nature portfolio

Peer Review File

Widespread biochemical reaction networks enable Turing patterns without imposed feedback

Reviewer #1 (Remarks to the Author):

Please see attached comments.

Reviewer #1 (Remarks on code availability):

Broadly the code looks good. A medium-level summary detailing the computational methods with more clarity, as requested in my comments, would help make the framework much more useful.

Review of NCOMMS-24-09225: Widespread biochemical reaction networks enable Turing patterns without imposed feedback

Andrew L. Krause, Durham University, UK*

The authors explore a range of biochemically-motivated reaction-diffusion systems to explore the question of Turing-type pattern formation outside of the classical 'activator-inhibitor' framework. They analyze a large number of systems involving 4 subunits leading to 11 complexes, and the various reaction schemes needed to form all such complexes. Within this space of elementary biochemical interactions, they used simple analytical criteria and extensive computational explorations to elucidate which reaction pathways led to systems able to form patterns within suitable biological parameter ranges. They also compared the topology of their reaction schema and complexes to those found in bioinformatics datasets, suggesting that many existing pathways in human cell lines exhibit the right topological motifs to admit pattern formation via Turing-like instabilities.

Overall I find the topic important, and the core ideas of the paper very interesting. It is clear that the classical activator-inhibitor paradigm is far too simplistic. Broadly, I find the systematic analysis in the paper well done, and some of the insights gained very promising. However I have a number of technical concerns, and a few suggestions regarding wider aspects of modelling that may (or may not) be helpful to relate to ongoing efforts for moving beyond these classical paradigms. Overall the manuscript has a bit of an exhaustive *tour de force* approach in terms of the number of systems studied, and the methods employed. However it does lack polish in some ways which more narrow studies would have because they do not intend to fit so many things into one study. Below are a number of comments aimed at helping the authors improve their story, arranged within a few categories. It should be noted that I do not think the authors must address *every* comment below in copious detail – rather, I feel they should consider these largely as opportunities to think about the strengths and weaknesses of their approach more critically, and to consider changes that they feel would help them make a stronger scientific argument.

Comments on Modelling/Interpretation

1. **Scope of Modelling:** It is a bit unclear precisely what scale of systems the authors are intending to study. They mention both intracellular and tissue-level patterning, but use parameter ranges largely for the latter (with most of the discussion also focusing on tissue-scale phenomena). Importantly, it appears like all of their complexes are modelled as diffusible on relatively large length scales. My understanding is that most presumptive pattern-forming systems at this scale are composed of diffusible signalling molecules coupled to more complex intracellular gene expression, rather than the purely chemical scenario of systems which can react and diffuse in the same space. There have been a number of studies on this more complicated spatial arrangement using either non-diffusible reactions coupled to diffusible signalling molecules (Klika et al., 2012; Korvasová et al., 2015) or gene-expression time delays (Gaffney and Monk, 2006; Sargood et al., 2022) which represent the intracellular dynamics but only strictly model the diffusible morphogens. I think in both cases, the resulting models may be more complicated, and less amenable to the kind of systematic analysis done in this manuscript. Nevertheless, I think the authors should at least consider that much of the reaction machinery for tissue-level patterning does not diffuse outside of the cell, and so the proposed framework is a bit of a crude approximation to a more detailed model of intercellular signalling and intracellular reactions. Some discussion or justification around the scope of the modelling here would be helpful.

2. **Type 2 instabilities are ill-posed:** Related to this, the authors cite reference 23 in terms of modelling

*By signing this review, I waive my right to anonymity.

with non-diffusible morphogens leading to dispersion curves that are not negative for large wavenumber (i.e. those for which $\lambda(q) > 0$ as $q \to \infty$). While reference 23 is a good paper, I'd recommend reading (Klika et al., 2012) carefully, as such 'Type 2' instabilities really lead to a breakdown of the continuum hypothesis needed to justify a PDE model in the first place. That is, if $\lambda(q) > 0$ for arbitrarily large values of q, the model is not mathematically well-posed, and one would expect cell-level or 'salt-and-pepper' patterning for models posed on the tissue level. Effectively this means the model is no longer valid, and such instabilities should be disregarded.

3. **Clearer Modelling Caveats:** The study seems to go between quantitative and qualitative in its scope at various points. For example, as above the parameters are chosen to match sensible biological ranges for tissue-level structures. But the broad outcome of the analysis is more qualitative, focusing on reaction pathways which can exhibit pattern formation. In the Section "Omics-level prediction of potential biochemical complexes enabling Turing patterns" the authors find that many human signalling systems are of the form of the identified pattern-forming systems. It would be worth qualifying this to clearly denote that this is in reaction structure, but not rate parameters (i.e. the authors did not fit their models to the datasets to show quantiative recapitulation). It is also important to note if the signalling molecules being described from these datasets are appropriately diffusible, or what precisely they represent in terms of intercellular signalling/expression. Being more careful in making clear that the outcomes here are largely qualitative, rather than trying to definitively state that certain motifs are strictly necessary for pattern formation, would improve the study. I liked the discussion of motifs, but the precise statements on what we learn about them could be made much more precise.

4. **Alternatives to Turing:** The authors note that many biological systems exhibit patterns, hence motivating the search for Turing-type signalling systems. It is worth noting that many physical mechanisms can give qualitatively similar symmetrical patterns, and that other mechanisms (e.g. positional information (Sharpe, 2019), clock-and-wavefront (Dziekan et al., 2014), or mechanochemical mechanisms Veerman et al. (2021)) also exhibit pattern-formation. Quoting Goerge Oster from the introduction of (Economou and Green, 2014): "So what good are models? The foregoing discussion may seem a bit depressing to theoretical biologists: all manner of models produce the same spatial patterns. Thus it is not generally possible to distinguish between models solely from the patterns they generate, and the question I raised at the beginning begs an answer: what good are models of pattern formation?" I feel the authors could consider a broader discussion of their motivation in terms of operationalizing a specific mechanism (Turing systems) for pattern formation, noting importance constraints about plausible biochemical pathways. This clear molecular constraining of models is exactly what is discussed in (Woolley et al., 2021) (as well as in (Economou et al., 2020)) about theoretical models being able to recapitulate patterns without actually justifying any particular mechanism unless they are constrained carefully to plausible molecular pathways. It would also be worth taking a look at a recent systematic analysis of simple reaction-diffusion models by Waters et al. (2024), which takes an altogether different nbut complementary view to such a systematic analysis from the present manuscript.

Mathematical/Technical Comments

5. The authors should be careful in the formulation of their mass-action models and subsequent linearizations. In particular, in Equation (S.5), should there be a factor of 2 in front of the terms involving $[AA]$ and $[A][A]$ in the first equation, but only a single factor of 2 in the second equation? This does not lead to mass conservation of the reaction with rate given by $K_{AA}\kappa_{\text{off}}$ for example. Similarly, in Equation (S.19), as well as many others, there are factors of 3 on some tetrameric terms, but not others (e.g. (S.32) has no integer factors?). Perhaps more worrying, in the linearization in (S.6), I would expect additional factors of 2 coming from the quadratic terms, but these have been dropped. In particular, an equation of the form

$$\frac{d[A]}{dt} = ... - 2K_{AA}\kappa_{\text{off}}[A][A] ...,$$

should lead to a linearized system of the form,

$$\frac{d\delta a}{dt} = ... - 4K_{AA}\kappa_{\text{off}}[A]_0 ...,$$

but a factor of 2 has disappeared from the corresponding Jacobian element a_{11}. Possibly this is just a typo, but please do check this carefully for any nonlinear term (higher-order reaction beyond a single monomer).

6. I am also somewhat concerned with the notion of steady-state or equilibrium solution (about which one does the linearization). As is well known for systems of nonlinear equations coming from mass-action kinetics, we may expect many equilibria, or even no equilibria, rather than a single one. For example, (S.5) can admit zero, one, or two equilibria, depending on the parameters, as these satisfy equations for the intersection points of two quadratics. Later in the supplement it is stated that " These steady-state concentrations are determined using the outcomes of numerical continuation analysis." However, this is still unclear in terms of uniqueness and dependence on the parameters... Multiple equilibria can radically change the landscape of pattern formation, for example by destroying all patterns created by a Turing instability as shown in Krause et al. (2024).

7. What do the authors mean by the "Hopf-Turing bifurcation"? There is a technical instability known as a "Turing-Hopf" bifurcation, which is a codimension-2 bifurcation coinciding with parameters crossing both Turing and Hopf thresholds for the same value (there is a related instability known as a "wave" or "Turing-wave" instability where the crossing of the dispersion curve at a Turing bifurcation occurs as a complex conjugate pair of growth rates (Villar-Sepúlveda and Champneys, 2023)). But I do not know what is meant in this section. More generally, I find the study of Hopf bifurcations in these systems a bit of a red herring; indeed there may be some correlation between these types of instabilities as the authors point out, but my impression is that it makes the analysis more cumbersome and less interpretable to include a search for Hopf bifurcations at all.

8. The authors discuss the use of Tellurium to perform continuation analysis. Do they mean numerical continuation of equilibria with respect to one or more parameters? Can they provide more details? Looking through the Tellurium documentation, I can't quite determine if it performs standard numerical continuation (e.g. as described in (Seydel, 2009) or in the software packages MatCONT (Dhooge et al., 2003), pde2path (Uecker et al., 2014), or the older Auto (Doedel and Oldeman, 1998); see (Krauskopf et al., 2007) for a wider review). Reading the file "te_bifurcation.py" it appears like Tellerium is using AUTO to perform a one-parameter continuation from an initial equilibrium point, but I can't easily spot where the original equilibrium is found (or which parameter(s) are used in the continuation). Can the authors describe how equilibria were found, and what kinds of continuation studies were performed? Was it looking for Hopf bifurcations only, or were higher codimension (or global) bifurcations also pursued? Many systems can admit oscillations without a Hopf bifurcation, and similarly Turing bifurcations can occur in systems without any Hopf. I appreciate this is a technical point, but high-throughput analysis is not something typically done with numerical continuation as far as I'm aware.

9. "Due to limitations in our numerical PDE simulations, we have confined the dimensionless diffusion coefficient" if the authors use an implicit timestepping scheme (e.g. by using scipy.integrate or any other off-the-shelf stiff methods), they will be able to explore a much larger range in diffusion coefficients. Any standard stiff method works very well for reaction-diffusion systems without any restrictions on diffusion parameters or timestepping.

10. More generally, the authors could consider a more algebraic approach to determining suitability of systems for pattern formation using matrix methods such as in (Satnoianu et al., 2000) or more recently in (Villar-Sepúlveda and Champneys, 2023). This is more in line with what Waters et al. (2024) does, though they only look at two-species systems. I suspect that a more analytical approach would make the computational search vastly more efficient than the current method.

11. Many details regarding the computational pipeline are not very clear from the supplement/methods/code itself. In particular, precisely what distributions of random parameters were used? Were these always Uniformly distributed in the ranges described? How were equilibria sought (and if initial data were used to search for equilibria, were these also randomized?) For the dispersion analysis, how exactly was q discretized and checked? How do the authors know they used a sufficiently fine resolution of q including sufficiently dense and high frequency modes? In general it would help if a more detailed description of the computational pipeline was provided, with all of the numerical values given.

Minor Comments/Typos

There are quite a few typos in the manuscript, so a more careful read through may help polish the text. Below are a few examples alongside very minor comments.

12. "can either protects" should be "can either protect".

13. "first-order diffusion" is not a term I'm familiar with, or one that appears searching around. Presumably the authors mean Fickian diffusion (i.e. Fick's first law)? The trouble with the term 'order' being that it has a distinct meaning for derivatives (where diffusion is a second-order operator).

14. "nearly more than 9000" is bad phrasing. "just under 9000" or "almost 9000" is more accurate presumably? One could simply be precise and give the number as well...

15. "particularly ratios the degradation rate constants" I think an "of" is missing before "the degradation rate constants".

16. "Together we other four models" Something is missing here.

17. Supplemental Section 1: "encounter Turing pattern is to add an addition degree of" likely the authors mean "additional".

18. Supplemental Section 1: "terms of it's potential to generate Turing pattern" no apostrophe needed in "its" here. Similar uses of "its" elsewhere as a possessive noun.

19. Punctuation missing in many equations, e.g. a period is needed in (S.8).

20. Supplemental Section 1: "the degree three polynomial we the table" delete "we".

21. The equation numbering in the Supplement is inconsistent, because it restarts within each section. The majority of what I've written refers to the first section, but repeated numbering makes it hard to be precise!

22. In Supplemental Section 3, equation (S.2) is typeset somewhat poorly. The authors could consider using block matrix notation to make it clearer, e.g.,

$$\begin{matrix} \boldsymbol{A} & \boldsymbol{B} \\ \boldsymbol{C} & \boldsymbol{D} \end{matrix} \quad , \quad \boldsymbol{A} = \dots$$

or similar.

NB: The manuscript already cites many references, so please do not feel the need to include any of the references I have here (especially any I am an author on). Rather, I am merely hoping the author considers some of the perspectives presented in these papers which may have been overlooked.

References

A. Dhooge, W. Govaerts, and Y. A. Kuznetsov. Matcont: a matlab package for numerical bifurcation analysis of odes. *ACM Transactions on Mathematical Software (TOMS)*, 29(2):141–164, 2003.

E. J. Doedel and B. Oldeman. Auto-07p: continuation and bifurcation software. *Montreal, QC: Concordia University Canada*, 1998.

P. Dziekan, B. Nowakowski, and A. Lemarchand. Reaction-diffusion scheme for the clock and wavefront mechanism of pattern formation. *The European Physical Journal B*, 87:1–7, 2014.

A. D. Economou and J. B. Green. Modelling from the experimental developmental biologists viewpoint. In *Seminars in cell & developmental biology*, volume 35, pages 58–65. Elsevier, 2014.

A. D. Economou, N. A. Monk, and J. B. Green. Perturbation analysis of a multi-morphogen turing reaction-diffusion stripe patterning system reveals key regulatory interactions. *Development*, 147(20):dev190553, 2020.

E. A. Gaffney and N. Monk. Gene expression time delays and turing pattern formation systems. *Bulletin of mathematical biology*, 68:99–130, 2006.

V. Klika, R. E. Baker, D. Headon, and E. A. Gaffney. The influence of receptor-mediated interactions on reaction-diffusion mechanisms of cellular self-organisation. *Bulletin of mathematical biology*, 74:935–957, 2012.

K. Korvasová, E. Gaffney, P. Maini, M. Ferreira, and V. Klika. Investigating the turing conditions for diffusion-driven instability in the presence of a binding immobile substrate. *Journal of theoretical biology*, 367:286–295, 2015.

A. L. Krause, E. A. Gaffney, T. J. Jewell, V. Klika, and B. J. Walker. Turing instabilities are not enough to ensure pattern formation. *Bulletin of Mathematical Biology*, 86(2):21, 2024.

B. Krauskopf, H. M. Osinga, and J. Galán-Vioque. *Numerical continuation methods for dynamical systems*, volume 2. Springer, 2007.

A. Sargood, E. A. Gaffney, and A. L. Krause. Fixed and distributed gene expression time delays in reaction–diffusion systems. *Bulletin of Mathematical Biology*, 84(9):98, 2022.

R. A. Satnoianu, M. Menzinger, and P. K. Maini. Turing instabilities in general systems. *Journal of mathematical biology*, 41(6):493–512, 2000.

R. Seydel. *Practical bifurcation and stability analysis*, volume 5. Springer Science & Business Media, 2009.

J. Sharpe. Wolpert's french flag: what's the problem? *Development*, 146(24):dev185967, 2019.

H. Uecker, D. Wetzel, and J. D. Rademacher. pde2path-a matlab package for continuation and bifurcation in 2d elliptic systems. *Numerical Mathematics: Theory, Methods and Applications*, 7(1):58–106, 2014.

F. Veerman, M. Mercker, and A. Marciniak-Czochra. Beyond turing: far-from-equilibrium patterns and mechano-chemical feedback. *Philosophical Transactions of the Royal Society A*, 379(2213):20200278, 2021.

E. Villar-Sepúlveda and A. R. Champneys. General conditions for turing and wave instabilities in reaction-diffusion systems. *Journal of Mathematical Biology*, 86(3):39, 2023.

F. R. Waters, C. A. Yates, and J. H. Dawes. Minimal reaction schemes for pattern formation. *Journal of the Royal Society Interface*, 21(211):20230490, 2024.

T. E. Woolley, A. L. Krause, and E. A. Gaffney. Bespoke turing systems. *Bulletin of Mathematical Biology*, 83(5):1–32, 2021.

Reviewer #2 (Remarks to the Author):

This study screened through a comprehensive set of simple biochemical networks without any explicit feedback for those that can generate Turing pattern. The work identified how network topology, reaction rate constants and diffusion coefficients affect the possibility of generating Turing pattern. Because the suggested pattern-enabling networks only contain complex formation and constitutive synthesis/degradation, thousands of protein and RNA complexes could match the network topology and hence could be candidates of Turing pattern generators. This study brings new and exciting insights into a classical topic in mathematical biology. Pattern formation is prevalent in development and other crucial biological processes. The widely accepted activator-inhibitor and substrate-depletion models for Turing pattern formation, although insightful, cannot easily find a matching example in reality. This study potential bridges this gap. The topic is of broad interest to the audience in biology, biophysics and bioengineering.

Major comments:

1. In the discussion about the required network topology, the authors stated: “Each of the 10 pattern-enabling reaction networks has at least two molecules whose associated parameter values (synthesis and degradation rate constants) can differ between the two.” How different should their parameter values be? Btw, this sentence is a bit confusing upon first glance. Suggested edit: “Each of the 10 pattern-enabling reaction networks contain at least two different molecules (Figure 2b, black and white circles). Note that in the model, different molecules specifically refer to molecules with different parameters.”
2. What is the unit of the diffusion coefficients shown in Fig. 5? An important reason why the classical activator-inhibitor and substrate-depletion models cannot easily find examples in reality is the required large difference between the diffusion coefficients of the two components of the system. Do pattern-enabling networks in this work have as stringent requirement on the diffusion coefficients?
3. In the omics-study, is there any possibility to further screen for the rate constants and diffusion coefficients even if they are just estimated numbers? There can’t be thousands of Turing pattern generators.
4. The Routh-Hurwitz analysis should be cited and described (could be in the Supplementary text).

Minor comments:

1. Typo: “It should be noted that the networks in our study are hypergraphs (each edge that represents binding involves 3 nodes) rather regular graphs commonly used for defining feed-forward loops”. “rather” ◊ "rather than”.
2. “In Set II (Figure 5c, left), Subunit A serves as a diffusion facilitator”. The diffusion coefficient of A is smaller than that of B and AB. So, B sounds more like the diffusion facilitator in this case?
3. Typo: “our analysis showed that the choice of parameter values, particularly ratios the degradation rate constants of protein/RNA subunits in various complexes, can be crucial for pattern formation.” Missing “between” after “ratios”.
4. Typo: “In addition to the direct interpretation of molecular degradation, the first-order decay rates of the molecules in our models are be generalized to rates of removal from a specific location (e.g. removal of molecules from plasma membrane via endocytosis) where patterns can arise.” “are be generalized” ◊ “can be generalized”.
5. Typo: “Together we other four models, we analytically proved that Eq 1 cannot have any unstable steady state with arbitrary positive rate constants,” “Together we” ◊ “Together with”

Black text: Reviewers' comments.

Blue text: Authors' responses (**Bold**: highlights of edits made to manuscript, where all edits are marked red in both main text and supplementary text).

Reviewer #1 (Remarks to the Author)

The authors explore a range of biochemically-motivated reaction-diffusion systems to explore the question of Turing-type pattern formation outside of the classical 'activator-inhibitor' framework. They analyze a large number of systems involving 4 subunits leading to 11 complexes, and the various reaction schemes needed to form all such complexes. Within this space of elementary biochemical interactions, they used simple analytical criteria and extensive computational explorations to elucidate which reaction pathways led to systems able to form patterns within suitable biological parameter ranges. They also compared the topology of their reaction schema and complexes to those found in bioinformatics datasets, suggesting that many existing pathways in human cell lines exhibit the right topological motifs to admit pattern formation via Turing-like instabilities.

Overall I find the topic important, and the core ideas of the paper very interesting. It is clear that the classical activator-inhibitor paradigm is far too simplistic. Broadly, I find the systematic analysis in the paper well done, and some of the insights gained very promising. However I have a number of technical concerns, and a few suggestions regarding wider aspects of modelling that may (or may not) be helpful to relate to ongoing efforts for moving beyond these classical paradigms. Overall the manuscript has a bit of an exhaustive tour de force approach in terms of the number of systems studied, and the methods employed. However it does lack polish in some ways which more narrow studies would have because they do not intend to fit so many things into one study. Below are a number of comments aimed at helping the authors improve their story, arranged within a few categories. It should be noted that I do not think the authors must address every comment below in copious detail – rather, I feel they should consider these largely as opportunities to think about the strengths and weaknesses of their approach more critically, and to consider changes that they feel would help them make a stronger scientific argument.

Author response: We thank the Reviewer for the thorough and insightful review. The comments helped us think more carefully about the scope and significance of our study in a broader context. We have now addressed each comment with additional discussion and more accurate interpretation of our results. In this revision, we also made corrections and clarifications on a few technical descriptions in our manuscript that were confusing to readers, and we performed additional analyses to address some concerns on the computational pipeline. Overall, we feel that the resulting revised manuscript has significantly improved in several aspects.

Comments on Modelling/Interpretation

1) **Scope of Modelling:** It is a bit unclear precisely what scale of systems the authors are intending to study. They mention both intracellular and tissue-level patterning, but use parameter ranges largely for the latter (with most of the discussion also focusing on tissue-scale phenomena). Importantly, it appears like all of their complexes are modelled as diffusible on relatively large length scales. My understanding is that most presumptive pattern-forming systems at this scale are composed of diffusible signalling molecules coupled to more complex intracellular gene expression, rather than the purely chemical scenario of systems which can react and diffuse in the same space. There have been a number of studies on this more complicated spatial arrangement using either non-diffusible reactions coupled to diffusible signalling molecules (Klika et al., 2012; Korvasov´a et al., 2015) or gene-expression time delays (Gaffney and Monk, 2006; Sargood et al., 2022) which represent the intracellular dynamics but only strictly model the diffusible morphogens. I think in both cases, the resulting models may be more

complicated, and less amenable to the kind of systematic analysis done in this manuscript. Nevertheless, I think the authors should at least consider that much of the reaction machinery for tissue-level patterning does not diffuse outside of the cell, and so the proposed framework is a bit of a crude approximation to a more detailed model of intercellular signalling and intracellular reactions. Some discussion or justification around the scope of the modelling here would be helpful.

Author response: We agree with the Reviewer that our original manuscript had a weakness in the connection between the tissue-scale pattern formation that often involves non-diffusive components and the assumption that all molecules are diffusive in our models. In addition, our intention was to use our models to explain both intracellular and tissue-level patterning, as noted by the Reviewer, but there were limited examples of and emphasis on intracellular patterning that we discussed in our manuscript. We have now discussed the limitation of our modeling scope (i.e. we did not consider immobile components in pattern formation), and we also added new text to the manuscript for building a stronger connection between our assumption of a continuum system and realistic biological patterns at both intracellular and multicellular levels. For the latter purpose, we included a reference to a recent experimental study that showed spontaneous, tissue-level self-organization in the absence of preformed cellular boundaries (Cheng and Ferrell Jr, 2019), highlighting the importance of continuum-like reaction-diffusion system without any preformed immobile component in development. We also included a reference to a recent modeling work of using Turing-pattern to explain intracellular patterning of Caulobacter (Xu et al., 2023). Finally, while intracellular patterning may be more consistent with the continuum assumption made in our models, we think that the fact that cells are immobile in many cases does not necessarily invalidate the assumption of systems with only diffusive molecules. The constant exchange of molecules via extracellular vesicles or symplastic transport, for example, makes the multicellular, cytoplasmic components in some systems closer to the continuum assumption. Of course, we agree with the Reviewer that if gene regulation at the transcriptional level is involved in initiating patterns, then assumption can be unrealistic. The relevant revision was performed in **the first paragraph of Introduction**, and **the last paragraph of Discussion**. All the suggested references have been cited.

2) **Type 2 instabilities are ill-posed:** Related to this, the authors cite reference 23 in terms of modelling with non-diffusible morphogens leading to dispersion curves that are not negative for large wavenumber (i.e. those for which $\lambda(q) > 0$ as $q \rightarrow \infty$). While reference 23 is a good paper, I'd recommend reading (Klika et al., 2012) carefully, as such 'Type 2' instabilities really lead to a breakdown of the continuum hypothesis needed to justify a PDE model in the first place. That is, if $\lambda(q) > 0$ for arbitrarily large values of q, the model is not mathematically well-posed, and one would expect cell-level or 'salt-and-pepper' patterning for models posed on the tissue level. Effectively this means the model is no longer valid, and such instabilities should be disregarded.

Author response: We thank the Reviewer for suggesting a more accurate description of Type 2 Turing instability. This helped us make a better connection between the absence of Type 2 instability in our results to the continuum assumption that we have made. To address the Reviewer's comment, we have revised both the main text (**the last paragraph of Results section 'Network topology required for Turing pattern-enabling reactions'**) and supplementary text (**B.4**) to highlight the importance of the continuum hypothesis and its validity, given the absence of Type 2 Turing instability from the analysis of our PDE models. The suggested reference was cited accordingly.

3) **Clearer Modelling Caveats:** The study seems to go between quantitative and qualitative in its scope at various points. For example, as above the parameters are chosen to match sensible biological ranges for tissue-level structures. But the broad outcome of the analysis is more qualitative, focusing on reaction pathways which can exhibit pattern formation. In the Section "Omics-level prediction of potential biochemical complexes enabling Turing patterns" the authors find that many human signalling systems are of the form of the identified pattern-forming systems. It would be worth qualifying this to clearly

denote that this is in reaction structure, but not rate parameters (i.e. the authors did not fit their models to the datasets to show quantitative recapitulation). It is also important to note if the signalling molecules being described from these datasets are appropriately diffusible, or what precisely they represent in terms of intercellular signalling/expression. Being more careful in making clear that the outcomes here are largely qualitative, rather than trying to definitively state that certain motifs are strictly necessary for pattern formation, would improve the study. I liked the discussion of motifs, but the precise statements on what we learn about them could be made much more precise.

Author response: We agree with the Reviewer that we should be more precise about the interpretation of the inferred, specific pattern formation systems. The Reviewer rightly pointed out that the conclusions in the last Results section are qualitative rather than quantitative. We have added the following sentences to **the first paragraph of the section** '**Omics-level prediction of potential biochemical complexes enabling Turing patterns**' for a more accurate interpretation of our results. "Due to the lack of measured rate constants in these predicted systems, we were not able to further constrain the list of gene products that enable Turing patterns with experimental data. This qualitative inference of complexes is therefore not sufficient to identify pattern-enabling molecules definitively, and the pool of complex-associated gene products should be viewed as a preliminary set for subsequent screening in future studies. It should be noted, however, that this limitation also applies to the classical activator-inhibitor paradigm for identifying systems for Turing patterns. Our findings on the broad range of proteins and RNAs whose network topologies permit Turing patterns suggest the need of measurements for key parameters, such as degradation rate constants and diffusion coefficients as shown in the previous section, for predicting Turing pattern-enabling systems more accurately."

4) **Alternatives to Turing:** The authors note that many biological systems exhibit patterns, hence motivating the search for Turing-type signaling systems. It is worth noting that many physical mechanisms can give qualitatively similar symmetrical patterns, and that other mechanisms (e.g. positional information (Sharpe, 2019), clock-and-wavefront (Dziekan et al., 2014), or mechanochemical mechanisms Veerman et al. (2021)) also exhibit pattern-formation. Quoting Goerge Oster from the introduction of (Economou and Green, 2014): "So what good are models? The foregoing discussion may seem a bit depressing to theoretical biologists: all manner of models produce the same spatial patterns. Thus it is not generally possible to distinguish between models solely from the patterns they generate, and the question I raised at the beginning begs an answer: what good are models of pattern formation?" I feel the authors could consider a broader discussion of their motivation in terms of operationalizing a specific mechanism (Turing systems) for pattern formation, noting importance constraints about plausible biochemical pathways. This clear molecular constraining of models is exactly what is discussed in (Woolley et al., 2021) (as well as in (Economou et al., 2020)) about theoretical models being able to recapitulate patterns without actually justifying any particular mechanism unless they are constrained carefully to plausible molecular pathways. It would also be worth taking a look at a recent systematic analysis of simple reaction-diffusion models by Waters et al. (2024), which takes an altogether different but complementary view to such a systematic analysis from the present manuscript.

Author response: The Reviewer pointed out an important and challenging question to all modelers: given a phenomenon (pattern formation in this case), can we accurately infer its underlying mechanism? The Reviewer's comment and the references have prompted us to think more deeply into this question for a better description of our work's scope and significance. We think that there are two parts for addressing this question: 1) Enumeration of mathematically and biologically plausible models/mechanisms, and 2) Model selection and identifiability. Our work does not contribute to the second part directly, as implied by this comment: our work does not aim to provide an evaluation of Turing instability over other pattern-formation mechanisms on their ability to explain similar observations, nor does it select a molecular network based on pattern specifics (strips vs spots, in-phase vs out-of-phase etc.). Instead, our work significantly expanded the list of mathematically and biologically plausible molecular mechanisms under the category of Turing pattern, and we think that these mechanisms were underappreciated previously. Indirectly, our work may

help future work to identify specific mechanisms with constraints on molecular pathways and patterning specifics.

We agree that it is important to mention alternatives mechanisms for pattern formation. We therefore added a sentence to **the first paragraph of Introduction**. We also discussed the referenced papers in relation to the model selection and identifiability problem that the Reviewer mentioned with **a new paragraph of Discussion** (the second last paragraph). To compare our work with the work of minimal mass-action system mentioned by the Reviewer, we added a sentence to **the last paragraph of Introduction**, and a sentence to **the first paragraph of Results**.

Mathematical/Technical Comments

5) The authors should be careful in the formulation of their mass-action models and subsequent linearizations. In particular, in Equation (S.5), should there be a factor of 2 in front of the terms involving [AA] and [A][A] in the first equation, but only a single factor of 2 in the second equation? This does not lead to mass conservation of the reaction with rate given by KAAκoff for example. Similarly, in Equation (S.19), as well as many others, there are factors of 3 on some tetrameric terms, but not others (e.g. (S.32) has no integer factors?). Perhaps more worrying, in the linearization in (S.6), I would expect additional factors of 2 coming from the quadratic terms, but these have been dropped. In particular, an equation of the form

$$\frac{d[A]}{dt} = \cdots - 2K_{AA}\,\kappa_{\text{off}}[A][A]\cdots$$

should lead to a linearized system of the form,

$$\frac{d\delta a}{dt} = \cdots - 4K_{AA}\kappa_{\text{off}}[A]_0\cdots$$

but a factor of 2 has disappeared from the corresponding Jacobian element a11. Possibly this is just a typo, but please do check this carefully for any nonlinear term (higher-order reaction beyond a single monomer).

Author response: We thank the Reviewer for the careful read. The error in the linearized equation S6, as pointed out by the Reviewer, was indeed a typo and it has been corrected in this revision. We also checked our code that generates the ODEs and performs algebraic analysis requiring Jacobian matrix (available at the GitHub site), and confirmed that the stoichiometry was properly used. **An additional textbox** explaining the stoichiometric coefficients (integer factors) has been included in the supplementary file (at **the end of Section A**).

6) I am also somewhat concerned with the notion of steady-state or equilibrium solution (about which one does the linearization). As is well known for systems of nonlinear equations coming from mass-action kinetics, we may expect many equilibria, or even no equilibria, rather than a single one. For example, (S.5) can admit zero, one, or two equilibria, depending on the parameters, as these satisfy equations for the intersection points of two quadratics. Later in the supplement it is stated that “These steady-state concentrations are determined using the outcomes of numerical continuation analysis.” However, this is still unclear in terms of uniqueness and dependence on the parameters... Multiple equilibria can radically change the landscape of pattern formation, for example by destroying all patterns created by a Turing instability as shown in Krause et al. (2024).

Author response: We agree with the Reviewer’s comment on the possibility that multistability can result in changes of pattern formation landscape. This issue is relevant to our work because all 10 pattern-enabling reaction networks are capable of producing bistability and Turing pattern related oscillation in our study, although they likely occur in different parameter regions.

First, to avoid this situation of multistability we have chosen the parameter space for Turing bifurcation analysis in the vicinity of limit cycle (Hopf bifurcation), because coexistence of limit cycle and stable steady state in a single parameter set is not very common in general. In specific systems, this approach of searching synthetic Turing patterns near Hopf bifurcation was used as an established protocol for similar reasons (references provided in main text: (Cross and Hohenberg, 1993; Jensen et al., 1993; Lengyel and Epstein, 1992). To further address the Reviewer's concern, we used a more stringent criterion to select ODE parameter sets for subsequent dispersion analysis: we focused on those sets that produced Hopf bifurcation point, but not saddle-node bifurcation point (a commonly used hallmark for finding bistability). With this more selective pool of parameter sets, we confirmed that the 10 reaction networks, but not others, can generate Turing patterns (Figure R1, same as newly added Figure S7). As shown in Figure R1 A, all 10 reaction networks produced Turning patterns with parameter sets free of saddle-node bifurcation in numerical continuation. The same conclusion was reached with parameter sets with both Hopf bifurcation and saddle-node bifurcation (Figure R1 B). It should be noted that in the latter case, although these parameter sets contain saddle-node bifurcation points, the Hopf bifurcation points do not typically overlap with a bistable region in term of the control parameter used for continuation (Figure R1 C).

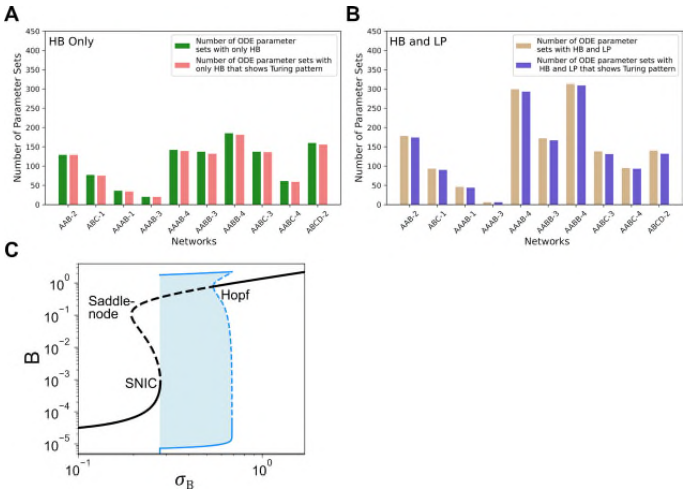

Figure R1. Bar charts depicting the comparison between bifurcation-containing and Turing pattern-enabling ODE parameter sets:(**A**) Scenario where ODE parameter sets with only HB points are considered. (**B**) Scenario where ODE parameter sets with both HB and LP points are considered (HB=Hopf Bifurcation and LP=Limit Point representing saddle-node bifurcation). (**C**) A typical scenario in B where a Hopf bifurcation point does not overlap with any bistable region even though saddle-node and Hopf bifurcations both appear in a continuation run.

In summary, while we cannot eliminate the possible but uncommon cases where Turing pattern-enabling parameter sets from our study are susceptible to the destructive effect of coexistence of a limit cycle and a stable steady state. Our prior and new evidence provides a strong support for the validity of our approach of searching for pattern-enabling networks. In the revised version, appropriate changes have been included in the supplementary file highlighted by red font (e.g. **Figure S7** and text in **Section B.3**).

7) What do the authors mean by the "Hopf-Turing bifurcation"? There is a technical instability known as a "Turing-Hopf" bifurcation, which is a codimension-2 bifurcation coinciding with parameters crossing both Turing and Hopf thresholds for the same value (there is a related instability known as a "wave" or "Turing-wave" instability where the crossing of the dispersion curve at a Turing bifurcation occurs as a complex conjugate pair of growth rates (Villar-Sep´ulveda and Champneys, 2023)). But I do not know what is meant in this section. More generally, I find the study of Hopf bifurcations in these systems a bit of a red herring; indeed there may be some correlation between these types of instabilities as the authors

point out, but my impression is that it makes the analysis more cumbersome and less interpretable to include a search for Hopf bifurcations at all.

Author response: We thank the Reviewer for pointing out the error and for the correct reference for Turing-Hopf bifurcation. The term “Hopf-Turing” bifurcation was indeed an incorrectly chosen term in our original manuscript intended to describe the combined process of Hopf bifurcation analysis and subsequent Turing bifurcation analysis using parameter sets exhibiting Hopf bifurcation. We have now corrected the error, and appropriate modifications marked by red font were made in **Section B5** of the supplementary text.

The Reviewer’s concern about using Hopf bifurcation is also related to Comment 6 and Comment 8. Here, we clarify that we used Hopf bifurcation as an intermediate step and a proxy to find Turing pattern-enabling systems for two reasons. First, we believe that the correlation between Hopf bifurcation and Turing instability is strong enough to justify the usage of Hopf bifurcation for convenience. In a 2-variable system, for example, the sign patterns of the Jacobian matrix necessary for supporting Hopf bifurcation and Turing instability are identical. They both require either $[+ +, - -]$ or $[+ -, + -]$, although Turing instability needs to be diffusion driven (Tyson, 2002; Tyson et al., 2003; Waters et al., 2024). It is therefore not surprising that analyzing specific chemical systems’ Turing instability in the vicinity of Hopf bifurcations has been routinely performed for several decades (Cross and Hohenberg, 1993; Jensen *et al.*, 1993; Lengyel and Epstein, 1992; Li et al., 2013; Mu and Lo, 2023; Zhao et al., 2021). In addition, high-throughput screening studies based on graphs showed that every pattern-enabling network topology contains a negative feedback loop with at least two variables, a network motif considered necessary and sufficient for oscillation in the absence of diffusion (Diego et al., 2018; Scholes et al., 2019). Our approach of using Hopf bifurcations to find systems with Turing instability is a natural extension of these studies. Secondly, we believe that Hopf bifurcation was underutilized for searching Turing instability in previous studies only because of the technical challenges of performing high-throughput numerical continuations (as pointed out by the Reviewer in Comment 8). With the implementation of high-throughput screening for Hopf bifurcations, our technique can accelerate the discovery of pattern-enabling system in the future.

In addition to correcting the wrong term ‘Hopf-Turing bifurcation’ that we used in the original manuscript, we performed additional dispersion analysis for parameter sets that did not produced any local (including Hopf) bifurcation from one-parameter continuation with ODE systems. We found that, in the 10 pattern-enabling reaction networks, a small fraction (<3% compared to about 99% in those with Hopf-bifurcations, as shown in Figure R1 A and B) of these bifurcation-free parameter sets can produce Turing patterns. Furthermore, we did not find any pattern-enabling parameter set in those 8 reaction networks originally identified to be non-pattern-enabling computationally (These networks are in addition to those 5 networks that we eliminated algebraically. See Comment 10). Because performing this additional dispersion analysis took 50-100 times more time than our original approach with Hopf bifurcation as an intermediate, and we did not find any new biological insight with the extended dispersion runs, we did not discuss the result in detail in the revised manuscript. Instead, we briefly mentioned it at **the end of the first Results section**.

8) The authors discuss the use of Tellurium to perform continuation analysis. Do they mean numerical continuation of equilibria with respect to one or more parameters? Can they provide more details? Looking through the Tellurium documentation, I can’t quite determine if it performs standard numerical continuation (e.g. as described in (Seydel, 2009) or in the software packages MatCONT (Dhooge et al., 2003), pde2path (Uecker et al., 2014), or the older Auto (Doedel and Oldeman, 1998); see (Krauskopf et al., 2007) for a wider review). Reading the file “te bifurcation.py” it appears like Tellerium is using AUTO to perform a one-parameter continuation from an initial equilibrium point, but I can’t easily spot where the original equilibrium is found (or which parameter(s) are used in the continuation). Can the authors describe how equilibria were found, and what kinds of continuation studies were performed? Was it looking for Hopf bifurcations only, or were higher codimension (or global) bifurcations also pursued? Many systems can admit oscillations without a Hopf bifurcation, and similarly Turing bifurcations can occur in

systems without any Hopf. I appreciate this is a technical point, but high-throughput analysis is not something typically done with numerical continuation as far as I'm aware.

Author response: The Reviewer is correct that we used one-parameter continuation from an initial equilibrium point. The initial equilibrium was first obtained by simulating the ODE system at $\sigma_B = 0$ (or $\sigma_A = 0$ for single subunit system without B), where the system always has a unique equilibrium point, using randomized initial conditions. Numerical continuation was subsequently performed and local bifurcation points (e.g. Hopf bifurcation and saddle-node bifurcations) were obtained through scanning the control parameter σ and updating equilibrium points. The continuation was performed with Tellurium, which includes an efficient implementation of AUTO that enabled us to perform the computation in a high-throughput, automated manner (screening massive parameter sets and many reaction networks). We are not aware of any high-throughput approach for detecting global bifurcation. Although the Tellurium package was not developed in our group, we feel that the community can widely benefit from using this high-throughput pipeline for finding local bifurcation points for various types of studies, so we think our published code and this manuscript will provide an example of this utility. The Reviewer's comment on the relationship between Hopf bifurcation and Turing instability was addressed in responses to Comments 6 and 7. We agree with the Reviewer that one cannot equate Hopf bifurcation to oscillation in general because there are global bifurcations that can lead to oscillation. However, due to practicality, detecting Hopf bifurcation is commonly used to evaluate/prove systems' ability to oscillate (Obatake et al., 2019). In this work, we extended the strategy and showed the practicality of using Hopf bifurcation to facilitate the discovery of Turing instability. We have now taken the Reviewer's suggestion to provide more details about the numerical continuation method. An additional paragraph with appropriate references was added to the **Methods section** 'ODE parameter sampling and numerical bifurcation analysis'.

9) "Due to limitations in our numerical PDE simulations, we have confined the dimensionless diffusion coefficient" if the authors use an implicit timestepping scheme (e.g. by using scipy.integrate or any other off-the-shelf stiff methods), they will be able to explore a much larger range in diffusion coefficients. Any standard stiff method works very well for reaction-diffusion systems without any restrictions on diffusion parameters or timestepping.

Author response: We thank the Reviewer for the suggestion. Our original statement needed to be corrected in that the choice of the diffusion coefficient range was both for biological reasons and for avoiding possible numerical problems. Restricting the diffusion coefficients to a moderate, rather than a wide, range around a realistic median value (estimation described in B.1.2 of supplementary text) help us focus on finding pattern-enabling systems containing molecules with typical, but not extreme diffusivity, with sufficient samples, which is more meaningful for discovering realistic biological systems in the future.

In terms of the potential numerical issue, the high stiffness partly comes from faster binding/unbinding reactions compared to production/degradation. In addition, the quadratic terms are the highest-order nonlinear terms in our system, which also features an abundance of linear terms. This abundance of linear terms and absence of higher-order nonlinearity beyond quadratic make the ODE system stiff. We had two options to handle the stiffness of the equations. First, using implicit methods for simulation, which are extremely slow (e.g. Figure R2) and cumbersome for larger networks, hence we avoided using implicit methods. To handle the stiffness and complexity of the networks with considerable computational efficiency, our only remaining option was utilizing simple explicit method (to deal with network complexity) implemented in a low-level language (to achieve computational efficiency) and employ a sufficiently small time-step to avoid divergence (prevention for stiffness). Moreover, we have implemented RK4 in FORTRAN for extremely stiff cases, and the gain in computational efficiency was lower than that of forward Euler with smaller time steps. In this revision, we also attempted to use the suggested method (scipy), and we found that with a stiff Gierer-Meinhardt reaction diffusion system (Figure R2), the scipy-based methods are too slow for large-scale computation. Furthermore, a FORTRAN-based stiff method LSODA failed to produce any

simulation result. We had the same observations with our AAB-2 network. Based on these observations, we did not change our numerical integration method for this study.

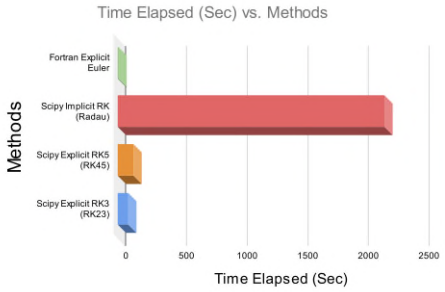

Figure R2. Integration method efficiency comparison for a stiff PDE system. Four chosen methods were used to simulate a stiff Gierer-Meinhardt reaction diffusion system. In addition, an implementation with the LSODA method failed to produce simulation results.

Nonetheless, using our current simulation method, it is possible to sample a wider range of diffusion coefficients by fine-tuning the spatial gridding appropriately, but we do not feel that this is biologically important for the scope of this work. To address the Reviewer's comment, we have now expanded the reasoning for choosing the diffusion coefficient range in **B.1.2 of supplementary text.** We also added text to **Section B.4** to explain the method of numerical integration in the supplementary text and included other pertinent modifications marked by red fonts.

10) More generally, the authors could consider a more algebraic approach to determining suitability of systems for pattern formation using matrix methods such as in (Satnoianu et al., 2000) or more recently in (Villar-Sepulveda and Champneys, 2023). This is more in line with what Waters et al. (2024) does, though they only look at two-species systems. I suspect that a more analytical approach would make the computational search vastly more efficient than the current method.

Author response: We agree with the Reviewer that algebraic methods can be more rigorous and more efficient than computational approaches. We clarify that we prioritized using algebraic approaches in our study. We have read the referenced analytical treatment leading to necessary and sufficient conditions for s-stability in the mentioned papers (Satnoianu et al., 2000; Villar-Sepulveda and Champneys, 2023; Waters et al. 2024) which are based on the stability conditions imposed by the Routh Hurwitz criterion. We would like to reiterate that, as shown in Figure 2c (now also included as a part of the new illustration in our GitHub site), during our systematic search for networks capable of pattern formation we first took the algebraic approach based on the Routh Hurwitz criterion, which successfully eliminated 5 reaction networks in terms of possible Turing instability (i.e. these systems are always stable with arbitrary positive parameters). When the algebraic approach gave inconclusive result due to the complexity of the networks, we utilized the computational pipeline to filter the Turing instability. With the remaining 18 networks we performed the computational dispersion analysis and 10 networks among them were predicted to give pattern formation. The remaining 8 networks could not exhibit Turing pattern formation with sampled, biologically meaningful parameter sets. While it is not in the scope of this work, future development of algebraic methods may provide additional insights into these 18 networks. We have provided the Routh-Hurwitz results in the supplementary data (Fig.S1-S3) and the GitHub repository [filename: Routh-Hurwitz.ipynb]. In this revision, we improved the **README file** (i.e. the front page) of the GitHub site to clarify our approach containing algebraic methods. We also added a brief comment on this issue under the **Methods section 'algebraic analysis'** in this revision.

11) Many details regarding the computational pipeline are not very clear from the supplement/methods/code itself. In particular, precisely what distributions of random

parameters were used? Were these always Uniformly distributed in the ranges described? How were equilibria sought (and if initial data were used to search for equilibria, were these also randomized?) For the dispersion analysis, how exactly was q discretized and checked? How do the authors know they used a sufficiently fine resolution of q including sufficiently dense and high frequency modes? In general, it would help if a more detailed description of the computational pipeline was provided, with all of the numerical values given.

Author response: We agree with the Reviewer that some important details of our computational pipeline were either missing or difficult to find in the original manuscript. We have been trying to increase the accessibility of the computational pipeline throughout the writing process, and as a result we developed the supplementary text and GitHub repository in a comprehensive way. However, the shear vastness of the computational work still made it challenging to compactly articulate the pipeline with all necessary details. We have now addressed the Reviewer's comment by further enhancing the clarity and reproducibility of our work in several aspects.

We have now improved the **detailed computational map** to clearly demonstrate the computational pipeline in the GitHub repository. We now clarified that we have used the Sobol quasi-random sequence to sample the parameter values. The sequence is uniformly distributed random numbers irrespective of the sample size, which is not the case for pseudorandom numbers. The distributions of the reactions parameter values were log-uniform (now specified in **Methods** and ranges provided in **Table S1**), whereas the those for the diffusion coefficients were uniform (**Section B.1** in supplementary text).

The initial equilibrium point was obtained with numerical simulation at $\sigma_B = 0$ using randomized initial conditions. The numerical continuation runs then update the equilibrium point iteratively and give the steady state concentrations, and we have used the steady states residing near the Hopf bifurcation to carry out the dispersion analysis. We have used $\Delta q = 0.05$ for the purpose of dispersion analysis and this implies that the performed dispersion analysis can capture unstable modes with wavelength less than $4.75 \times 10^3 \mu\mathrm{m}$ (now mentioned in **Methods**) and detailed reasoning pertaining to the inquiry is provided in the **supplementary text** (under Dispersion Analysis). Other necessary modifications were also carried out in the supplementary text and are marked by red font.

To further address the Reviewer's comment, we created a **new table** listing all important model and algorithm parameters in our **GitHub README file** (at the bottom of the front page), where readers can find the locations of text referencing the parameter values as well as the code files for tuning those parameters, if desired.

Minor Comments/Typos

There are quite a few typos in the manuscript, so a more careful read through may help polish the text. Below are a few examples alongside very minor comments.

12) "can either protects" should be "can either protect".

Author response: Thank you for catching the typo. We have now corrected it and marked it with red font.

13) "first-order diffusion" is not a term I'm familiar with, or one that appears searching around. Presumably the authors mean Fickian diffusion (i.e. Fick's first law)? The trouble with the term 'order' being that it has a distinct meaning for derivatives (where diffusion is a second-order operator).

Author response: Thank you for catching the typo. We have now taken the suggestion and replaced 'first-order' by 'Fickian' and marked it with red font.

14) “nearly more than 9000” is bad phrasing. “just under 9000” or “almost 9000” is more accurate presumably? One could simply be precise and give the number as well...

Author response: Thank you for catching the error. We have now changed the phrase to ‘almost 9000’ and marked it with red font.

15) “particularly ratios the degradation rate constants” I think an “of” is missing before “the degradation rate constants”.

Author response: Thank you for catching the typo. We have now corrected it by adding ‘between’ after ‘ratios’ and marked it with red font.

16) “Together we other four models” Something is missing here.

Author response: Thank you for catching the typo. We have now replaced ‘we’ by ‘with’ and marked it with red font.

17) Supplemental Section 1: “encounter Turing pattern is to add an addition degree of” likely the authors mean “additional”.

Author response: Thank you for catching the typo. We have now corrected it and marked it with red font.

18) Supplemental Section 1: “terms of it’s potential to generate Turing pattern” no apostrophe needed in “its” here. Similar uses of “its” elsewhere as a possessive noun.

Author response: Thank you for catching the typo. We have now corrected this and other instances and marked them with red font.

19) Punctuation missing in many equations, e.g. a period is needed in (S.8).

Author response: Thank you for catching the error. We have now corrected it and marked it with red font.

20) Supplemental Section 1: “the degree three polynomial we the table” delete “we”.

Author response: Thank you for catching the error. We have now corrected it and marked it with red font.

21) The equation numbering in the Supplement is inconsistent, because it restarts within each section. The majority of what I’ve written refers to the first section, but repeated numbering makes it hard to be precise!

Author response: This is a good point. We have addressed the issue by redefining the subsection numbering which now includes the section index, e.g., previously subsection 1 of section A was numbered as (1); in current version the subsection appears as (A.1).

22) In Supplemental Section 3, equation (S.2) is typeset somewhat poorly. The authors could consider using block matrix notation to make it clearer, e.g.,

$$\begin{pmatrix} A & B \\ C & D \end{pmatrix}, \quad A = \dots$$

or similar.

Author response: Thank you for the great suggestion. We have now incorporated this notation for this equation (Eq S.6 with the current numbering).

NB: The manuscript already cites many references, so please do not feel the need to include any of the references I have here (especially any I am an author on). Rather, I am merely hoping the author considers some of the perspectives presented in these papers which may have been overlooked.

Author response: We thank the Reviewer for all the excellent references and for the flexibility. We have now included all the suggested references in the revision (except one replacement with the original paper for the clock-and-wavefront model). We do not think the list of references is excessive, but we will confirm this with the Editor later if the manuscript is accepted.

Comments regarding code: Broadly the code looks good. A medium-level summary detailing the computational methods with more clarity, as requested in my comments, would help make the framework much more useful.

Author response: As mentioned in responses above, we have now improved the documentation in the README file of the GitHub repository. This includes an **additional visual illustration** showing how our computational pipeline (Figure 2c) is connected to the code files, and a **new table** listing all model and algorithm parameters.

References from Reviewer 1

A. Dhooge, W. Govaerts, and Y. A. Kuznetsov. Matcont: a matlab package for numerical bifurcation analysis of odes. ACM Transactions on Mathematical Software (TOMS), 29(2):141–164, 2003.

E. J. Doedel and B. Oldeman. Auto-07p: continuation and bifurcation software. Montreal, QC: Concordia University Canada, 1998.

P. Dziekan, B. Nowakowski, and A. Lemarchand. Reaction-diffusion scheme for the clock and wavefront mechanism of pattern formation. The European Physical Journal B, 87:1–7, 2014.

A. D. Economou and J. B. Green. Modelling from the experimental developmental biologists viewpoint. In Seminars in cell & developmental biology, volume 35, pages 58–65. Elsevier, 2014.

A. D. Economou, N. A. Monk, and J. B. Green. Perturbation analysis of a multi-morphogen turing reaction- diffusion stripe patterning system reveals key regulatory interactions. Development, 147(20):dev190553, 2020.

E. A. Gaffney and N. Monk. Gene expression time delays and turing pattern formation systems. Bulletin of mathematical biology, 68:99–130, 2006.

V. Klika, R. E. Baker, D. Headon, and E. A. Gaffney. The influence of receptor-mediated interactions on reaction-diffusion mechanisms of cellular self-organisation. Bulletin of mathematical biology, 74:935–957, 2012.

K. Korvasov´a, E. Gaffney, P. Maini, M. Ferreira, and V. Klika. Investigating the turing conditions for diffusion- driven instability in the presence of a binding immobile substrate. Journal of theoretical biology, 367:286–295, 2015.

L. Krause, E. A. Gaffney, T. J. Jewell, V. Klika, and B. J. Walker. Turing instabilities are not enough to ensure pattern formation. Bulletin of Mathematical Biology, 86(2):21, 2024.

Krauskopf, H. M. Osinga, and J. Gala´n-Vioque. Numerical continuation methods for dynamical systems, volume 2. Springer, 2007.

A. Sargood, E. A. Gaffney, and A. L. Krause. Fixed and distributed gene expression time delays in reaction– diffusion systems. Bulletin of Mathematical Biology, 84(9):98, 2022.

R. A. Satnoianu, M. Menzinger, and P. K. Maini. Turing instabilities in general systems. Journal of mathe- matical biology, 41(6):493–512, 2000.

R. Seydel. Practical bifurcation and stability analysis, volume 5. Springer Science & Business Media, 2009.

J. Sharpe. Wolpert's french flag: what's the problem? Development, 146(24):dev185967, 2019.

H. Uecker, D. Wetzel, and J. D. Rademacher. pde2path-a matlab package for continuation and bifurcation in 2d elliptic systems. Numerical Mathematics: Theory, Methods and Applications, 7(1):58–106, 2014.

F. Veerman, M. Mercker, and A. Marciniak-Czochra. Beyond turing: far-from-equilibrium patterns and mechano-chemical feedback. Philosophical Transactions of the Royal Society A, 379(2213):20200278, 2021.

Villar-Sepu´lveda and A. R. Champneys. General conditions for turing and wave instabilities in reaction- diffusion systems. Journal of Mathematical Biology, 86(3):39, 2023.

R. Waters, C. A. Yates, and J. H. Dawes. Minimal reaction schemes for pattern formation. Journal of the Royal Society Interface, 21(211):20230490, 2024.

T. E. Woolley, A. L. Krause, and E. A. Gaffney. Bespoke turing systems. Bulletin of Mathematical Biology, 83(5):1–32, 2021.

References from Authors

Cheng, X., and Ferrell Jr, J.E. (2019). Spontaneous emergence of cell-like organization in Xenopus egg extracts. Science *366*, 631-637.

Cross, M.C., and Hohenberg, P.C. (1993). Pattern formation outside of equilibrium. Reviews of Modern Physics *65*, 851-1112. 10.1103/RevModPhys.65.851.

Diego, X., Marcon, L., Müller, P., and Sharpe, J. (2018). Key features of Turing systems are determined purely by network topology. Physical Review X *8*, 021071.

Jensen, O., Pannbacker, V.O., Dewel, G., and Borckmans, P. (1993). Subcritical transitions to Turing structures. Physics Letters A *179*, 91-96.

Lengyel, I., and Epstein, I.R. (1992). A chemical approach to designing Turing patterns in reaction-diffusion systems. Proc Natl Acad Sci U S A *89*, 3977-3979. 10.1073/pnas.89.9.3977.

Li, X., Jiang, W., and Shi, J. (2013). Hopf bifurcation and Turing instability in the reaction–diffusion Holling–Tanner predator–prey model. The IMA Journal of Applied Mathematics *78*, 287-306.

Mu, Y., and Lo, W.-C. (2023). Hopf and Turing bifurcation for a competition and cooperation system with spatial diffusion effect. Journal of Computational and Applied Mathematics *422*, 114924.

Obatake, N., Shiu, A., Tang, X., and Torres, A. (2019). Oscillations and bistability in a model of ERK regulation. J. Math. Biol. *79*, 1515-1549.

Scholes, N.S., Schnoerr, D., Isalan, M., and Stumpf, M.P.H. (2019). A comprehensive network atlas reveals that turing patterns are common but not robust. Cell systems *9*, 243-257.

Tyson, J.J. (2002). Biochemical oscillations. In Computational cell biology, (Springer), pp. 230-260.

Tyson, J.J., Chen, K.C., and Novak, B. (2003). Sniffers, buzzers, toggles and blinkers: dynamics of regulatory and signaling pathways in the cell. Curr. Opin. Cell Biol. *15*, 221-231.

Waters, F.R., Yates, C.A., and Dawes, J.H.P. (2024). Minimal reaction schemes for pattern formation. Journal of the Royal Society Interface *21*, 20230490.

Xu, C., Tyson, J.J., and Cao, Y. (2023). Turing-pattern model of scaffolding proteins that establish spatial asymmetry during the cell cycle of Caulobacter crescentus. Iscience *26*.

Zhao, Q., Liu, S., and Niu, X. (2021). Turing instability and Hopf bifurcation for a diffusion-plankton system with cell size. International Journal of Computer Mathematics *98*, 480-501.

Reviewer #2 (Remarks to the Author)

This study screened through a comprehensive set of simple biochemical networks without any explicit feedback for those that can generate Turing pattern. The work identified how network topology, reaction rate constants and diffusion coefficients affect the possibility of generating Turing pattern. Because the suggested pattern-enabling networks only contain complex formation and constitutive synthesis/degradation, thousands of protein and RNA complexes could match the network topology and hence could be candidates of Turing pattern generators. This study brings new and exciting insights into a classical topic in mathematical biology. Pattern formation is prevalent in development and other crucial biological processes. The widely accepted activator-inhibitor and substrate-depletion models for Turing pattern formation, although insightful, cannot easily find a matching example in reality. This study potential bridges this gap. The topic is of broad interest to the audience in biology, biophysics and bioengineering.

Author response: We thank the Reviewer for the positive evaluation of our manuscript in terms of significance and for the constructive suggestions. In this revision, we have taken all the Reviewer's suggestions to revise the manuscript. Two new figures were added to the supplementary materials to illustrate the new analyses that we performed to address the Reviewer's comment.

Major comments:

1. In the discussion about the required network topology, the authors stated: "Each of the 10 pattern-enabling reaction networks has at least two molecules whose associated parameter values (synthesis and degradation rate constants) can differ between the two." How different should their parameter values be? Btw, this sentence is a bit confusing upon first glance. Suggested edit: "Each of the 10 pattern-enabling reaction networks contain at least two different molecules (Figure 2b, black and white circles). Note that in the model, different molecules specifically refer to molecules with different parameters."

Author response: We agree with the Reviewer that the differences in parameters between molecules needed to be better clarified and illustrated. The description of parameter ranges was not very clear, especially in the main text, so it caused considerable confusion to reviewers. During our computational screening, we sampled most parameters from log-uniform distributions with estimated, biologically plausible ranges covering two orders of magnitude (Table S1). As an example, we use the figure below (Figure R2) to illustrate the distributions of degradation rate constants for molecules A and C in two pattern-enabling models (ABC-1 and ABCD-2). Between these two molecules, the rate constants can differ by up to two orders of magnitude, because of the sampling scheme (Figure R2, gray). In each of the pattern-enabling parameter sets, the two parameters can still differ significantly: many of them differ by more than an order of magnitude (Figure R2, blue). The selection of these two models is significant in the sense that molecules A and C are symmetrical in the network: they can bind to molecule B independently. There are two special cases of the two models respectively: AAB-1 and AAAB-4, in which A and C can be viewed as the same molecules with identical parameters. Interestingly, both the kinetically symmetrical versions AAB-1 and AAAB-4 and the asymmetrical versions ABC-1 and ABCD-2 are pattern-enabling.

To address the reviewer's comment, we took the suggestion to revise the text at the beginning of **the second paragraph of the result section 'Network topology required for Turing pattern-enabling reactions'**. We also added brief descriptions of the parameter sampling ranges to the text introducing Figure 2C and Methods. In addition, we added **a new Figure S9** (same as Figure R3 shown here) to the Supplementary Information.

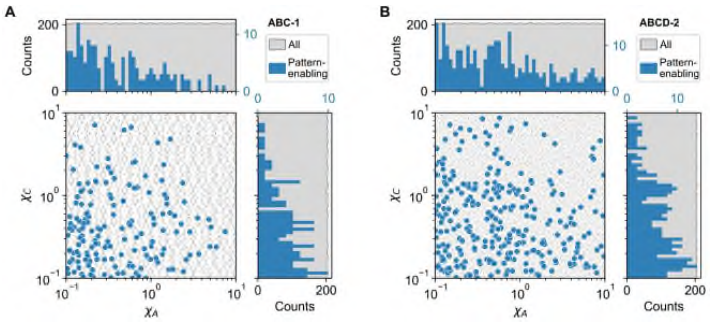

Figure R3. An illustration of sampled parameters for two models. Degradation rate constants for the free form of molecule A and molecule C in ABC-1 model (**A**) and ABCD-2 model (**B**) were sampled randomly and independently (gray dots in scatter plots and gray histograms) from log-uniform distributions spanning two orders of magnitude. The same parameters in those pattern-enabling sets are shown in blue. Although low degradation rates favor pattern formation, the values of each parameter can still differ significantly.

2. What is the unit of the diffusion coefficients shown in Fig. 5? An important reason why the classical activator-inhibitor and substrate-depletion models cannot easily find examples in reality is the required large difference between the diffusion coefficients of the two components of the system. Do pattern-enabling networks in this work have as stringent requirement on the diffusion coefficients?

Author response: The Reviewer has brought an important point that we needed to clarify further. One unit of dimensionless diffusion coefficients in Figure 5 corresponds to $\sim 4.6\ \mu m^2 s^{-1}$ physical units with a representative length scale in our models. We have now included this unit in the **legend of Figure 5.** A detailed description regarding the unit of diffusion coefficient in addition to the unit of space-time is also added in **Sec.B.1.2 of the supplementary text.**

The requirement of differential diffusion rates is a glaring obstruction in the way of attaining synthetic Turing patterns in biological systems. We have sampled the diffusion coefficient in the range of 1-20 nondimensional units, e.g. in the physical range of ~4.6 to ~92 $\mu m^2 s^{-1}$ in the tissue-level scale scenario. This corresponds to the diffusion coefficient of the macromolecules (mRNA, miRNA, Proteins). Hence, our cautious choice of sampling range of diffusion coefficients ensures that the values do not spill the biologically acceptable barrier and reinforce the experimental viability of our model. We have included the explanation in **Sec.B.1.2 of the supplementary text.**

To address the Reviewer's comment on the comparison of our models and conventional models in terms of the requirement of the diffusion coefficients, we also conducted a detailed study to investigate the condition of differential diffusion rates for our system and included the result in the supplementary data with **a new figure (Figure S10).** The result reveals the possibility of Turing pattern formation for certain networks under constraints on differential diffusion rates that are less stringent compared to conventional models (moderate standard deviations and the diffusion coefficients are within an order of magnitude). It should be noted that some studies on classical systems for Turing patterns formation allowed an immobile molecule (0 diffusion rate) and other diffusive molecules with the equal diffusion rate (e.g. Marcon et al. 2016). We think that our models still have less stringent requirements compared to these studies for 'equally diffusing signals'. We also added a description of this point in **the last paragraph of 'Kinetic rate constant requirement for Turing pattern-enabling reactions.**

3. In the omics-study, is there any possibility to further screen for the rate constants and diffusion coefficients even if they are just estimated numbers? There can't be thousands of Turing pattern generators.

Author response: We agree with the Reviewer that the screen for network complex configuration alone cannot provide sufficient constraints on Turing pattern-enabling reaction networks in the most rigorous manner. Unfortunately, due to the limitations of experimental techniques,

we are not able to estimate the rate constants and diffusion coefficients to further constrain the preliminary list of networks. It should be noted that, to our best knowledge, such estimates on rate constants were not available in most classically defined activator-inhibitor systems for Turing pattern formation.

We also agree with Reviewer that there can't be thousands of systems for generating Turing patterns. We think that the primary value of our work including the omics-level prediction is to demonstrate the breath of network topologies and their associated gene products that potentially enable Turing patterns, such that future experimentalists should focus on measuring rate constants rather than seek for matching network structures. This shift of attention will help to fill the gap mentioned in the paragraph above. Nonetheless, our high-throughput screen can still provide a meaningful list of candidate proteins and RNAs for Turing pattern formation, and our gene ontology analysis provided some evidence to this.

To address the Reviewer's comment, we have now **described the limitations** and the **specific significance** of our omics-level prediction to the **last Results section**.

4. The Routh-Hurwitz analysis should be cited and described (could be in the Supplementary text).

Author response: We agree with the Reviewer. We have now mentioned and cited the Routh-Hurwitz Criterion at its first appearance in the main figure (Figure 2c) (the third paragraph of Results section). We also cited the original papers in Methods. In addition, a textbox (in **Sec.A.2**) containing the **new citations** and **new description** of Routh-Hurwitz analysis for a 3-component system is included in the latest version of the supplementary text.

Minor comments:

1. Typo: "It should be noted that the networks in our study are hypergraphs (each edge that represents binding involves 3 nodes) rather regular graphs commonly used for defining feed-forward loops". "rather" --> "rather than".

Author response: Thank you for catching the typo. We have now corrected it and marked it with red font.

2. "In Set II (Figure 5c, left), Subunit A serves as a diffusion facilitator". The diffusion coefficient of A is smaller than that of B and AB. So, B sounds more like the diffusion facilitator in this case?

Author response: Thank you for catching the error. We have now corrected it and marked it with red font.

3. Typo: "our analysis showed that the choice of parameter values, particularly ratios the degradation rate constants of protein/RNA subunits in various complexes, can be crucial for pattern formation." Missing "between" after "ratios".

Author response: Thank you for catching the error. We have now corrected it and marked it with red font.

4. Typo: "In addition to the direct interpretation of molecular degradation, the first-order decay rates of the molecules in our models are be generalized to rates of removal from a specific location (e.g. removal of molecules from plasma membrane via endocytosis) where patterns can arise." "are be generalized" --> "can be generalized".

Author response: Thank you for catching the typo. We have now corrected it and marked it with red font.

5. Typo: “Together we other four models, we analytically proved that Eq 1 cannot have any unstable steady state with arbitrary positive rate constants,” “Together we” --> “Together with”

Author response: Thank you for catching the typo. We have now corrected it and marked it with red font.

References for Response to Reviewer 2

Marcon, L., Diego, X., Sharpe, J., & Müller, P. (2016). High-throughput mathematical analysis identifies Turing networks for patterning with equally diffusing signals. *Elife*, 5, e14022.

Reviewer #1 (Remarks to the Author):

The authbors have done an excellent job of addressing my comments, and vastly improving their manuscript. I congratulate them on a nice piece of work.

I have one outstanding comment that could be worth tweaking a sentence or two to address, though I do not think this comment has a significant impact on the manuscript, which I strongly recommend for publication in any case.

The remaining comment relates to the very detailed response regarding multistability of the reaction networks. The authors suggest (in the section "ODE parameter sampling and numerical bifurcation analysis") that when sigma_B=0 (or sigma_A=0), there is a single unique stable equilibrium. Is this always the case? Is there a simple mathematical reason why we shouldn't expect the base parameters to admit more than one stable equilibria? Apologies if this is obvious, but it could be worth (briefly) justifying why there should only be a monostable equilibrium in this case. In SI section B.3 there is a discussion of using an equilibrium near a Hopf threshold to prevent multistability, but again one has to a priori start with a system that only has a monostable equilibrium I think to ensure this. It also does not preclude other "far away" bifurcations from generating stable equilibria (e.g. saddle-node bifurcations which do not involve the original stable equilibrium).

I appreciate that the high-throughput analysis really focuses on the local picture, studying only bifurcations of equilibria, but there are many systems where other stable equilibria exist and can spoil the potential for pattern formation in complicated ways depending on subtle details of the nonlinearities and parameters (e.g. see comment 6 in my first set of comments, and the cited work there). The direct numerical simulations of the PDE are a good validation that the parameters found do not exhibit these pathologies, but it could help to show that there are no far-away stable equilibria in general (though this may not be possible away from sigma=0). Despite the verbosity, this is really a minor comment!

Reviewer #1 (Remarks on code availability):

The authors have done an excellent job making their methods clearer, and their high-throughput pipeline accessible. Very well done!

Reviewer #2 (Remarks to the Author):

The authors have addressed all my comments.

Reviewer #2 (Remarks on code availability):

I did not install and run the code. But the instructions look very clear.

Black text: Reviewers' comments.

Blue text: Authors' responses (**Bold**: highlights of edits made to manuscript, where all edits are marked red in both main text and supplementary text).

Reviewer #1 (Remarks to the Author)

The authors have done an excellent job of addressing my comments, and vastly improving their manuscript. I congratulate them on a nice piece of work.

I have one outstanding comment that could be worth tweaking a sentence or two to address, though I do not think this comment has a significant impact on the manuscript, which I strongly recommend for publication in any case.

The remaining comment relates to the very detailed response regarding multistability of the reaction networks. The authors suggest (in the section "ODE parameter sampling and numerical bifurcation analysis") that when sigma_B=0 (or sigma_A=0), there is a single unique stable equilibrium. Is this always the case? Is there a simple mathematical reason why we shouldn't expect the base parameters to admit more than one stable equilibria? Apologies if this is obvious, but it could be worth (briefly) justifying why there should only be a monostable equilibrium in this case. In SI section B.3 there is a discussion of using an equilibrium near a Hopf threshold to prevent multistability, but again one has to a priori start with a system that only has a monostable equilibrium I think to ensure this. It also does not preclude other "far away" bifurcations from generating stable equilibria (e.g. saddle-node bifurcations which do not involve the original stable equilibrium).

I appreciate that the high-throughput analysis really focuses on the local picture, studying only bifurcations of equilibria, but there are many systems where other stable equilibria exist and can spoil the potential for pattern formation in complicated ways depending on subtle details of the nonlinearities and parameters (e.g. see comment 6 in my first set of comments, and the cited work there). The direct numerical simulations of the PDE are a good validation that the parameters found do not exhibit these pathologies, but it could help to show that there are no far-away stable equilibria in general (though this may not be possible away from sigma=0). Despite the verbosity, this is really a minor comment!

Author response: We thank the Reviewer again for the insightful review. Our revised manuscript benefited significantly from the constructive suggestions.

Regarding the current comment, we have now added justifications of a monostable system at $\sigma_B = 0$ (or $\sigma_A = 0$ for single subunit system without B) for each of the reaction network subject to numerical continuation (i.e. 18 reaction networks without triangles shown in Figure 2b). Overall, the monostability of these systems can be mathematically proved with change of variable and considering subsystems amenable for analytical approaches. Here we describe the main idea of the proofs. Among these 18 networks, 17 involve binding of subunit B to another subunit (only AAAA-2 does not have B). We consider a new variable $B_{\mathrm{Total}} \equiv \sum_1^{n_B} C_{i,B}$, where $C_{i,B}$ represents the concentration of each B-containing complex (including its free form) and n_B is the number of B-containing complexes, for each system. The dynamics of B_{Total} is governed by $dB_{\mathrm{Total}} = \sigma_B - \sum_1^{n_B} k_{i,B} C_{i,B}$, which is an open system with a single 'source' and multiple 'sinks'. Because the degradation rate constants ($k_{i,B}$) are positive for B in each complex (i.e. B always has 'sinks') and the concentrations are nonnegative, the condition $\sigma_B = 0$ will make all B-bound species and free B relaxed to zero at steady state (i.e. the only equilibrium point). Therefore, the only possibility for multistability must arise from species not bound by B. For AAAB-2 network, the subsystem without B has a trimerization network of A (i.e. the AAA-1 network), which was proven to be monostable with arbitrary positive rate constants using the Routh-Hurwitz criterion (the uniqueness of positive steady state can also be ensured by the chemical reaction network theory). For the same reason, AAAB-1, AAAB-3, AABB-1, AABB-3, AABC-1 and AABC-2 involve only dimerization of A in the absence of B, and this subnetwork (AA-1) was stable with arbitrary positive rate constants. ABCD-1 involves dimerization of C and D in the absence of B, and this subnetwork was also stable (equivalent to AB-1) with the proof using the Routh-Hurwitz criterion. For all the above 8 networks still containing di- or tri-merization and all other 9 networks free of dimerization without B at $\sigma_B = 0$, each of the isolated subsystems

containing only free subunits can only have a single steady state because they have the form $dx/dt = p - qx$, which is monostable. For AAAA-2, the system has only sinks but no source when $\sigma_A = 0$ as it has the form $dA_{\mathrm{Total}}/dt = -\sum_1^{n_A} k_{i,A} C_{i,A}$ (by changing of variables), which is monostable and each species must equal zero at the equilibrium point.

We have now added this justification paragraph to **B.3 of Supplementary Information**, and we mentioned it briefly in **Methods**, where we have also cited the suggested reference on the disruption of pattern formation by far-away equilibria.

Reviewer #1 (Remarks on code availability):

The authors have done an excellent job making their methods clearer, and their high-throughput pipeline accessible. Very well done!

Reviewer #2 (Remarks to the Author):

The authors have addressed all my comments.

Reviewer #2 (Remarks on code availability):

I did not install and run the code. But the instructions look very clear.

We thank both Reviewers for their positive evaluations of our manuscript in the second round of revision.