not discussed in the text. This is related to one of the major points, but it is not clear that it is possible to extrapolate dynamics within a single mother machine chamber (typically less than 10 cells and length in the micrometer scale) to colonies (thousands of cells and diameter in millimeter scale). This is at the discretion of the authors, but my sense is that Fig. 7D is not fully justified and could be removed. If it remains, please clarify in the caption what the color intensity of the red, green, and blue represent.

17. Line 504: Please clarify whether the data for Prna1 used elsewhere in the manuscript corresponds to the mKate2 reporter version or if it is a GFPmut2 (or SCFP3A) version.

18. Line 575-576: a few typos in this line.

19. Line 598: "Peaky" may be a typo

20. Line 600: This is referred to as "steady state" in the figures, not "steady"

Authors' response to the reviewers' first round comments

Attached.

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

Dear Dr. Uphoff,

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 review these notes along with the detailed formatting requirements listed in the [Final Files Checklist](#). We've also put together this [FAQ](#) (click the Final Formatting Checks tab) for your convenience. Please ask any questions you may have, make any necessary changes to your manuscript files, and then upload your final files into Editorial Manager. Once we receive your formatted files, we will go through our formatting checks and let you know if further changes are needed.

We hope to receive your formatted files within 5 business days. Please email me directly if this timing is a problem or you're facing extenuating circumstances. Alternatively, if this manuscript needs to be officially accepted by a particular date because of grant deadlines, applications, or because it will help your trainees, please let me know.

Introducing new referencing style

To standardize the referencing style across Cell Press journals, starting from October 2022, we ask that all in-text citations be formatted as superscripted numbers (e.g. "Multiple reports support this observation.^{1,2}"). Moving away from the Harvard referencing style (e.g. Smith *et al.*, 2020) will improve author and reader experiences. All manuscripts accepted from now on must use **the superscript numbered Cell Press referencing style**. Make sure to use this numbered referencing style for all new and revised submissions as well. Switching is easy. Just use the updated [CSL](#) and [EndNote](#) referencing styles for Cell Press articles.

Below my signature, you'll find specific information about what to expect next regarding formatting checks and working with our Production Department after acceptance. It's been a pleasure working with you, please feel free to contact our journal team with 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: Your title is a bit too general, as remarked by Reviewer #2. I suspect it could be more effective. 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.

Abstract: The abstract is unfortunately too long. Please condense to 150 words or less. Note that most effective abstracts have the following structure:

[One sentence of background.] However, X. [X essentially presents the problem that you will solve.] Here, we [solve X]. [A compound sentence that begins with a methodological phrase and ends with a phrase that describes the results that the methodology produces (e.g. "Using a combination of method Y and method Z, we show something new about X.").] [Sentences that describe key results and include carefully selected details that allow the reader to place the key results within a broader context.]

For a complementary view on how to write abstracts, read: <http://crosstalk.cell.com/blog/how-to-hook-an-audience-with-a-great-abstract>.

Manuscript Text:

- 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.
- 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.

- 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.

Resource Availability: Please note that Cell Press has recently changed the way it approaches "availability" statements for the sake of ease and clarity. Please revise your resource availability section as follows, noting that the examples used might not pertain to your study. Please note that the Resource Availability section should immediately follow the Discussion section in the main manuscript.

RESOURCE AVAILABILITY

Lead Contact: Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Jane Doe (janedoe@qwerty.com).

Materials Availability: This study did not generate new materials. -OR- Plasmids generated in this study have been deposited at [Addgene, name and catalog number]. -OR- etc.

Data and Code Availability:

- **Source data statement** (described below)
- **Code statement** (described below)
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

Data and Code Availability statements **have three parts and each part must be present. Each part should be listed as a bullet point, as indicated above.**

Instructions for section 1: Data. The statements below may be used in any number or combination, but at least one must be present. They can be edited to suit your circumstance. ***Please ensure that all datatypes reported in your paper are represented in section 1.*** For more information, please consult [this list of standardized datatypes and repositories recommended by Cell Press](#).

- [Standardized datatype] data have been deposited at [datatype-specific repository] and are publicly available as of the date of publication. Accession numbers are listed in the key resources table.

- [Adjective] data have been deposited at [general-purpose repository] and are publicly available as of the date of publication. DOIs are listed in the key resources table.
- [De-identified human/patient standardized datatype] data have been deposited at [datatype-specific repository]. They are publicly available as of the date of publication until [date or delete “until”]. Accession numbers are listed in the key resources table.
- [De-identified human/patient standardized datatype] data have been deposited at [datatype-specific repository], and accession numbers are listed in the key resources table. They are available upon request until [date or delete “until”] if access is granted. To request access, contact [insert name of governing body and instructions for requesting access]. [Insert the following when applicable] In addition, [summary statistics describing these data/processed datasets derived from these data] have been deposited at [datatype-specific repository] and are publicly available as of the date of publication. These accession numbers are also listed in the key resources table.
- Raw [standardized datatype] data derived from human samples have been deposited at [datatype-specific repository], and accession numbers are listed in the key resources table. Local law prohibits depositing raw [standardized datatype] datasets derived from human samples outside of the country of origin. Prior to publication, the authors officially requested that the raw [adjective] datasets reported in this paper be made publicly accessible. To request access, contact [insert name of governing body and instructions for requesting access]. [Insert the following when applicable] In addition, [summary statistics describing these data/processed datasets derived from these data] have been deposited at [datatype-specific repository] and are publicly available as of the date of publication. These accession numbers are also listed in the key resources table.
- The [adjective] data reported in this study cannot be deposited in a public repository because [reason]. To request access, contact [insert name of governing body and instructions for requesting access]. [Insert the following when applicable] In addition, [summary statistics describing these data/processed datasets derived from these data] have been deposited at [datatype-specific or general-purpose repository] and are publicly available as of the date of publication. [Accession numbers or DOIs] are listed in the key resources table.
- This paper analyzes existing, publicly available data. These accession numbers for the datasets are listed in the key resources table.
- [Adjective or all] data reported in this paper will be shared by the lead contact upon request.

Instructions for section 2: Code. The statements below may be used in any number or combination, but at least one must be present. They can be edited to suit your circumstance. ***If you are using GitHub, please follow [the instructions here](#) to archive a “version of record” of your GitHub repo at Zenodo, then report the resulting DOI. Additionally, please note that the Cell Systems strongly recommends that you also include an explicit reference to any scripts you may have used throughout your analysis or to generate your figures within section 2.***

- All original code has been deposited at [repository] and is publicly available as of the date of publication. DOIs are listed in the key resources table.
- All original code is available in this paper's supplemental information.
- This paper does not report original code.

Instructions for section 3. Section 3 consists of the following statement: Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

STAR Methods:

- STAR Methods follows a standardized structure. Please reorganize your experimental procedures to include these specific headings in the following order: EXPERIMENTAL MODEL AND SUBJECT DETAILS (when appropriate); METHOD DETAILS (required); QUANTIFICATION AND STATISTICAL ANALYSIS (when appropriate); ADDITIONAL RESOURCES (when appropriate). We're happy to be flexible about how each section is organized and encourage useful subheadings, but the required sections need to be there, with their headings. They should also be in the order listed. Please see the STAR Methods [guide](#) for more information or contact me for help.
- If your data are not standardized, we recommend that you deposit them in a [general purpose repository recommended by Cell Press](#). Please provide your datasets' accession numbers/DOIs in Deposited Data section of the Key Resources Table.
- Please ensure that original code has been archived in a [general purpose repository recommended by Cell Press](#) and that its DOI is provided in the Software and Algorithms section of the Key Resources Table. If you've chosen to use GitHub, please follow [the instructions here](#) to archive a "version of record" of your GitHub repo at Zenodo, complete with a DOI. Thank you!

Thank you!

Reviewer comments:

Reviewer #1: I am happy with authors' response. I recommend publication.

Reviewer #2: Choudhary, et al. have improved the manuscript in various ways including a more comprehensive description of the model, corrections at various points in the text and figures, inclusion of both fluorescence and promoter activity data, and addition of relevant references. I have some small remaining thoughts:

1. I tend to agree with Reviewer 1's comment that the title is too general and I have this same impression even in its revised form. It is not clear that this applies to all master regulators and it would be better to focus on OxyR, for which the manuscript presents results.
 2. The manuscript does now clearly state when mother cells are used and when frontier cells are used, however it is still not always clear why mother cells are used, given that the effect is most pronounced in frontier cells. It would be helpful to provide additional text, for example near L240, that justifies this choice.
 3. L138: it would be useful to also reference Fig. S1 here because Fig. 2C alone does not show the expression pulse, just the constant promoter activity.
-

Reviewers' comments:

Reviewer #1: *In this interesting article, authors described diverse gene expression profiles to oxidative stress. The expression of 31 genes were followed, which showed distinct patterns (down-regulation, pulsate and graduate upregulation), which have distinct roles (sudden and prolonged stress). I am not an expert in oxidative stress, but was able to follow the manuscript. However, I believe the improvements can help general readers.*

Thank you for this summary of our paper. We are pleased you found it interesting.

1. *The title is too general: it does not properly summarize what the paper is about.*

We went back and thought more about the title. Considering that stress response regulons are often controlled by one master transcription factor, we think a general title is appropriate and we would like to highlight the central point about spatio-temporal regulation. We rephrased the title as follows:

‘A master regulator orchestrates bacterial stress response genes in space and time’

2. *The effects of growth rate on gene expression can vary depending on how growth was inhibited. Therefore, testing a constitutive promoter is critical. It was nice that authors did that using Prna1. However, I don't think many people would have a priori knowledge about the nature of this promoter. The fact that this promoter is outside of the OxyR regulon does not prove it is constitutive. There could be other indirect effects. Please provide a reference or evidence that shows that this promoter is indeed constitutive.*

Thank you for the suggestion. We have modified text (L134-137) as follows:

‘These conclusions are supported by using the replication control promoter *Prna1* of plasmid PBR322 as a control, which is constitutively expressed and not part of the OxyR regulon. Our analysis shows that this promoter displays a similar expression pulse but maintains constant promoter activity in response to H₂O₂ treatment [Figure 2C, Movie S3].’

Reference added: Liang, S-T., et al. "Activities of constitutive promoters in Escherichia coli." *Journal of molecular biology* 292.1 (1999): 19-37.

3. *Authors tracked 31 genes. How large is oxidative stress regulon? How was this set of 31 genes chosen? Are they representative genes? Do they have important functions?*

We modified the text to address this valid point (L78-81):

“Specifically, we chose promoters with OxyR binding sites according to chromatin immunoprecipitation measurements by Seo et al. 36 and promoters that show H₂O₂-induced changes in transcription according to RNA-seq measurements by Roth et al 4. The resulting 31 promoters encompass the currently known OxyR regulon (Table 1).”

Table 1 lists the selected promoters, the gene functions and relevant references. In addition, the introduction also includes an overview of the known functions of the most important and best characterised genes.

4. *Details of the model were alluded to the previous publication. As it is written now, the model feels*

like a black box. It reads as if something happens in the model and what comes out explains the data. There are many models of gene expression out there. How is the model used here different from these models? What are the unique features of this model that are critical to explaining the data?

We have modified the section about the model to explain it better and highlight specifically the role of the dissociation constant of OxyR in determining expression dynamics. We agree that readers should not need to look up our previous papers to understand the results here, so we provide all equations that were used for modelling in this paper in the materials and methods section. We also added all modelling parameters and constants. Furthermore, Figure 4A shows a graphical illustration of the model. We updated the legends for **Figure 4** and **7** to provide information that will aid readers in understanding the modeling results.

Reviewer #2: *In this study, Choudhary et al. use single-cell time-lapse imaging to understand the Escherichia coli response to oxidative stress. Their results reveal diversity in the expression dynamics of genes in the oxidative stress response network, with differences in timing, magnitude, and direction of expression exhibited between 31 genes in the network. However, responses follow a consistent pattern where all genes show a transient increase in protein levels due to slowed cell growth under oxidative stress. After controlling for this effect, the authors focus on three classes of gene regulation—pulsatile upregulation, gradual upregulation, and downregulation—primarily emphasizing the first two and the tradeoffs they offer in speed versus precision of the response. This study builds upon previous work from the authors, which characterized the effect of cell-to-cell interactions on the concentration of H₂O₂ across cells growing within mother machine chambers and how this relates to the induction of oxidative stress response via OxyR. In this manuscript, Choudhary et al. determine how the dynamics of genes regulated by OxyR can differ. They explain these differences using a mathematical model that describes the effects of OxyR-dependent gene regulation via changes in two key parameters. Their model is able to explain the differences in expression dynamics and predict the response of cells to gradual versus sudden addition of H₂O₂. Further testing using dual reporters and a gene knock out also support the model. The authors then use data from expression levels versus number of barrier cells to suggest how spatial positioning of cells can affect the oxidative stress response.*

Overall, the authors have carried out a thorough examination of the single-cell dynamics associated with the expression of genes involved in the oxidative stress response. Their model provides insights into how different expression patterns can result from promoters acted upon by the same transcription factor. The experimental data are high quality and these are challenging measurements to conduct. However, there are various places where the manuscript requires improvement which I describe in greater detail below.

Thank you for this summary of our project and the helpful suggestions. We address all points below.

Major

1. *The manuscript switches between analysis of frontier cells and mother cells for reasons that are not clearly justified. Fig. 3 and 6 are focused on mother cells while other figures are focused on frontier cells. This switch is not explained, and more importantly the frontier cells would be expected to have a stronger response so it seems the proper set of cells to focus on. I understand that the authors cannot show multi-hour lineages of the same frontier cell since they are flushed out of the chamber so there*

may be places where it is necessary to focus on the mother cell, but this should be clearly explained and justified.

We have now added text to explicitly mention the rationale for analysing frontier cells or mother cells (L88-92, L153-157, L24-242, L263-265):

‘Unless stated otherwise, our analysis focused on the ‘frontier cells’, which are located at the open end of the growth trenches where they are directly exposed to the H₂O₂ treatment from the media flow channel. Due to efficient absorption and scavenging of H₂O₂, the cells located beneath the frontier cells experience diminishing concentrations of H₂O₂ and thus a lower stress response.’

‘After classifying the up-regulated genes into two groups based on the *peak/steady-state* ratio, we analysed the mother cells located at the closed end of the growth trenches to follow the gene expression dynamics of the same cells for longer durations. We found that the pulsatile genes are generally activated earlier after the onset of H₂O₂ stress, ...”

‘For this, we followed the expression dynamics of mother cells and found that the time of induction after the onset of stress was shorter and less variable across cells for the pulsatile genes [Figure 5G].’

‘Focusing on the dual reporter strain, we found that *PkatG* and *PgrxA* expression in the same mother cell showed relatively little correlation during the transient expression pulse [Figure 6D, Movie S5].’

The figure captions state for each plot if frontier cells or mother cells were analysed.

2. The model presents an interesting and simple explanation on how *Kd* and *Kind* for *OxyR* can result in differential expression from the promoters examined, but many of these genes are regulated by other transcription factors. Could some results be explained by other transcription factors interacting with these promoters differentially during oxidative stress? This likely possibility is not discussed in the text.

This is a good point. Our manuscript raises this possibility:

“The oxidative stress response involves feedbacks from various metabolic pathways^{4,32,36,41,48,49,53–55} and several genes in the *OxyR* regulon are co-regulated by more than one transcription factor^{36,64–66,69,70}. Differences in the expression of genes in the *OxyR* regulon, therefore, may arise in multiple ways.”

Regarding the promoters that we analysed in most detail: *PtrxC*, *PgrxA*, *PahpC*, *PhemH*, *PkatG*, *PyaaA* are primarily thought to be regulated by *OxyR*. *PyaiA*, *PclpS* are predicted to be regulated by ppGpp and PhoP respectively, which will be relevant under stationary phase or acid stress conditions respectively. *Pfur* in addition to being regulated by *OxyR*, is negatively regulated by itself. *PpoxB* and *PuxuA* have multiple transcriptional regulators.

We added the following conclusion in the manuscript (L205-208):

“Furthermore, although other transcription factors are known to control some of the genes in the *OxyR* regulon^{36,64–66,69,70}, we found that *OxyR* alone was sufficient in our model for explaining the expression patterns seen under the conditions of our measurements (H₂O₂ treatment during exponential growth).”

3. There are several places where the results described in the text do not line up with the data in the figures.

a. Line 88-89 indicates that all 31 genes showed an initial expression pulse within 10 min and references Fig. 1C, but Fig. 1C starts at 30 min, and this pulse is not clear in all the genes.

Thank you for noticing this. We have changed the text (L96-98) as follows:

‘With this setup, we were able to observe that all 31 genes showed an initial expression pulse within 30 min after the start of treatment [Figure 1C], but expression of different genes diverged after this initial pulse.’

b. Please include the fluorescence data showing the expression pulse for the control *Prna1* to help make the comparison mentioned in lines 122-123. Movie S3 does not show a pulse in fluorescence of *Prna1* due to reduced growth that is described in the manuscript text.

We have added supplementary **Figure S1** (in response to Minor comment 7) which includes the promoter activity and fluorescence data for *Prna1*.

c. Are the data in Fig. S1 fluorescence levels or promoter activity? The y axis label suggests fluorescence levels. However, the text says that cells with no change in promoter activity (black lines) still show a pulse in fluorescence due to decreased cell growth, and this is not visible in these plots.

The data in **Figure S2** (previously figure S1) display the expression levels as stated in the legend and the figure axes. The discrepancy stems from the difference in response between mother cells and frontier cells. The figure plots the expression traces for mother cells under H₂O₂ treatment. Our previous study demonstrated that cells protect each other by actively scavenging local H₂O₂, creating steep micro-meter scale gradients of oxidative stress response. Consequently, the mother cells experience lower stress levels than frontier cells. Thus, the reduction in growth rate for mother cells is smaller, making the gene expression pulse in mother cells (Figure S2) less pronounced compared to the frontier cells (Figure S1).

We explained this difference in the figure caption.

d. Line 132 references Fig. 3D as a plot of basal versus peak expression, but Fig. 3D is a kymograph. Perhaps this is a typo and should be Fig. 3A?

Thank you for noticing this, the figure references for Figure 3 have been corrected (L144-145), (L148-149), (L152-153), and (L158-159) as follows:

‘we plotted the reporter expression level during the transient peak versus the level at steady-state [Figure 3A].’

‘while the other upregulated genes (*PgrxA*, *PtrxC*, *Pfur*, *PahpC*) share a lower ratio of *peak/steady-state* ~ 1.5 [Figure 3B].’

‘gradual induction to an elevated steady-state level (hence termed “gradually induced”) [Figure 3C, D, Movie S1-S2].’

‘37.5±7.8 minutes post H₂O₂ treatment for the non-pulsatile genes [Figures 3E, S1, Movie S1-S2].’

e. Lines 192-193 discusses KD versus H₂O₂ sensitivity in relation to Fig. 5A, but the plots in 5A show no information about KD. This should be clarified in the plot legend or caption.

Legend for **Figure 5A** has been modified as follows:

‘(A) The response of pulsatile genes (high K_D) is more sensitive to changes in H₂O₂ concentration than gradually induced genes (low K_D).’

4. Although the manuscript purports to provide insight into dynamics across cell populations, the mother machine is limited to ~10 cells in a chamber in a single line. While the relationship between mother cells, barrier cells, and frontier cells provides interesting insights, it is a bit of a stretch to call this population or community level structure. I recommend revising the language throughout the manuscript to accurately reflect the experiments.

We agree that while the microfluidics chips provide clear advantages for this study, they do not fully reflect the conditions in larger cell populations. To address this, we previously conducted experiments on cell colonies and found that H₂O₂ treatment of colonies generated similar spatial gradients in stress response as in microfluidic channels. We also showed how the response gradients scale with increasing numbers of cells, both in microfluidic channels and in colonies meaning that results from microfluidics can be meaningfully extrapolated to larger cell populations.

We added this point in the discussion section.

Minor

1. Although the authors strive to simplify the different categories of dynamic responses into clean groups, this is sometimes an oversimplification. For example, in Fig. 2C the Pflu and PiscS cases exhibit downregulation, but then end up with steady state values that are higher than the baseline. I appreciate that it is more straightforward to discuss distinct groups, but it could be more balanced to acknowledge that these distinctions are not always clear cut.

We have added the following text (**L123-127**):

‘While most genes display consistent up- or down-regulation throughout H₂O₂ treatment, a few genes show initial downregulation followed by upregulation at the steady state. For simplicity, we categorized the direction of regulation based on the promoter activity changes immediately after treatment.’

2. It would be helpful to include more x-axis tick marks in Figs. 1D and 2C since much of the focus is on the initial pulse period.

Figures 1D, 2C and S1 modified with more x-axis ticks.

3. Fig. 1D: how is darker shaded region determined? It may be best to remove this.

Darker shades were initially used to indicate the CV values are higher at peak response than steady state levels, but have now been removed for simplicity since the data clearly show the peak in gene-gene variation.

4. Line 117-121: when discussing how there is a positive pulse in protein levels of negatively regulated genes, is the referenced protein the fluorescent reporter from Fig. 1C or is it the inferred protein level for the gene of interest?

The referenced protein is the fluorescent reporter, we have modified the text to make it clear (**L129-131**).

‘An interesting consequence of the effects of growth arrest on expression levels in the cell is that the genes that we identify as negatively regulated still show a positive pulse in reporter expression levels after treatment [Figure 2B, C].’

5. Fig. 2C: It took me a little while to understand what the statistical significance bars were comparing. It would be helpful to describe in the caption what timepoints were actually used for the comparisons.

The legend for **Figure 2C** has been updated to specify which data points were used to compute the statistical significance values.

6. Fig. 3A and C are not referenced in the text.

This has now been corrected in the text. Please refer to Major comment 3d.

7. It would be useful to show promoter activity and fluorescence levels together for each reporter (for example, in the supplement).

We added **Figure S1** for this purpose.

8. In Fig. 3A's caption there are three levels of H₂O₂ (25, 50, and 100 μ M), however it is not clear which points these different values correspond to on the plot.

The expression levels for different concentrations have now been depicted by different markers, and the legend for **Figure 3A** has been updated accordingly.

9. The phrase "gradually induced" is a bit confusing in that it could be interpreted as a difference in the introduction rate of H₂O₂. A potential alternative would be "gradual response."

We agree with this suggestion, as we experiment with a gradually increasing dose of H₂O₂. The term ‘gradually induced’ may confuse the reader. In line with your suggestion, we have clarified the wording where it may be confusing which are **L152-153, L202-203, L217, L218-219, L227-228, L239-240, L242-244, L248-250, L268-269, L289-291, L333-340** as follows. And, the legends of **Movie S1** and **Figures 3, 4, 5, 7** and **S3** have been changed to ‘gradually responding’.

‘peak/steady-state ratio showed a gradual induction to an elevated steady-state level (hence termed “gradually responding”)’

‘higher than for the *PahpC* promoter which shows a gradual response⁷² [Figure 4F].’

‘promoter activity compared to the *PgrxA* promoter which shows a gradual response’

‘Similarly, other pulsatile genes showed a higher sensitivity to H₂O₂ compared to gradually responding genes [Figure 5B, S3].’

‘These analyses suggest that pulsatile genes are important in response to sudden stress, whereas gradually responding genes are more important during prolonged stress’

‘We next asked to what extent the expression of pulsatile and gradually responding genes is coordinated within the same cell’

‘However, the amplitude of the expression peak reached by individual cells was more variable for pulsatile genes compared to gradually responding genes’

‘Next, we constructed a dual-reporter strain with two fluorescently labelled transcriptional reporters: the pulsatile *PkatG*-YFP reporter expressed from a plasmid and the gradually responding *PgrxA*-CFP reporter inserted on the chromosome’

‘Together, our single-cell analysis revealed that pulsatile and gradually responding genes are tightly coordinated’

‘Turning to upregulated genes, we see a steeper decay in expression in space as one moves away from the source of stress for pulsatile genes as compared to the gradually responding genes’

‘Being more sensitive to local H₂O₂ fluctuations, pulsatile genes therefore display high cell-cell variability in expression magnitude and steeper spatial gradients compared to gradually responding genes. Of the two H₂O₂ scavenging enzymes, pulsatile catalase (*katG*) is important to protect against immediate stress experienced by cells on the periphery of a population while gradually responding alkyl hydroperoxidase (*ahpCF*) efficiently scavenges low H₂O₂ levels that reach the rest of the population. Gradually responding thioredoxins (*grxA*⁷⁸, *trxC*⁴⁶) regulate the redox status of proteins, including OxyR.’

10. Fig. 4E: *It would be helpful to plot actual values of K_{ind} and K_d from the model and show the lines they fall along as a supplementary figure.*

Figure 4E has been updated and the legend has been updated accordingly.

11. Line 182-184: *"experiments showed that the dissociation constant of oxidised OxyR from the pulsatile gene promoter *PkatG* is an order of magnitude higher than for the gradually induced *PahpC* promoter" and references citation 72 and Fig. 4F. I see that reference 72 measures dissociation constants for the two promoters, but as written the text suggests that the values are measured and presented in Fig. 4F. Please clarify this wording.*

We have clarified it now by editing the text and adding the reference clearly. The modified text reads as follows (L200-203):

‘In support of this, experiments by Tartaglia et al. showed that the dissociation constant of oxidised OxyR from the pulsatile gene promoter *PkatG* is an order of magnitude higher than for the gradually induced *PahpC* promoter⁷² [Figure 4F].’

12. *There is a typo in the caption for Fig. 5A that labels both the peak and steady state plots as (bottom).*

Thank you for noticing. The legend for **Figure 5A** has been corrected.

13. *The fluorescence levels of PgrxA in delta_katG cells with gradual versus step increase in H₂O₂ look opposite in Movie S4 and Fig. 5E. The figure shows brighter response of the fluorescent reporter to a step in H₂O₂ in delta_katG cells, but the legend for Movie S4 says the step in H₂O₂ is the bottom set of cells, which show growth defects and dimmer fluorescence than the cells that received gradual H₂O₂ addition in the top of the video.*

The discrepancy was due to a difference in how the images in the figure and the movie were normalised. This has now been corrected in the figure, showing dimmer fluorescence for the step treatment, consistent with the movie.

14. *Line 208: The text describes delta_katG cells as being more sensitive to the step of H₂O₂. Does this refer to the induction of the PgrxA promoter or to growth rate/survival?*

We changed the text to clarify this (**L229-232**):

‘deletion of the pulsatile *katG* gene makes cells extremely sensitive to sudden H₂O₂ treatment, leading to complete growth arrest and inability to induce the oxidative stress response [Figure 5E-F, Movie S4]. However, $\Delta katG$ cells were still able to survive and adapt to a gradually increasing dose of H₂O₂ reaching the same final concentration’

15. *Line 243: My understanding is that Fig. 6F uses fluorescence. If promoter activity is used instead (which would be preferred), how much of the correlation remains?*

We attempted to perform correlation analysis on the promoter activity traces. However, the promoter activity traces are very noisy at a single-cell level (since they are based on time-derivatives of both the fluorescence levels and the cell size) such that the resulting correlation traces were not easily interpretable.

16. *Fig. 7D is not discussed in the text. This is related to one of the major points, but it is not clear that it is possible to extrapolate dynamics within a single mother machine chamber (typically less than 10 cells and length in the micrometer scale) to colonies (thousands of cells and diameter in millimeter scale). This is at the discretion of the authors, but my sense is that Fig. 7D is not fully justified and could be removed. If it remains, please clarify in the caption what the color intensity of the red, green, and blue represent.*

We appreciate the point and have added a statement with evidence that the extrapolation is sensible (**L292-297**), as described in our response to point 4. We referenced Figure 7D at the end of the results section (**L280**) as follows:

‘pulsatile genes are strongly upregulated in relatively few cells that are close to the H₂O₂ source, which generates significant cross protection, such that only the most stressed cells show expression. Conversely, negatively regulated genes are more strongly expressed in the interior of a population. The gradually upregulated genes then activate more evenly in larger numbers of cells to provide lasting protection. Figure 7D illustrates the resulting pattern of gene expression in a cell colony.’

We modified the legend of **Figure 7D** to clarify that the schematic shows the “expected” pattern of gene expression.

17. Line 504: Please clarify whether the data for *Prna1* used elsewhere in the manuscript corresponds to the *mKate2* reporter version or if it is a *GFPmut2* (or *SCFP3A*) version.

Prna1 has been updated to *Prna1-mKate2* (L377,421, 531) to avoid confusion.

18. Line 575-576: a few typos in this line.

Typos have been corrected and the text reads as follows (L610-613):

‘BACMMAN generated output in separate excel files containing cell growth characteristics, *Prna1-mKate2* intensity data and one file for each fluorescence data. These files were then analyzed using a custom python pipeline to compute cell parameters as described in the following section.’

19. Line 598: "Peaky" may be a typo

Corrected in L634.

20. Line 600: This is referred to as "steady state" in the figures, not "steady"

Corrected in L636.