

Toward Single-Cell Control: Noise-Robust Perfect Adaptation in Biomolecular Systems

Corresponding Author: Professor Jae Kyoung Kim

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

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

In recent years, the Antithetic Integral Feedback (AIF) motif has emerged as a fundamental biomolecular controller for enabling Robust Perfect Adaptation (RPA) in arbitrary intracellular networks—even under inherently stochastic reaction dynamics. RPA ensures that the steady-state output of the controlled network is maintained at the population-average level, despite disturbances that may alter system parameters.

AIF has been successfully implemented in living cells and extensively studied theoretically. However, a well-known limitation is its tendency to increase intrinsic noise, which can be detrimental to cellular function and poses challenges for synthetic biology applications. Previous efforts to address this issue have primarily focused on augmenting the AIF to suppress noise.

In contrast, this paper presents a novel approach. Rather than merely attempting to reduce fluctuations, it introduces a Noise Controller (NC)—an additional motif that complements AIF by regulating both the mean and variance of the system's output. By employing a dimerisation-based sensor, the NC enables targeted control of noise levels, subject to the constraint that the variance must exceed the mean. The utility of this framework is demonstrated through an *in silico* study of an *E. coli* DNA repair system model, highlighting the potential of the NC-AIF architecture for achieving more reliable cellular control.

The paper addresses an important problem in the design of intracellular controllers, one that is likely to be of broad interest to the synthetic biology community. However, I have several concerns regarding the design of the Noise Controller and its practical applicability. I would suggest the authors undertake a major revision to address the following issues and make it more suitable for Nature Communications.

1. Case for controlling the variance: The paper does not make a compelling case for the necessity of controlling variance. While it is evident that large variance can be detrimental to cellular function, it is not clear why the objective should be to regulate variance to a specific set-point, rather than to minimise it as much as possible. Although this paper reports that the Fano factor (variance divided by the mean) cannot be reduced below 1, other studies have proposed modifications to the antithetic controller that achieve Fano factors significantly less than 1 (see Ref. 14, Ref. 15 in the manuscript or ref [1] mentioned below). What distinguishes this paper, however, is that the steady-state variance is regulated to a precise, controller-determined value, whereas in the other studies, the variance depends on the characteristics of the open-loop network being controlled. To strengthen the case for this approach to noise regulation, the authors should include numerical case studies where existing enhanced versions of the antithetic controller perform poorly—i.e., where the Fano factor exceeds 1—following disturbances such as parameter perturbations, while their proposed design maintains the Fano factor at or near 1.

2. Checking Ergodicity: Since the paper lacks any experimental results, it must, in my view, strengthen its theoretical contributions to be more suitable for Nature Communications. A clear way to do this would be to prove that adding the noise controller to the antithetic controller does not compromise ergodicity. This is not trivial, as the system includes two integrators—one from the original antithetic controller and another from the noise controller—that may compete with each other. It is well known that the presence of multiple integrators can easily undermine system stability. While I fully acknowledge that verifying ergodicity in stochastic reaction networks is a highly challenging problem, any results the authors can provide,

even for a subclass of open-loop networks, would significantly strengthen the paper's theoretical foundation.

3. Issues with Ergodicity: The authors state that the proposed motif functions work as intended provided the combined dynamics are ergodic with finite second moments. However, they also note that if the target Fano factor is set below 1, certain controller species (notably Z1 and Z4) accumulate indefinitely over time, indicating a breakdown of ergodicity. Given that RPA systems are designed to reject disturbances that arise from parameter perturbations, it is important to consider scenarios where such perturbations drive the Fano factor set-point below 1. In such cases, the resulting accumulation of controller species could be more harmful to the cell than the very noise the noise controller is designed to suppress. I recommend that the authors explicitly address this issue and discuss potential strategies to mitigate or avoid such pathological behaviour.

4. Checking for Bimodality: While controlling intrinsic noise via output variance is a valid objective, this strategy is meaningful only if the output distribution remains unimodal (e.g., Poisson-like). If the output distribution becomes bimodal, then variance alone no longer offers a reliable measure of single-cell control, as individual cells may still exhibit large deviations from the mean. It would strengthen the paper considerably if the authors could demonstrate, through simulation or theoretical analysis, that the proposed Noise Controller does not induce bimodality in the output distribution, which would undermine its intended purpose.

5. Effect of Sensor Saturation: The derivation of the Fano factor assumes mass-action kinetics for the sensing mechanism. However, when implemented using gene expression components—as suggested in the paper—sensor saturation is a realistic concern. In practical terms, the sensing rate cannot increase indefinitely and is likely to plateau at high copy-numbers of the output species. This saturation could significantly affect the performance of the Noise Controller. I encourage the authors to assess this effect by replacing the mass-action kinetics of the dimer sensing reaction with a saturating Hill function in their computational model. Such an analysis would provide valuable insight into the practical feasibility and robustness of the proposed design.

6. Relation to Existing literature: The classical paper by Lestas et al. [2] derives fundamental limits to noise suppression in biomolecular systems. I was surprised that this influential work was not cited and discussed. It would be highly valuable for the authors to explore whether the hard lower bound on noise identified in this study is related to these known theoretical limits. Including such a discussion would not only strengthen the theoretical grounding of the paper but also provide a compelling justification for why the observed noise floor cannot be reduced further.

Minor Points:

1. On line 296, "sequesterate" should be "sequester"

2. In the results reported in Figure 4, it is not clear how the authors are computing the copy-numbers from the fluorescence units.

References

[1] M. Filo, S. Kumar, and M. Khammash, "A hierarchy of biomolecular proportional-integral-derivative feedback controllers for robust perfect adaptation and dynamic performance," *Nature communications*, vol. 13, no. 1, p. 2119, 2022.

[2] I. Lestas, G. Vinnicombe, and J. Paulsson, "Fundamental limits on the suppression of molecular fluctuations," *Nature*, vol. 467, no. 7312, pp. 174–178, 2010.

Reviewer #2

(Remarks to the Author)

The authors proposed augmenting a noise controller (NC) motif to existing AIF controller motif, which they called mean controller (MC) to achieve not only perfect adaptation on mean level but also at perfect adaptation at noise level. The paper is well-written, I enjoyed reading as it easy to follow and the proposed idea though simple in structure, has been demonstrated through simulation to work well. While reading I made note to ask the authors to clarify my questions but as I progressed, the paper begin to answer majority of my queries, that I began crossing out all those notes that I have made. Well done to the authors.

Here are some of my comments.

- Figures 1a and 1d. There are arrows pointing into Z2 and Z4 but no label/legend of what these arrows should be. Can the author clarify what those arrows are?

- Why k is increased 8 times? Why this specific number 8? What happened if this number is increased to 20 times? Is there a limit on the value of k that ensure noise RPA?

- All parameter values have no unit. For example, η_1 and η_2 in Figure 3 are set to 50. What unit is this 50. Also, isn't 50 very large for a sequestration rate? Please include relevant unit to those parameters. Also, are those parameters taken from literature or arbitrary assigned. If it is the later, are those parameters biologically realistic? If not, why not do the analysis from the start with values that are biologically feasible?

- Fano factor less than 1 showing Z1 and Z4 diverge. Any insight on why these two species diverge?
- In line 225. Shouldn't (Fig. 4c, d Red) be (Fig. 4c, d, Blue)? And similarly in line 227 shouldn't (Fig. 4c, d Blue) be (Fig. 4c, d Red)?
- Figure 5. In the NC structure (Fig. 1d), there is an autoregulation of Z4, but this is not accounted for in Figure 5 (at least it's not obvious to me). Any comment on this?
- Equation (2). If cross referencing to Figure 1a, why is that the degradation involving k_1 is not included in the ODE?
- Equation (4). If cross referencing to Figure 1d, why is the autoregulation of Z4 and the arrow with no variable entering into Z4 is not included in the ODE?
- The chemical reactions shown from lines 453-460. For the second reaction, instead of writing Gamma, why not use the symbol Γ ?
- References. Ensure consistency. There are mixture of using title case, journal not abbreviated, etc. Specifically, Refs. 1, 6, 31 uses title case. Refs. 13 uses uppercase for the journal title, Refs. 20 and 28, journals are not abbreviated. Ref. 25, the alignment is more to the left compared to other references.
- Data availability. I would highly recommend making the code available instead of the statement made in the manuscript for transparency and reproducibility.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors have done a commendable and thorough job in addressing the concerns raised in my previous review, providing substantial additional analysis and insightful discussion. Although certain theoretical aspects I mentioned earlier, such as checks for ergodicity, consistency with existing noise suppression limits etc., have only been partially explored, the manuscript nevertheless offers valuable perspectives and identifies promising directions for future research. Overall, I consider this an important and well-executed paper, and I find it acceptable for publication in Nature Communications in its current form.

Minor comment:

In the analysis of saturation effects presented in Supplementary Text S5, the authors state that as $K \rightarrow \infty$, the propensity $f_{\text{Hill}}(X_2)$ approaches $\theta_2 X_2^2$. This does not appear to be correct, as the limit seems to approach zero unless V_{max} increases proportionally with K^2 to keep θ constant. I recommend that the authors clarify this point.

(Remarks on code availability)

Reviewer #2

(Remarks to the Author)

The authors have clearly and successfully addressed all my queries. I am happy for the manuscript to be accepted in the current form.

(Remarks on code availability)

The provided codes seem complete and contain readme file for user to use and reproduce.

Open Access This Peer Review File is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made.

In cases where reviewers are anonymous, credit should be given to 'Anonymous Referee' and the source.

The images or other third party material in this Peer Review File are included in the article's Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder.

To view a copy of this license, visit <https://creativecommons.org/licenses/by/4.0/>

We thank the reviewers for their thorough and constructive comments. In light of these comments, we have made substantial revisions to our manuscript, resulting in a significant enhancement of its readability and impact.

Below, you will find a point-by-point response to each comment. Our responses are indicated in blue, while the revised portions of the manuscript are highlighted in green (with italic font indicating the modified section).

Reviewer #1:

Remarks to the Author:

In recent years, the Antithetic Integral Feedback (AIF) motif has emerged as a fundamental biomolecular controller for enabling Robust Perfect Adaptation (RPA) in arbitrary intracellular networks—even under inherently stochastic reaction dynamics. RPA ensures that the steady-state output of the controlled network is maintained at the population-average level, despite disturbances that may alter system parameters.

AIF has been successfully implemented in living cells and extensively studied theoretically. However, a well-known limitation is its tendency to increase intrinsic noise, which can be detrimental to cellular function and poses challenges for synthetic biology applications. Previous efforts to address this issue have primarily focused on augmenting the AIF to suppress noise.

In contrast, this paper presents a novel approach. Rather than merely attempting to reduce fluctuations, it introduces a Noise Controller (NC)—an additional motif that complements AIF by regulating both the mean and variance of the system's output. By employing a dimerisation-based sensor, the NC enables targeted control of noise levels, subject to the constraint that the variance must exceed the mean. The utility of this framework is demonstrated through an *in silico* study of an *E. coli* DNA repair system model, highlighting the potential of the NC-AIF architecture for achieving more reliable cellular control.

The paper addresses an important problem in the design of intracellular controllers, one that is likely to be of broad interest to the synthetic biology community. However, I have several concerns regarding the design of the Noise Controller and its practical applicability. I would suggest the authors undertake a major revision to address the following issues and make it more suitable for Nature Communications.

Major:

1. Case for controlling the variance: The paper does not make a compelling case for the necessity of controlling variance. While it is evident that large variance can be detrimental to cellular function, it is not clear why the objective should be to regulate variance to a specific set-point, rather than to minimise it as much as possible. Although this paper reports that the Fano factor (variance divided by the mean) cannot be reduced below 1, other studies have proposed modifications to the antithetic controller that achieve Fano factors significantly less than 1 (see Ref. 14, Ref. 15 in the manuscript or ref [1] mentioned below). What distinguishes this paper,

however, is that the steady-state variance is regulated to a precise, controller-determined value, whereas in the other studies, the variance depends on the characteristics of the open-loop network being controlled. To strengthen the case for this approach to noise regulation, the authors should include numerical case studies where existing enhanced versions of the antithetic controller perform poorly—i.e., where the Fano factor exceeds 1—following disturbances such as parameter perturbations, while their proposed design maintains the Fano factor at or near 1.

We thank the reviewer for the valuable comment. We thank the reviewer for this valuable comment. As the reviewer correctly noted, several previous studies achieved a Fano factor below 1 by modifying Antithetic Integral Feedback (AIF) motifs. However, these enhanced versions of AIF do not guarantee noise RPA, leading to, after perturbation, an increased Fano factor or the accumulation of controller species.

To demonstrate this situation, we investigated how the Fano factor of an original network combined with other enhanced versions of AIF motifs—an aPI controller (Filo et al.; ref. 16 in the revised manuscript), AIF with negative action (Briat et al.; ref. 14 in the revised manuscript), and a sensor-actuated AIF controller (Kell et al.; ref. 15 in the revised manuscript)—changes after perturbations (Text. S4 and Fig. S6). The results show that the aPI controller suggested by Filo et al. (Fig. S6a), AIF controller with negative action from Briat et al. (Fig. S6c), and the sensor-actuated AIF controller proposed by Kell et al. (Fig. S6e) could not maintain a low noise level. After the parameter perturbation, the Fano factor rapidly increased above 1 (Fig. S6b, d, f). In contrast, our NC maintained the Fano factor close to 1 even after perturbation (Fig. S6b, d, f).

These results underscore a key advantage of our NC: it regulates noise to a precise, controller-determined set point that is robust to disturbances, unlike previous controllers whose noise properties depend on the open-loop network. We have incorporated these points into the revised manuscript.

Discussion:

...

Similar to the NC, *several previous studies have proposed AIF-based controllers for noise reduction*¹⁴⁻¹⁶ (Text S4). For example, output noise could be reduced by combining the MC and the negative feedback loop, where output species repress the production of input species¹⁴. Employing a similar negative feedback loop as an alternative to the MC's actuation achieved noise reduction below the original level^{15,16}. *Some of these achieved a Fano factor below 1, but after parameter perturbations, they typically failed to maintain low noise levels* (Text S4, Fig. S6). To resolve this limitation, we developed the NC, which senses the output levels via the dimerization of output species (Fig. 1d). This approach ensures the control of the second moment of the output (See Methods for more details), which leads to the noise RPA (Fig. S6b, d, f). ...

Supplementary Information:

Text S4. Comparison of NC with other enhanced versions of AIF controller.

In previous studies, modified AIF controllers were reported to achieve a Fano factor below 1 [3, 4, 5]. However, the performance of those controllers depend on reaction parameters, and thus they could fail to maintain noise RPA after parameter perturbations.

To compare this point with the performance of NC, we checked the performance of NC with three types of modified AIF controller (Fig. S6a, b, e), which achieved a Fano factor below 1. We perturbed one of the reaction parameters and checked whether the noise level and ergodicity was maintained or not. The results show that two AIF motifs suggested by [5] (Fig. S6a) and [3] (Fig. S6c) could not maintain the low noise level and increased the Fano factor to greater than 1 after perturbation (Fig. S6b, d). On the other hand, our NC maintains the same noise level after perturbation (Fig. S6b, d). When comparing the sensor-actuated controller [4] with NC, since NC does not contain a reaction identical to the actuation reaction in sensor-actuated controller (nu , Fig. S6e), we instead perturbed the reaction parameter of NC's actuation reaction ($X_1 + Z_4 \rightarrow Z_4$), which exerts a similar negative regulation. The results show that the sensor-actuated AIF failed to maintain a Fano factor below 1 after parameter perturbation, whereas NC continued to maintain a Fano factor close to 1 (Fig. S6f).

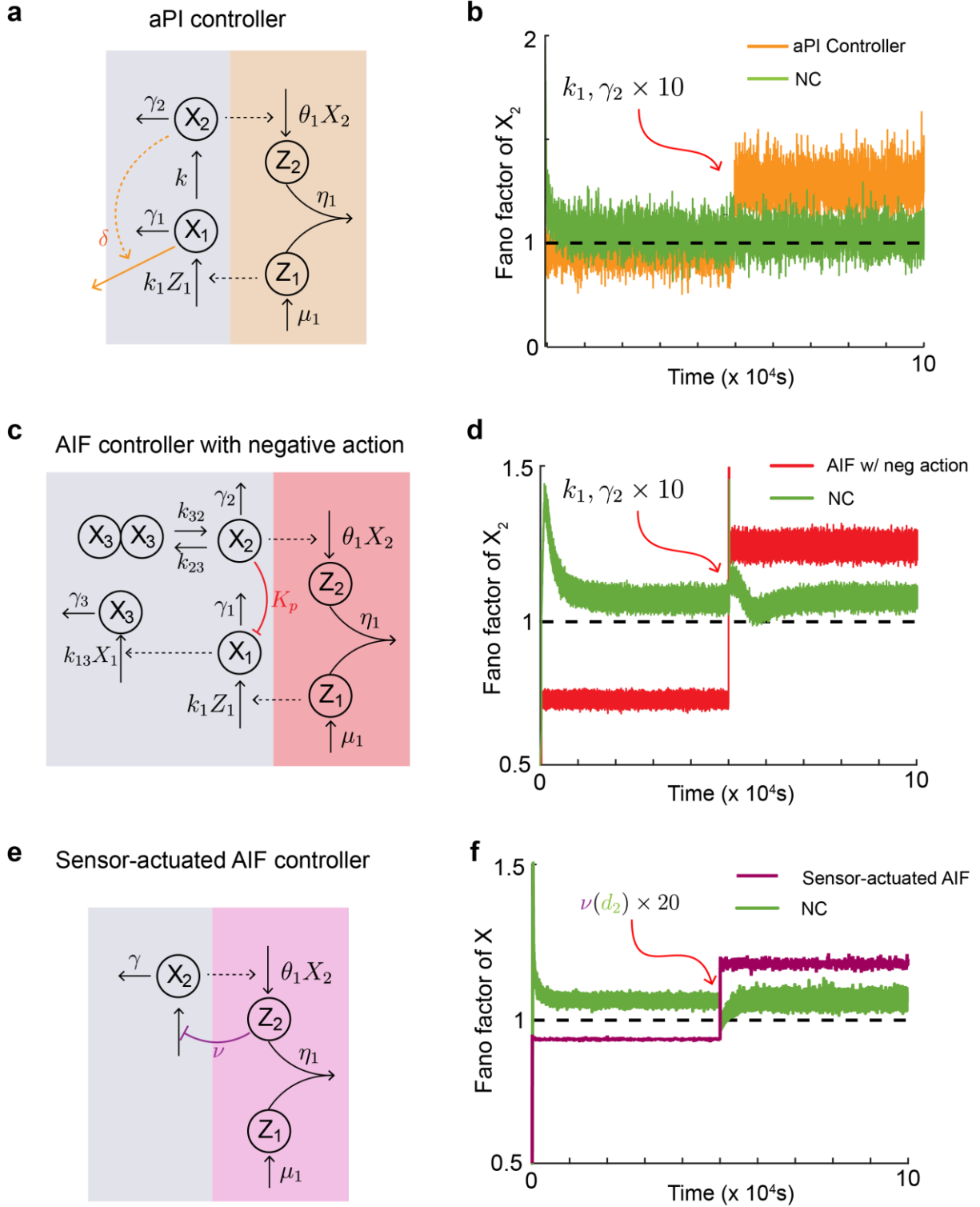


Fig. S6 Modified version of AIF controller does not have a noise RPA. (a) aPI Class 1 degradation-inhibition controller (aPI controller, orange box, [5]) attached to the sample original network (gray box). The additional negative feedback loop (delta) is also a part of aPI controller. (b) The Fano factor of 500 trajectories of X_2 in (a) and original network of (a) combined with MC and NC simulated with parameters $\mu_1 = 10, \theta_1 = 2, k = 3, \gamma_1 = 2, \gamma_2 = 7, \delta = 150, k_1 = 2, \eta_1 = 1000$, and k_1, γ_2 both increased 10-fold at time = 50,000s. The aPI controller (orange) could achieve a Fano factor of X_2 below 1. However, after the perturbation of k_1 ($t = 2,500$), the Fano factor increases rapidly. On the other hand, NC (green) maintains the Fano factor of X_2 close to 1, even after the parameter perturbation ($t = 50,000$). (c) AIF controller with negative action (red box, [3])

attached to the sample original network (gray box). The additional negative feedback loop (K_p) is also a part of the controller. (d) The Fano factor of 10,000 trajectories of X_2 in (c) and original network of (c) combined with MC and NC simulated with parameters $\mu_1 = 10, \theta_1 = 2, \gamma_1 = 2, \gamma_2 = 1, \gamma_3 = 1, K_p = 5, k_{13} = 1, k_{32} = 3, k_{23} = 1$, and k_1, γ_2 both increased 10-fold at time = 50,000s. Similar to (b), the AIF controller with negative action (red) could not maintain the Fano factor of X_2 after parameter perturbation ($t = 50,000$), while NC (green) maintains the Fano factor of X_2 after the perturbation. (e) Sensor-actuated AIF controller (purple box, [4]) attached to the sample original network (gray box). The negative feedback reaction (v) is also a part of the controller. (f) The Fano factor of 50,000 trajectories of X_2 in (e) and original network of (e) combined with MC and NC simulated with parameters $\mu_1 = 1.574, \theta_1 = 13.967, \eta_1 = 0.132, \gamma = 1, v = 0.125$, and v increased 20-fold at time = 50,000s. Since the reaction corresponding to ν does not exist in MC and NC, we instead increased the parameter of NC's actuation reaction (d_2), which exerts a similar negative regulation. The sensor-actuated controller (purple) could not maintain the Fano factor of X_2 after parameter perturbation ($t = 50,000$), while NC (green) maintains the Fano factor of X_2 after the perturbation.

2. **Checking Ergodicity:** Since the paper lacks any experimental results, it must, in my view, strengthen its theoretical contributions to be more suitable for Nature Communications. A clear way to do this would be to prove that adding the noise controller to the antithetic controller does not compromise ergodicity. This is not trivial, as the system includes two integrators—one from the original antithetic controller and another from the noise controller—that may compete with each other. It is well known that the presence of multiple integrators can easily undermine system stability. While I fully acknowledge that verifying ergodicity in stochastic reaction networks is a highly challenging problem, any results the authors can provide, even for a subclass of open-loop networks, would significantly strengthen the paper's theoretical foundation.

We thank the reviewer for this valuable comment. In this study, we observed the transience of some controller species when the target Fano factor was below 1 (Fig. 2c,d, Fig. 3), whereas ergodicity of the system was found when the target Fano factor was above 1 and less than the Fano factor of the original network with MC (Fig. 2c d, Fig. 3). While some previous studies introduced stochastic reaction networks admitting a Fano factor smaller than 1 {ref 31, 32 in manuscript}, we believe it is really interesting to theoretically verify that a system successfully controlled by MC+NC will not allow the Fano factor to go below 1. To provide a theoretical justification for this, we first established the transience of the network in a subregion of the parameter space where the target Fano factor is less than 1. Specifically, we proved the following theorem (see Text S3 for details):

Theorem. For any given open-loop network, if the parameters of MC and NC satisfy $\frac{\mu_1}{\theta_1} > \frac{1}{2}$ and $\frac{\mu_1}{\theta_1} + \frac{\mu_2}{\theta_2} - \left(\frac{\mu_1}{\theta_1}\right)^2 < \min\left(\frac{\mu_1}{\theta_1} - \left\lfloor \frac{\mu_1}{\theta_1} \right\rfloor, \left\lceil \frac{\mu_1}{\theta_1} \right\rceil - \frac{\mu_1}{\theta_1}\right)^2$, then the continuous-time Markov chain associated with the open-loop network combined with MC and NC is transient.

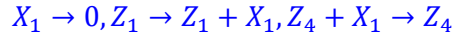
Note that under the conditions of the theorem, the target Fano factor of X_2 has to be less than 1 under the stationary distribution if it exists.

We then attempted to extend this analysis to the ergodicity when the parameters are set to make a Fano factor above 1. A typical approach for this is to construct a Lyapunov function V . That is, V should be norm-like and satisfy the drift condition: $\mathcal{A}V(\mathbf{x}) \leq -C$ for all but finitely many $\mathbf{x} \in \mathbb{Z}_{\geq 0}^n$ for some positive constant $C > 0$, where $\mathcal{A}V(\mathbf{x})$ is the infinitesimal generator of the associated continuous-

time Markov chain (Meyn, Sean P., and Richard L. Tweedie. "Stability of Markovian processes III: Foster–Lyapunov criteria for continuous-time processes." *Advances in Applied Probability* 25.3 (1993): 518-548; Hairer, Martin. "Convergence of Markov processes." *Lecture notes* 18.26 (2010): 11).

First, we considered the piecewise-linear Lyapunov function, a general approach for proving ergodicity in high-dimensional reaction networks also used in Briat et al. (ref. 6 in the revised manuscript). In Briat et al., the piecewise-linear function V was constructed by dividing the state-space according to the comparison between Z_1 and a positive linear combination of X_1 and X_2 , and each region took the form of a convex polyhedral cone. This construction made the boundaries of each region linear, allowing the coefficients of V for each region to be adjusted to ensure that the drift condition holds on the boundaries.

However, such a construction of a piecewise linear Lyapunov function is challenging for the NC. For example, in the example described in Fig. 1 of this manuscript, three reactions change the number of X_1 :



In the region where $Z_1 \rightarrow Z_1 + X_1$ dominates, we have to construct V as it decreases in X_1 to satisfy the drift condition. Conversely, in the region where $Z_4 + X_1 \rightarrow Z_4$ dominates, we have to construct V as it increases in X_1 . If V is piecewisely defined in the two regions with linear functions, then they should be compatible with each other along the boundary between the regions to satisfy the drift condition. Therefore, the boundary separating the two regions needs to be linear. However, due to the term $Z_4 X_1$, the boundary between the two regions presumably should be nonlinear. Hence, we concluded that a non-linear Lyapunov function needs to be constructed, which means that the approach shown in Briat et al. is no longer suitable for our case. One possible alternative is dividing the regions into smaller subregions $Z_4 > n$ and $Z_4 \leq n$ for some sufficiently large constant n , as done in Briat et al.; however, this is also challenging due to the high-dimensionality of the system. Consequently, we conjecture that this problem must be addressed using non-linear Lyapunov functions, which are highly non-trivial to construct and analyze.

We also attempted a ‘skeleton chain’ approach: For a finite stopping time τ , if there exists a function V such that $\mathbb{E}[V(X(\tau)) - V(X(0))] < -1$, then $X(t)$ is ergodic. By Dynkin’s formula, we have $\mathbb{E}[V(X(\tau)) - V(X(0))] = \mathbb{E}[\int_0^\tau \mathcal{L}V(X(s))ds]$. Hence, the goal is to show $\mathbb{E}[\int_0^\tau \mathcal{L}V(X(s))ds] < 0$, which is a weaker condition than $\mathcal{L}V(\mathbf{x}) < 0$ for all but finitely many $\mathbf{x}$. To do this, we can use negative drifts that eventually show up on the time interval $[0, \tau]$. For example, when $Z_2 = 0$ but Z_1 is large at the beginning, $\mathcal{L}V(X(t))$ has a dominant negative drift through $Z_1 + Z_2 \rightarrow 0$ for a short time once Z_2 is created, while a dominant positive drift appears for most of the time on $[0, \tau]$ through $Z_1 \rightarrow Z_1 + X_1$. These drifts, acting on two different time scales, can be counterbalanced by choosing a V that is linear in X_1 but highly non-linear in Z_1 and Z_2 , thereby ensuring $\mathbb{E}[\int_0^\tau \mathcal{L}V(X(s))ds] < 0$. However, such an approach is difficult to apply in the region where X_2 and Z_2 (or Z_4) are large but others are 0. In this region, both $X_2 \rightarrow X_2 + Z_2$ and $2X_2 \rightarrow 2X_2 + Z_4$ keep creating positive drifts. As these positive drifts cannot be offset by any reaction, Z_2 keeps increasing until X_2 becomes small (Fig. R1). Therefore, to ensure the negative drift on $[0, \tau]$, the τ should be set longer than the time for X_2 to converge near the origin. However, this time is expected to be $O(\log n)$ when $X_2(0) = n$, which has to be proven as well, and hence balancing the positive drift accumulated for $O(\log n)$

amount of time and a dominant negative drift appearing only afterwards will be difficult. While a skeleton chain approach was successfully employed in a previous study (David F. Anderson, Daniele Cappelletti, and Jinsu Kim, *Stochastically modeled weakly reversible reaction networks with a single linkage class*, *Journal of Applied Probability*, 57(3), 792–810, 2020), the class of reaction networks of interest in the paper allows the dominance of a positive drift at the boundary of the state space only for a very short time. Thus, showing ergodicity using a skeleton chain for a reaction network controlled by MC+NC presents a new type of challenge.

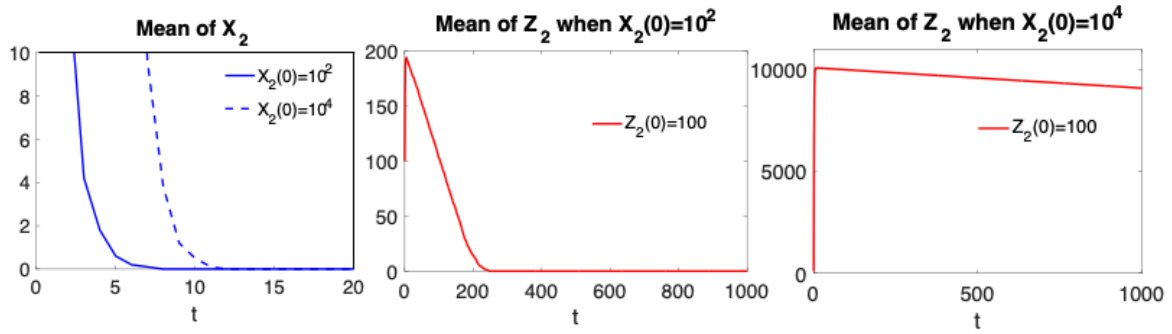


Fig. R1 Z_2 keeps increasing until X_2 drops near zero. The mean of 10 trajectories of X_2 (left) and Z_2 (middle and right) using reaction networks in Fig. 1 and parameters $\mu_1 = 1, \theta_1 = 1, \eta_1 = 50, k_1 = 1, \mu_2 = 3, \theta_2 = 1, \eta_2 = 50, d_2 = 1$. With initial value $X_2(0) = 10^2$ (left; blue line), X_2 reaches near zero at 6s, and Z_2 increases until time = 6s (middle). With $X_2(0) = 10^4$ (left; blue dotted line), X_2 reaches near zero at 12s, and Z_2 increases until time = 12s (right).

While we did not succeed in showing ergodicity, our insights on this problem highlight that proving ergodicity for reaction networks with NC is not just a technical challenge but a qualitatively new problem, distinct from typical proofs for ergodicity of reaction networks. This suggests that future work may require new types of proof techniques, potentially broadening theoretical developments in the study of stochastic reaction networks. We have clarified these points in the revised manuscript.

Discussion:

...

Through numerical simulations of several open-loop networks with various parameter values (Fig. 2, 3), we observed that the reaction network associated with NC is stable when the target Fano factor exceeds 1, and unstable when the target Fano factor is below 1. To establish this claim rigorously, we proved that in some parameter regions where the target Fano factor is less than 1, the associated continuous-time Markov chain is transient (Text. S3).

Furthermore, we found that showing sufficient conditions for ergodicity of the controlled system may lead to new theoretical approaches. One clear difficulty comes from the production-based control by $Z_1 \rightarrow Z_1 + X_1$ in MC and the degradation-based control by $Z_4 + X_1 \rightarrow Z_4$ in NC. As they give opposite drifts, one may think of using a piecewise Lyapunov function as shown in previous study⁶. However, due to the non-linearity of the degradation reaction in NC, it is challenging to control the drifts around the boundaries between the regions where a piecewise Lyapunov function is defined

differently. Another common approach is to use a skeleton chain, which is a discrete-time Markov chain obtained by sampling the original Markov chain at certain time points³⁵. This approach is often employed to exploit negative drifts that are not present initially but emerge after some time. However, this method does not appear to be easily applicable here. For example, when the positive drift induced by $2X_2 \rightarrow 2X_2 + Z_4$ dominates, this dominance is expected to last for $O(\log n)$ time if the initial amount of X_2 is n . It would be highly challenging to balance the positive drift accumulated over an $O(\log n)$ time interval with a dominant negative drift that appears only afterward. Consequently, our analysis indicates that proving the ergodicity of the NC requires a fundamentally new analytical framework rather than conventional techniques typically used for reaction networks. Future work toward proving the ergodicity and finiteness of the moment of a reaction network with NC may create a new theoretical foundation for future studies in stochastic reaction networks.

...

3. Issues with Ergodicity: The authors state that the proposed motif functions work as intended provided the combined dynamics are ergodic with finite second moments. However, they also note that if the target Fano factor is set below 1, certain controller species (notably Z_1 and Z_4) accumulate indefinitely over time, indicating a breakdown of ergodicity. Given that RPA systems are designed to reject disturbances that arise from parameter perturbations, it is important to consider scenarios where such perturbations drive the Fano factor set-point below 1. In such cases, the resulting accumulation of controller species could be more harmful to the cell than the very noise the noise controller is designed to suppress. I recommend that the authors explicitly address this issue and discuss potential strategies to mitigate or avoid such pathological behaviour.

We thank the reviewer for this valuable comment. As the reviewer pointed out, when the target Fano factor is set below 1, the controller species Z_1 and Z_4 tend to accumulate (Fig. 2d). To address this problem, one of the promising strategies is using the anti-windup topology proposed by Filo et al. (ref. 33 in the revised manuscript), which, when used together with MC, effectively prevented continuous accumulation of species in MC.

To assess whether this anti-windup topology can prevent the accumulation of NC species (i.e., Z_4) as well as MC species (i.e., Z_1), we examined the dynamics of Z_1 and Z_4 with and without anti-windup when the target Fano factor temporarily dropped below 1 (Text. S1 and Fig. S2). Without anti-windup, a temporary drop in the target Fano factor below 1 resulted in continuous accumulation of Z_1 and Z_4 (Fig. S2a). Even after the target Fano factor returned above 1, the excess Z_1 and Z_4 decayed slowly (Fig. S2a), delaying the convergence of the Fano factor to its target value (Fig. S2b). In contrast, with anti-windup, when the target Fano factor dropped below 1, Z_1 and Z_4 accumulated only up to a limited level (Fig. S2c). This allowed Z_1 and Z_4 to rapidly reach their stationary values once the target Fano factor returned above 1 (Fig. S2c), enabling the Fano factor to track the desired value more quickly (Fig. S2d).

Despite these advantages, using the anti-windup topology resulted in a slight deviation from the actual Fano factor to the target Fano factor (Fig. S2d). This is because adding the anti-windup topology alters the moment equations of the controller species Z_1, Z_2, Z_3 , and Z_4 (Text. S1), making the Fano factor

formula presented in Fig. 2a no longer hold. As this formula, which solely depended on the MC and NC parameters, is the key to guaranteeing noise RPA, it remains uncertain whether noise RPA would still hold when anti-windup is incorporated. Therefore, developing a method that can prevent controller species accumulation via anti-windup while also theoretically guaranteeing the noise RPA of MC and NC represents a promising avenue for future work. We have discussed these points in the revised manuscript.

Discussion:

...

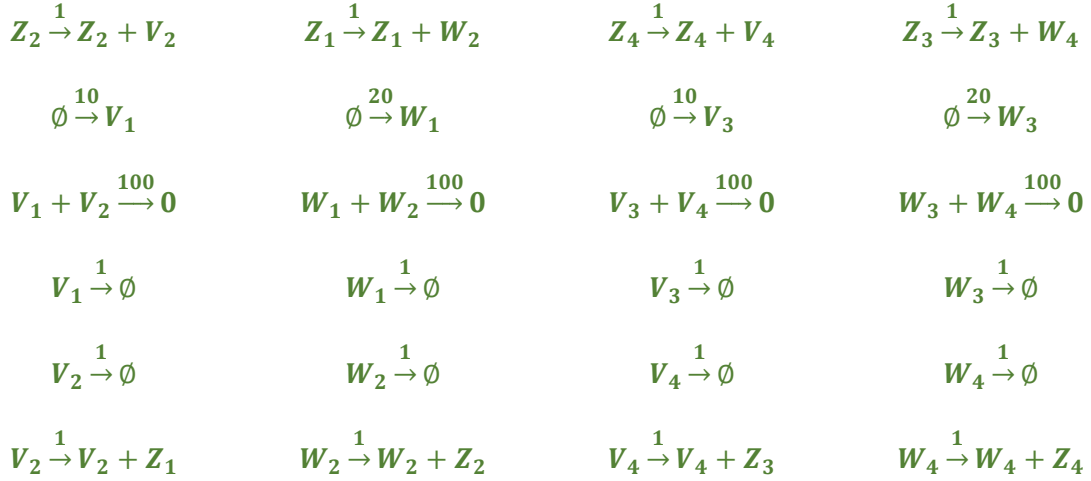
Despite the advantages of MC and NC, their species—particularly Z_1 and Z_4 —can accumulate indefinitely when the target Fano factor is below 1 (Fig. 2d). Such accumulation of controller species can be detrimental to cells and may impair the proper functioning of MC and NC. A promising strategy to prevent this issue is the anti-windup approach proposed by Filo et al.³³, which has been shown to effectively prevent the accumulation of MC species. Using this anti-windup topology, we confirmed that the accumulation of Z_1 and Z_4 can also be effectively prevented (see Text S1 and Fig. S2 for details). However, incorporating anti-windup led to a deviation between the actual and target Fano factors (Fig. S2), as it alters the Fano factor formula described in Fig. 2a (Text S1). As the altered Fano factor formula may depend on the parameters of original networks, it remains uncertain whether noise RPA would still hold when anti-windup is applied. Therefore, developing a method that prevents controller species accumulation via anti-windup while also theoretically ensuring the noise RPA of MC and NC represents a promising direction for future work.

...

Supplementary Information:

Text. S1 Anti-windup topology prevents the accumulation of controller species

When the target Fano factor is set below 1, certain controller species in MC and NC—specifically Z_1 and Z_4 in Fig. 1—accumulate indefinitely over time (Fig. 2d). This accumulation is detrimental to cellular function and possibly disrupts output control in both MC and NC. To address this issue, we incorporated the anti-windup topology proposed by Filo et al. (Fig. S2). In particular, we constructed the anti-windup topology as shown below and compared the dynamics of Z_1 and Z_4 with and without anti-windup when the target Fano factor temporarily dropped below 1 (Fig. S2):



Without anti-windup, a temporary drop in the target Fano factor below 1 resulted in continuous accumulation of Z_1 and Z_4 (Fig. S2a). Even after the target Fano factor returned above 1, the excess Z_1 and Z_4 decayed slowly (Fig. S2a), delaying the convergence of the Fano factor to its target value (Fig. S2b). In contrast, with anti-windup, when the target Fano factor dropped below 1, Z_1 and Z_4 accumulated only up to a limited level (Fig. S2c). This allowed Z_1 and Z_4 to rapidly reach their stationary values once the target Fano factor returned above 1 (Fig. S2c), enabling the Fano factor to track the desired value more quickly (Fig. S2d). However, the achieved Fano factor deviated slightly from the target (Fig. S2d), as the addition of anti-windup topology changes the moment equations of Z_1, Z_2, Z_3 , and Z_4 , as follows:

$$\mathbb{E}[Z_1] = \mu_1 - \eta_1 \mathbb{E}[Z_1 Z_2] + \mathbb{E}[V_2], \mathbb{E}[Z_2] = \theta_1 \mathbb{E}[X_2] - \eta_1 \mathbb{E}[Z_1 Z_2] + \mathbb{E}[W_2]$$

$$\mathbb{E}[Z_3] = \mu_2 - \eta_2 \mathbb{E}[Z_3 Z_4] + \mathbb{E}[V_4], \mathbb{E}[Z_4] = \theta_2 \mathbb{E}[X_2(X_2 - 1)] - \eta_2 \mathbb{E}[Z_3 Z_4] + \mathbb{E}[W_4]$$

where $\mu_1, \eta_1, \theta_1, \mu_2, \eta_2, \theta_2$ are the MC and NC parameters in Methods. This leads to the Fano factor depending on parameters other than those of the controller reactions:

$$\text{Fano factor of } X_2 = 1 - \frac{\mu_1}{\theta_1} + \frac{\theta_1 \mu_2}{\theta_2 \mu_1} + \frac{\theta_1}{\mu_1} \left(\mathbb{E} \left[\frac{V_4 - W_4}{\theta_2} - \frac{V_2 - W_2}{\theta_1} \right] \right)$$

Here, the term $\mathbb{E} \left[\frac{V_4 - W_4}{\theta_2} - \frac{V_2 - W_2}{\theta_1} \right]$ has no closed-form expression that depends solely on the controller parameters and may also rely on the parameters of the original networks. This raises uncertainty about whether noise RPA would still hold when anti-windup is incorporated.

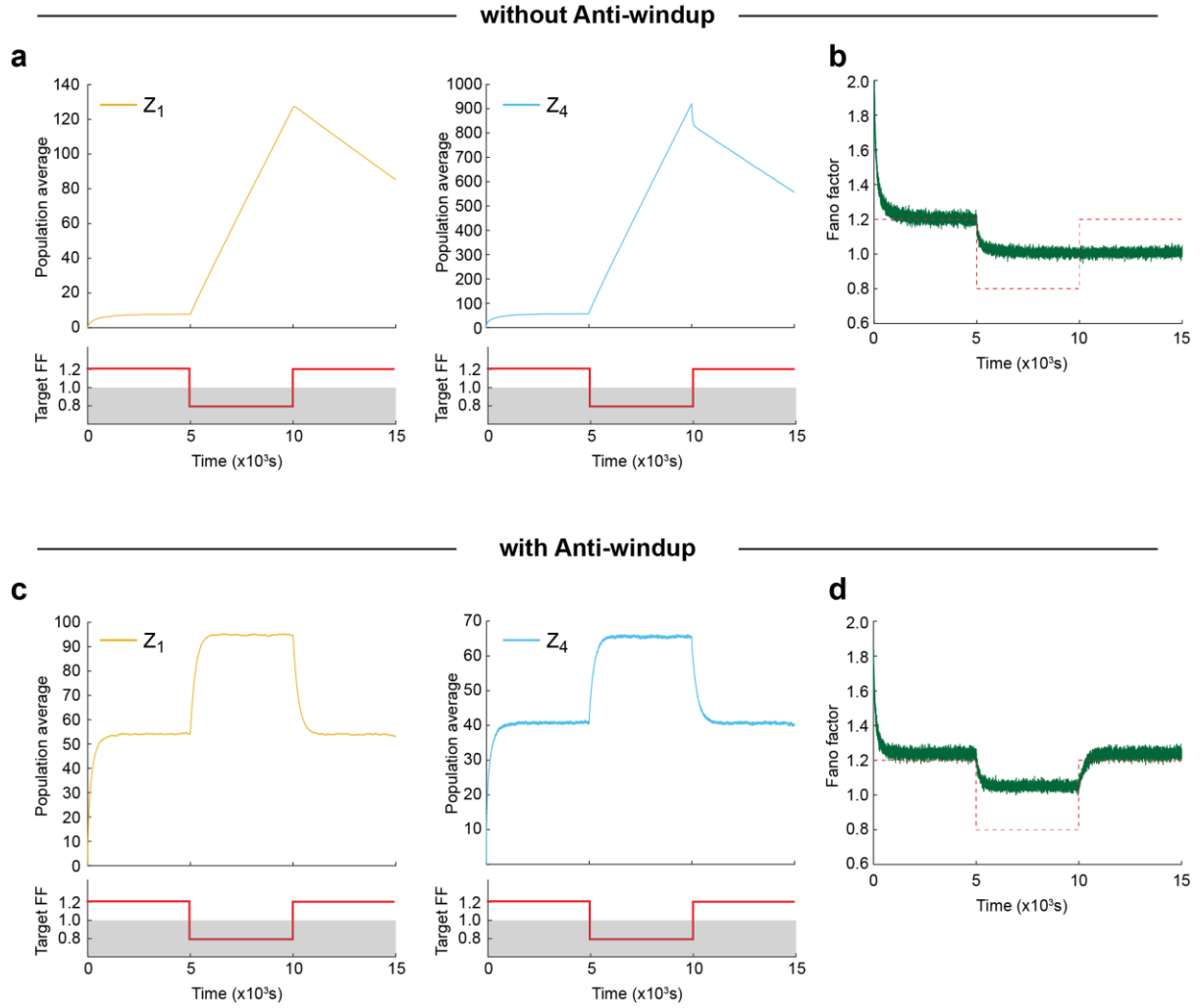


Fig. S2 Anti-windup topology suppresses the accumulation of controller species, yet it disturbs precise noise control. (a) We simulated 10,000 trajectories of Z_1 (left) and Z_4 (right) using the reaction networks and the parameters following those in Fig. 1e-f except for μ_2 . Specifically, μ_2 was set to 9.6 during 0 – 5000s and 10,000s – 15,000s and to 8.4 for 5000 – 10,000s, corresponding to target Fano factor (red lines) of 1.2 and 0.8, respectively. During the period when the target Fano factor falls below 1 (gray shaded regions), Z_1 and Z_4 were accumulated and reduced slowly to the original level after the target Fano factor rose up to 1. (b) The Fano factor of 10,000 trajectories of X_2 simulated under same conditions in (a). The Fano factor of X_2 failed to track the desired value even after the target Fano factor (red dotted line) rose above 1. (c) The mean of 10,000 trajectories of Z_1 (left) and Z_4 (right) from the same system as in (a-c), but combined with anti-windup topology described in Text. S2. Combining the anti-windup topology successfully prevents the accumulation of Z_1 and Z_4 , during the period when the target Fano factor (red lines) fell below 1 (gray shaded region) (d) The Fano factor of 10,000 trajectories of X_2 simulated under the same conditions in (c). While the Fano factor failed to track the desired value when the target value (red dotted line) was below 1, it quickly tracked the target once the desired Fano factor exceeded 1. However, the achieved Fano factor deviated slightly from the target, as the addition of anti-windup topology changes the moment equations of Z_1, Z_2, Z_3 , and Z_4 (see Text. S2 for more details).

4. Checking for Bimodality: While controlling intrinsic noise via output variance is a valid objective, this strategy is meaningful only if the output distribution remains unimodal (e.g., Poisson-like). If the output distribution becomes bimodal, then variance alone no longer offers a reliable measure of single-cell control, as individual cells may still exhibit large deviations

from the mean. It would strengthen the paper considerably if the authors could demonstrate, through simulation or theoretical analysis, that the proposed Noise Controller does not induce bimodality in the output distribution, which would undermine its intended purpose.

We thank the reviewer for highlighting the importance of checking for bimodality. Bimodality in the output distribution typically arises from the coexistence of a fast mode of output production (corresponding to the larger peak) and a slow mode of output production (the smaller peak). For example, in the telegraph model (Fig. 4a), stochastic switching between a fast production mode, when the gene is active and produces proteins rapidly, and a slow production mode, when the gene is inactive and produces proteins slowly, yields a bimodal output (i.e., protein) distribution. In light of this, if the difference between the fast and slow modes can be eliminated using MC and NC, the output distribution will not exhibit bimodality.

To test this, we investigated how the telegraph model's output distribution changes as MC and NC are combined (Fig. 4a-c). In the original telegraph model, or with MC alone, the protein level continued to transition between high and low states (Fig. 4b (i) and (ii), top), and the output stationary distribution remained bimodal (Fig. 4b (i) and (ii), bottom). In contrast, when both MC and NC were combined, protein levels fluctuated minimally around the desired set point (Fig. 4b (iii), top), yielding a unimodal output distribution (Fig. 4b (iii), bottom).

To explain these results, we investigated how the average protein production propensity (i.e., reaction α_p in Fig. 4a) in the fast and slow production modes changed with the addition of MC and NC (Fig. 4c; see Results and Methods in the revised manuscript). While the fast and slow production modes exhibited markedly different propensities in the original network (Fig. 4c, left), and again with MC alone (Fig. 4c, center), the difference between the two modes collapsed and their propensities became essentially indistinguishable when both MC and NC were combined (Fig. 4c, right).

The convergence of the two production modes by MC and NC arises from the simultaneous action of production-based actuation from MC and degradation-based actuation from NC (Fig. S1). Production-based actuation increases mRNA, raising the slow-mode propensity, while degradation-based actuation reduces mRNA, directly lowering the fast-mode propensity (see Results in the revised manuscript). This pattern was also evident in the toggle switch system—a model that exhibits a bimodal stationary distribution due to its fast and slow production modes. Taken together, these results show that the NC does not induce bimodality; rather, in combination with the MC, it suppresses the fast–slow mode disparity and maintains a unimodal output distribution. We have incorporated these analyses and clarifications in the revised manuscript.

Results:

MC and NC prevent bimodality in output distribution

Using MC and NC, the output noise of the original network can be reduced up to a Fano factor of 1. However, a reduced Fano factor value alone cannot guarantee our ultimate goal of single-cell control. For example, if the output distribution is bimodal, then the output level at the single-cell level may deviate from the desired set point even with low output noise. Therefore, we investigated whether MC and NC can also prevent bimodality in output distribution (Fig. 4).

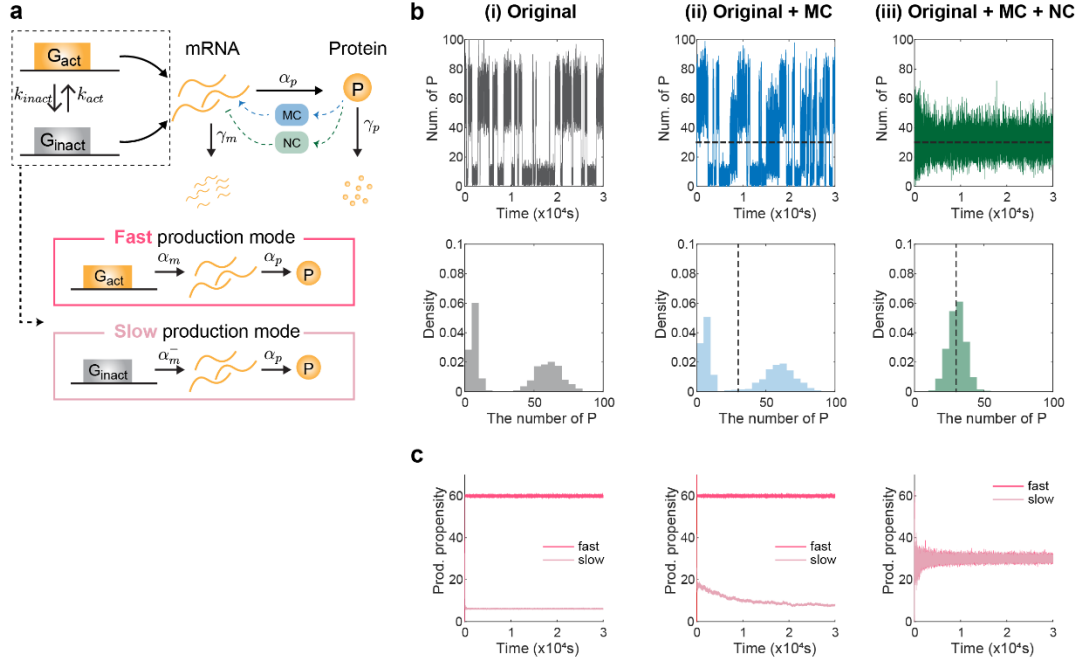
To do this, we combined MC and NC with a telegraph model¹⁷, which exhibits a bimodal stationary distribution in its output, and observed how the stationary distribution of the output changed (Fig. 4a–c). In the telegraph model, protein synthesis occurs in two modes: the fast production mode, where active gene produces protein rapidly, and the slow production mode, where the inactive gene produces protein slowly (Fig. 4a). The two modes alternately appear at the single-cell level, causing the protein amount to fluctuate between high and low states (Fig. 4b (i), top), leading to a bimodal stationary distribution (Fig. 4b (i), bottom). Even when MC was combined to control the target mean, protein levels at the single-cell level still alternated between high and low states with a large deviation from the target (Fig. 4b (ii), top), and the stationary distribution remained bimodal (Fig. 4b (ii), bottom). In contrast, when both MC and NC were combined, protein levels fluctuated only around the desired set point without sharp transitions (Fig. 4b (iii), top), and the stationary distribution became unimodal (Fig. 4b (iii), bottom).

To understand why the stationary distribution became unimodal when MC and NC were combined, we investigated how the protein production propensity (i.e., reaction α_p in Fig. 4a) in the fast and slow production modes changed with the addition of MC and NC (Fig. 4c). In particular, the propensity for the fast production mode was calculated from 1,000 simulated protein production propensity trajectories by averaging, at each time point, the values corresponding to instances in which the gene was active. Applying the same method to cases where the gene was inactive, we obtained the propensity in the slow production mode (see Methods for details). In the original telegraph model, the difference between the propensities of the fast and slow modes was pronounced (Fig. 4c, left), and this difference persisted even when MC was combined (Fig. 4c, center). However, when both MC and NC were combined, the fast-mode propensity decreased, while the slow-mode propensity increased, eliminating the gap between the two modes (Fig. 4c, right).

The convergence of the two production modes by MC and NC arises from the simultaneous action of production-based actuation from MC and degradation-based actuation from NC (see Fig. S1 and Discussion for details). Production-based actuation increases mRNA, raising the slow-mode propensity, while degradation-based actuation reduces mRNA, directly lowering the fast-mode propensity. As MC alone lacks degradation-based actuation, without NC, the fast-mode propensity can only decrease through natural mRNA degradation. As a result, the fast-mode propensity does not sufficiently decrease in the original telegraph model or with MC alone (Fig. 4c (i-ii)), which in turn constrains any increase in slow-mode propensity, as such an increase would otherwise raise the output mean beyond the target value. Thus, a large difference remains between the propensities of the two modes in the original telegraph model or with MC alone (Fig. 4c (i-ii)), leading to large protein-level differences during mode switching. In contrast, when both MC and NC are used, the two propensities become nearly identical (Fig. 4c (iii)), so protein levels show little difference during mode switching.

This pattern was also observed when MC and NC were combined with the toggle switch model¹⁸ (Fig. 4d–f)—another model that exhibits a bimodal stationary distribution due to fast and slow production modes. Taken together, for the systems with two output production modes, combining both MC and NC can prevent bimodality by making the two modes of the bimodal distribution identical.

Telegraph



Toggle switch

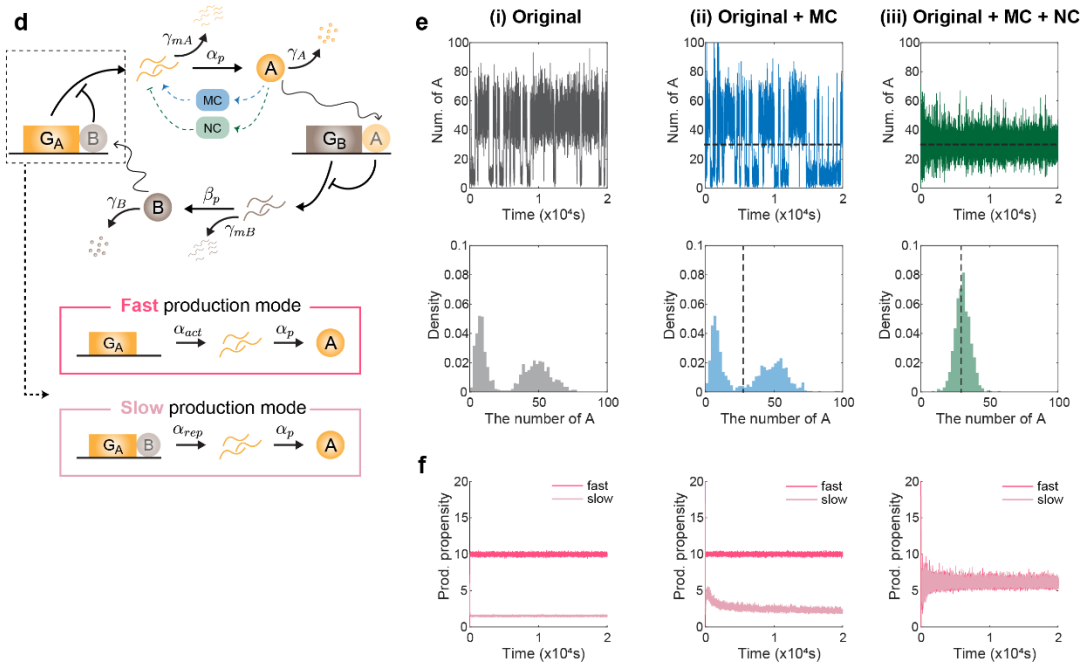


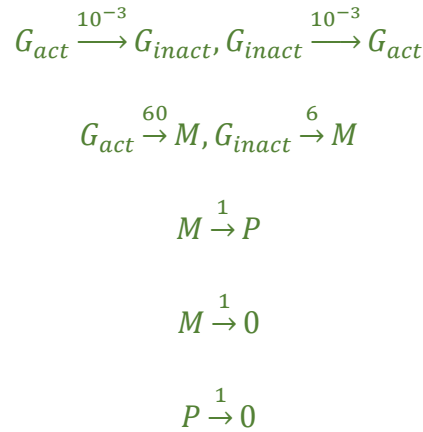
Figure 4. NC prevents bimodality in the output stationary distribution. (a) Schematic diagram of the telegraph model with MC (blue) and NC (green). The model alternates between two protein (P) production modes (dotted box): a fast production mode (pink box), in which an active gene (G_{act}) produces mRNA and subsequently protein, and a slow production mode (pale pink box), in which an inactive gene (G_{inact}) produces mRNA and subsequently protein at a lower rate. (b) A representative single-cell trajectory of P (top) and the histogram of its stationary distribution (bottom) simulated from the telegraph model (i), telegraph model with MC (ii), and the telegraph model with MC and NC (iii). Simulations were performed with parameters $k_{act} = 10^{-3}$, $k_{inact} = 10^{-3}$, $\alpha_m =$

60, $\alpha_m^- = 6$, $\alpha_p = 1$, $\gamma_m = 1$, $\gamma_p = 1$ and parameters of MC and NC following Fig. 1 except $\mu_1 = 30$, $\mu_2 = 906$. See Methods for more details on stochastic simulations. In the original telegraph model (i) and with MC alone (ii), P switches between high (~ 60) and low (~ 6) levels (i and ii, top), producing a bimodal stationary distribution (i and ii, bottom), whereas with both MC and NC (iii), P no longer switches between two distinct states and it shows decreased deviation from the target mean (dotted lines) value (iii, top), resulting in a unimodal stationary distribution (iii, bottom). (c) The average production propensity of P when the gene is active (pink) or inactive (pale pink), calculated over 1,000 trajectories simulated from the telegraph model (left), the telegraph model with MC (center), and the telegraph model with MC and NC (right). The difference between fast and slow production propensities is large for the telegraph model (left) and the telegraph model with MC (center), but this difference is eliminated when NC is additionally included (right). (d) Schematic diagram of the toggle switch model with MC (blue) and NC (green). The model alternates between two protein production modes (dotted box): a fast production mode (pink box), in which an active gene (G_A) produces mRNA and subsequently protein (A) at a higher rate, and a slow production mode (pale pink box), in which the repressed gene (G_A bind with B) produces mRNA and subsequently A at a lower rate. (e-f) The same analyses as (b-c), but with the telegraph model replaced by the toggle switch model. Simulations were performed with parameters $\alpha_{act} = 10$, $\alpha_{rep} = 1.5$, $\alpha_p = 1$, $\gamma_{mA} = 1$, $\gamma_A = 0.2$, $\beta_p = 1$, $\gamma_{mB} = 1$, $\gamma_B = 0.1$ and parameters of MC and NC following Fig. 1 except $\mu_1 = 30$, $\mu_2 = 906$. See Methods for more details on stochastic simulations. The results are consistent with those obtained from the telegraph model.

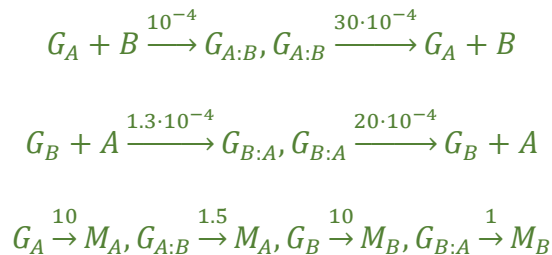
Methods:

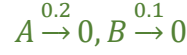
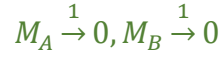
Details of bimodality analysis using telegraph and toggle switch models

We demonstrated the MC and NC's ability to remove the bimodality using the telegraph model (Fig. 4a-c) and toggle switch model (Fig. 4d-f), which exhibits the bimodal output stationary distribution from fast and slow production modes. Here, the telegraph model consists of the following reactions:



where G_{act} , G_{inact} , M , and P denote the active gene, inactive gene, mRNA, and protein, respectively. The output species was P , while the input species was M . The toggle switch model consists of the following reaction:





where $G_A, G_{A:B}, G_B, G_{B:A}, M_A, M_B, A$, and B denote the gene A, gene A bind with B, gene B, gene B bind with A, mRNA of A, mRNA of B, protein A, and protein B, respectively. The output species was A , while the input species was M_A .

For the telegraph model, we simulated 1,000 trajectories of output species (i.e., P) in the telegraph model (Fig. 4b (i)), the telegraph model with MC (Fig. 4b (ii)), and the telegraph model with MC and NC (Fig. 4b (iii)), initializing all species at zero except one active gene and running each simulation for 30,000 seconds. The output stationary distribution was obtained from the final state of P in each trajectory (value at 30,000 s). To define the fast-mode production propensity (Fig. 4c), at each time point t , we computed the mean protein level across those trajectories in which the gene was active at t ; for example, the fast-mode propensity at $t = 1,000s$ is the average of P over all trajectories with the gene active at 1,000s. Applying the same method to the cases where the gene was inactive, we obtained the propensity in the slow production mode.

For the toggle switch model, we simulated 1,000 trajectories of output species (i.e., A) in the toggle switch model (Fig. 4e (i)), the toggle switch model with MC (Fig. 4e (ii)), and the toggle switch model with MC and NC (Fig. 4e (iii)), initializing all species at zero except one unbound gene A and one unbound gene B and running each simulation for 20,000 seconds. The output stationary distribution was obtained from the final state of A in each trajectory (value at 20,000 s). To define the fast-mode production propensity (Fig. 4f), at each time point t , we computed the mean level of A across those trajectories in which the gene was not bound with B at t ; for example, the fast-mode propensity at $t = 1,000s$ is the average of B over all trajectories with the gene not bound with B at 1,000s. Applying the same method to the cases where the gene was bound with B , we obtained the propensity in the slow production mode.

5. Effect of Sensor Saturation: The derivation of the Fano factor assumes mass-action kinetics for the sensing mechanism. However, when implemented using gene expression components—as suggested in the paper—sensor saturation is a realistic concern. In practical terms, the sensing rate cannot increase indefinitely and is likely to plateau at high copy-numbers of the output species. This saturation could significantly affect the performance of the Noise Controller. I encourage the authors to assess this effect by replacing the mass-action kinetics of the dimer sensing reaction with a saturating Hill function in their computational model. Such an analysis would provide valuable insight into the practical feasibility and robustness of the proposed design.

We thank the reviewer for this valuable comment. As suggested, we replaced the NC's sensing propensity with a Hill function form (Text S5), combining it and MC with the original network, and

examined whether the Fano factor reaches its desired set point. As a result, the Fano factor converges to the target even when the actuator saturates if the dissociation constant, K , is large (Fig. S7)—the condition in which the Hill function mathematically approaches mass-action kinetics (Text. S5). In contrast, for small K , the NC fails to achieve the set point, underscoring the importance of operating with a high dissociation constant in practice.

To realize a high effective dissociation constant (i.e., lower binding affinity) in a biological circuit, the X_2 homodimer should bind weakly to the GH DNA operator (Fig. 6d). This can be implemented by weakening the operator sequence (e.g., base substitutions at the GH site) or by engineering the DNA-binding domain of X_2 to reduce affinity (e.g., targeted amino-acid substitutions). We have added this discussion to the revised manuscript.

Discussion:

...

One of the key challenges in implementing the NC as a biological circuit is the saturation effect. Although the sensing reaction follows mass-action kinetics in this study, biochemical reaction rates often follow a Hill-function form in reality. This issue can be addressed by tuning the dissociation constant of the sensing reaction (Text S5, Fig. S7). In the supplementary text, we checked that the NC with saturation effect could achieve the noise RPA and set-point tracking by larger dissociation constant (Fig. S7). To realize a large dissociation constant in practice, one could modify the base sequence of the GH DNA site (Fig. 6d) or engineer the DNA-binding domain of the X_2 homodimer (Fig. 6d) to make the binding affinity low.

...

Supplementary Information:

Text. S5 Effect of saturation effect

One of the obstacles in implementing the NC in an actual biological circuit is the saturation effect. While the propensity of the NC's sensing reaction is represented as a quadratic equation in our theoretical model, in reality there is a maximum allowable reaction rate. To model this, we replace the propensity of sensing reaction with a Hill function of the form:

$$f_{\text{Hill}}(X_2) = V_{\text{max}} \frac{X_2^2}{K^2 + X_2^2}$$

Here, K denotes the dissociation constant, where a larger K means weaker binding of the X_2 homodimer to the DNA site. And V_{max} is the maximal allowable reaction rate. To determine θ_2 in this reaction, we require the Hill function to closely approximate mass-action kinetics. Similar to the approach of S1.2 in [6], we do that by assuming that the propensity of sensing reaction, written in mass-action kinetics as $f(X_2) = \theta_2 X_2^2$, passes through the inflection point of the Hill function $\left(K, \frac{V_{\text{max}}}{2}\right)$. Under this assumption, θ_2 is determined by the ratio of V_{max} to K^2 :

$$\theta_2 = \frac{V_{max}}{2K^2}$$

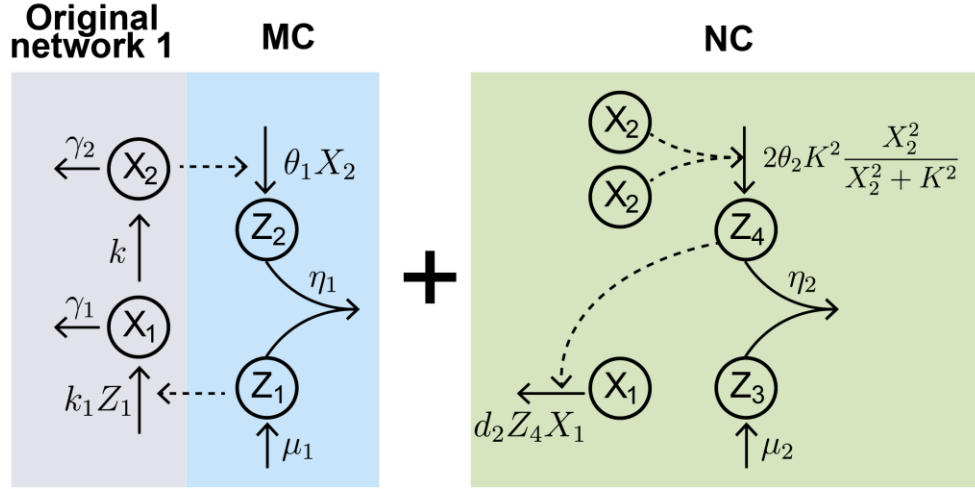
We see that as $K \rightarrow \infty$, the propensity $f_{Hill}(X_2)$ approaches $2\theta_2 X_2^2$. Under this condition, the variance and the set point Fano factor of X_2 changes as follows:

$$Var(X_2) = \frac{\mu_2}{2\theta_2} - \left(\frac{\mu_1}{\theta_1}\right)^2$$

$$FF(X_2) = \frac{\frac{\mu_2}{2\theta_2} - \left(\frac{\mu_1}{\theta_1}\right)^2}{\frac{\mu_1}{\theta_1}}$$

To examine how the saturation effect influences NC functionality, we varied the dissociation constant K and observed the resulting changes in the Fano factor (Fig. S7). As K increased, the Fano factor approached the set-point more closely (Fig. S7b), and the noise level was also maintained after parameter perturbation (Fig. S7b, $t = 300$). To implement such high dissociation constants in an actual biological circuit, one could alter the base sequence of the GH DNA site (Fig. 6d) or engineer the amino acids of the DNA-binding domain in the X_2 homodimer (Fig. 6d) to lower the binding affinity.

a



b

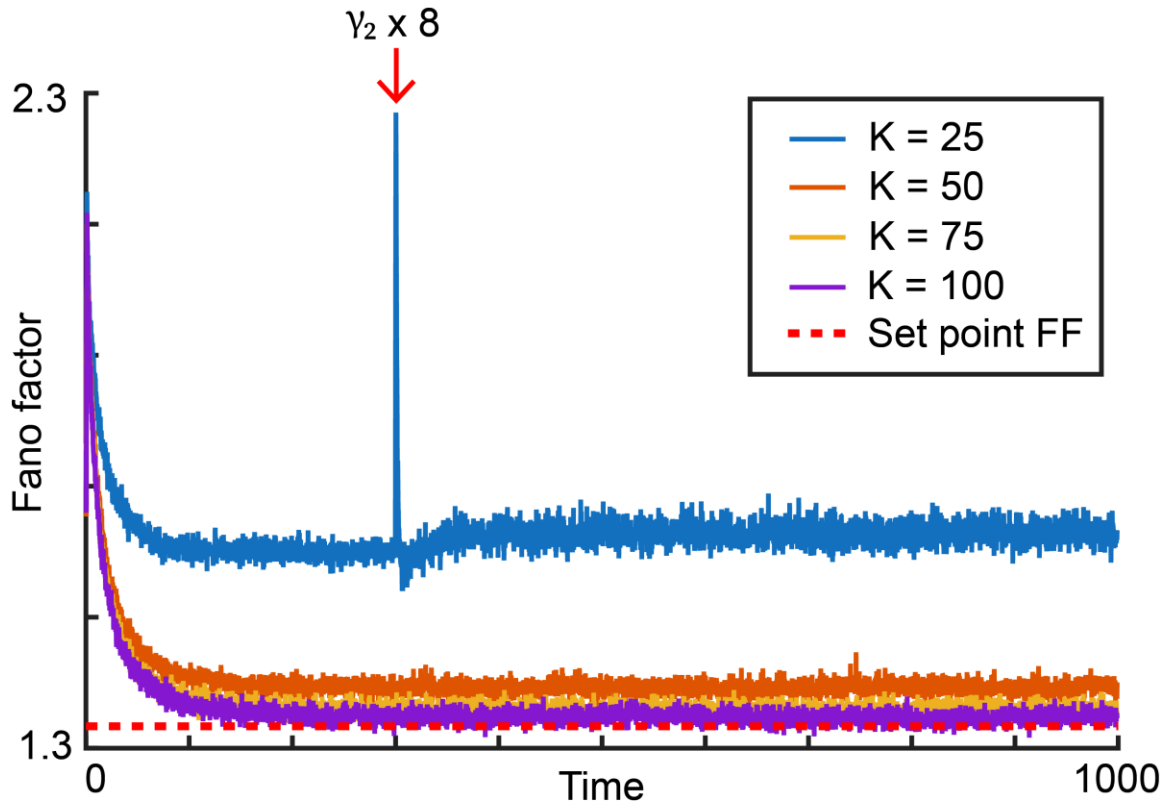


Fig. S7 Even in the presence of the saturation effect, the NC operates properly when the dissociation constant is sufficiently large. (a) NC with the saturation effect. Considering the saturation effect, the sensing reaction of NC is represented in Hill-function form. (b) The Fano factor of 50,000 trajectories of X_2 from the original network with MC and NC with the saturation effect. As the dissociation constant (K) increases, the Fano factor closely follows the set point (red dotted line) and maintains a stable noise level even after parameter perturbation ($t = 300$).

6. **Relation to Existing literature:** The classical paper by Lestas et al. [2] derives fundamental limits to noise suppression in biomolecular systems. I was surprised that this influential work was not cited and discussed. It would be highly valuable for the authors to explore whether the hard lower bound on noise identified in this study is related to these known theoretical limits. Including such a discussion would not only strengthen the theoretical grounding of the paper but also provide a compelling justification for why the observed noise floor cannot be reduced further.

We thank the reviewer for highlighting the important work of Lestas et al. In that study, the authors theoretically established a lower bound on the noise of a single species regulated by a feedback loop (i.e., X_1 affects the production of X_2 , and X_2 indirectly regulates X_1), as follows:

$$\frac{\sigma_1^2}{\langle x_1 \rangle^2} \geq \frac{1}{\langle x_1 \rangle} \frac{2}{1 + \sqrt{1 + \frac{4N_2}{N_1}}} \quad (\text{i})$$

where $\langle x_1 \rangle$ and σ_1^2 denote the mean and variance of X_1 , respectively, and N_1 and N_2 are the numbers of birth events of X_1 and X_2 during the average lifetime of X_1 (i.e., the reciprocal of its degradation rate). This inequality can be equivalently written as:

$$\frac{\sigma_1^2}{\langle x_1 \rangle} \geq \frac{2}{1 + \sqrt{1 + \frac{4N_2}{N_1}}} \quad (\text{ii})$$

which provides a lower bound on the Fano factor of X_1 . Interpreting X_1 as the output species of the original network and the feedback loop as the MC and NC, the main inequality (i) in Lestas et al. indeed establishes a theoretical lower bound for the Fano factor of the output.

However, directly applying this result to account for the $\text{FF} = 1$ limit observed in our study faces two obstacles. First, the lower bound (the right-hand side of inequality (ii)) is strictly less than 1, so it cannot explain why control fails when the target Fano factor lies between that theoretical bound and 1. Second, the derivation of (i) in Lestas et al. assumes that the reaction sensing the level of X_1 is linear (i.e., the reaction rate is proportional to X_1), whereas in our NC, the sensing reaction is quadratic—a case not explicitly treated in Lestas et al.. These differences imply that a new inequality tailored to our setting is required.

To derive a new inequality following the procedure of Lestas et al., we would compute the feedback capacity—the fundamental upper bound on the rate at which the controller can acquire information about X_1 —of MC and NC using (iii) (i.e., the last equation in Section 3 of Supplementary material in Lestas et al.), and then substitute it into inequality (iv) (i.e., the equation (14) in Section 4 of Supplementary material in Lestas et al.).

$$C = \langle f \rangle \log \left(1 + \frac{\sigma_f^2}{\langle f \rangle^2} \right) \quad (\text{iii})$$

$$\frac{\sigma_f^2}{\langle x_1 \rangle} \geq \frac{1}{C\tau_1 + 1} \quad (\text{iv})$$

Here, C denotes the capacity, τ_1 the average lifetime of X_1 , f the intensity of the reaction sensing the output, and $\langle f \rangle$ and σ_f^2 the mean and variance of f , respectively. When both MC and NC are present, the f is given by $f = \theta_1 X_1 + \theta_2 X_1(X_1 - 1)$ (i.e., the sum of the propensity of sensing reactions of MC and NC. Here, to maintain consistency with the notation used by Lestas et al., we use ‘ X_1 ’ to denote the output species, whereas in Figs. 1–3 of our manuscript the output species was denoted as ‘ X_2 ’). Substituting this expression into (iii) makes the feedback capacity depend on the fourth moment of X_1 . Consequently, the inequality bounding the Fano factor also involves the fourth moment, making it difficult to express a universal (i.e., constant) lower bound of the Fano factor.

Nevertheless, extending the framework of Lestas et al. to establish a rigorous theoretical explanation for the lower bound of Fano factor = 1 remains an important direction for future research. Motivated by the reviewer’s comment, we have added a discussion of this point in the revised manuscript.

Discussion:

...Thus, a Fano factor of 1 may represent the lower limit of noise in natural systems, meaning that NC is the module capable of reducing noise to this fundamental limit of natural systems. *Nevertheless, a deeper theoretical investigation of the Fano factor = 1 limit is warranted. A lower bound on the noise of an output species regulated by a feedback loop has been established³⁰, by using the theory of information transmission. Applying this theory to our scenario, where an output species is regulated by MC and NC, remains an important direction for future work.*

While the Fano factor of 1 may represent the natural lower bound of noise, recent studies have reported cases where the Fano factor drops below 1. ...

Minor:

1. On line 296, “sequestrate” should be “sequester”

We have revised this typo.

Figure legend:

Figure. 6 Schematic representation of a biological circuit implementing NC. (a) ..., because the expressed proteins (red circle and blue cup) sequester each other.

2. In the results reported in Figure 4, it is not clear how the authors are computing the copy-numbers from the fluorescence units.

Here, we scaled the copy number of Ada and fluorescence by aligning the thresholds used to distinguish Ada on/off cells. In Uphoff et al. (ref 19 in the revised manuscript), Ada-off cells accounted for 20% of the total population, corresponding to one bar in the fluorescence histogram shown in Fig. 1c of ref. 16. Since six bars spanned the fluorescence range of 200–400, the width of one bar is estimated to be $100/3$. This suggests that the fluorescence threshold for distinguishing Ada on/off cells is $100/3$. In our simulation, however, the threshold was defined as an Ada copy number below 900. Therefore, we equated an Ada copy number of 900 with a fluorescence value of $100/3$. We have added a description of this procedure to the Methods section.

Methods:

To scale the copy number of Ada to fluorescence, we matched the thresholds that distinguish Ada on/off cells. In Uphoff et al., Ada-off cells comprised 20% of the population, corresponding to one bar in the fluorescence histogram shown in Fig. 1c of the reference¹⁹. Because the six bars in that figure covered a fluorescence range of 200–400, the width of one bar can be estimated as $100/3$. This implies that the threshold distinguishing Ada-on/off cells is $100/3$ in fluorescence units. On the other hand, our simulations defined the threshold as an Ada copy number of fewer than 900 molecules (Fig. 5f legend). Accordingly, we set 900 Ada molecules to be equivalent to a fluorescence value of $100/3$. The scaled fluorescence distribution after 5 seconds from the MMS treatment was captured and visualized in Fig. 5f.

Reviewer #2:

Comments to the Author:

The authors proposed augmenting a noise controller (NC) motif to existing AIF controller motif, which they called mean controller (MC) to achieve not only perfect adaptation on mean level but also at perfect adaptation at noise level. The paper is well-written, I enjoyed reading as it easy to follow and the proposed idea though simple in structure, has been demonstrated through simulation to work well. While reading I made note to ask the authors to clarify my questions but as I progressed, the paper begin to answer majority of my queries, that I began crossing out all those notes that I have made. Well done to the authors.

Here are some of my comments.

1. Figures 1a and 1d. There are arrows pointing into Z_2 and Z_4 but no label/legend of what these arrows should be. Can the author clarify what those arrows are?

The arrows pointing into Z_2 and Z_4 should be interpreted together with those next to θ_1 and θ_2 , respectively. They are drawn in this way to indicate that X_2 promotes the reactions generating Z_2 and Z_4 without being consumed. We have revised Fig. 1a and 1d accordingly.

Figure:

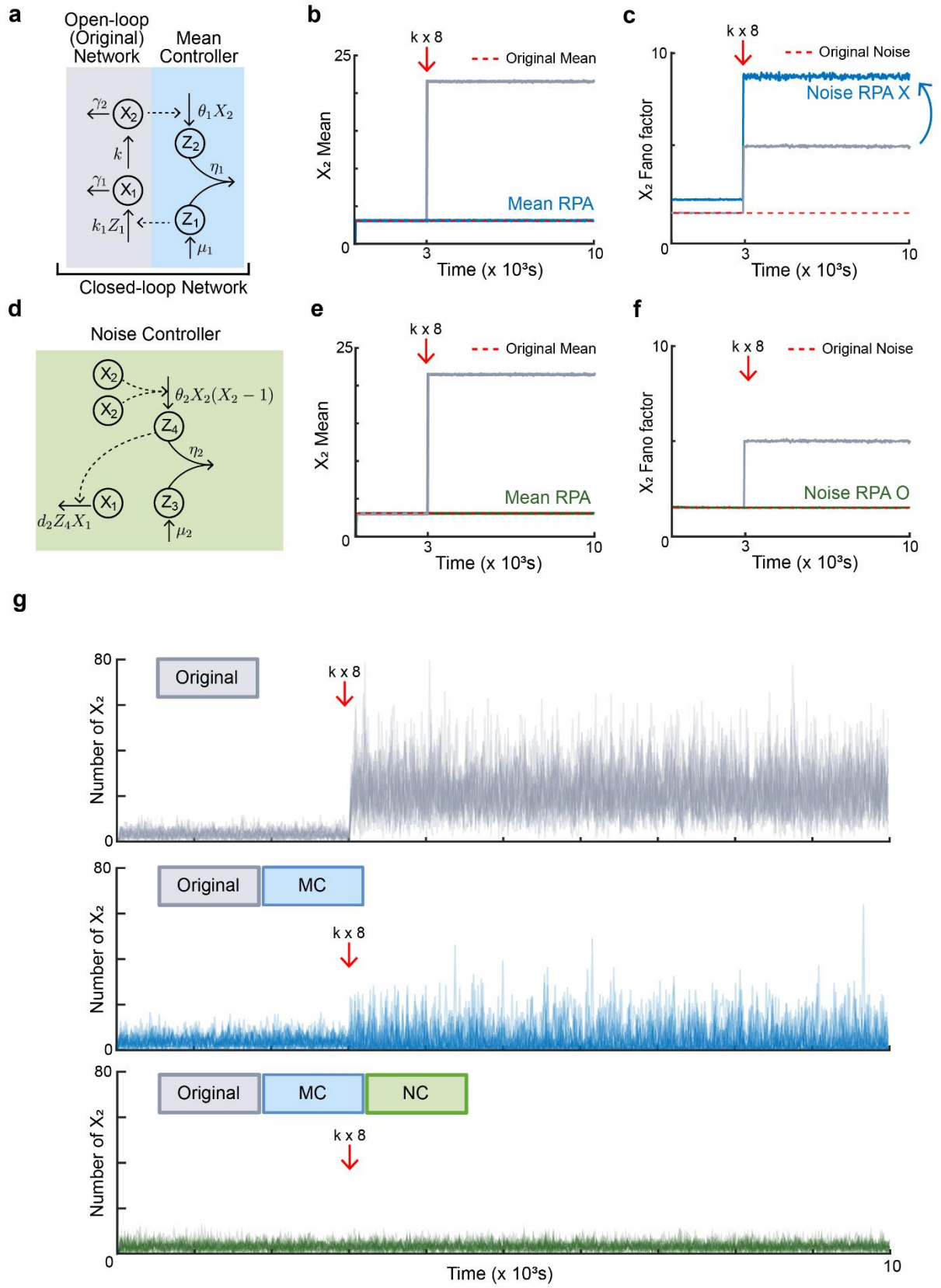


Figure 1. The noise controller achieves noise RPA.

2. Why k is increased 8 times? Why this specific number 8? What happened if this number is increased to 20 times? Is there a limit on the value of k that ensure noise RPA?

As suggested, we reproduced Fig. 1e-f with k increased to 20, 50, 100, and 10,000 times (Fig. S4). The results show that the output mean as well as the Fano factor remained at their desired level even after increases of 20, 50, and 100 times (Fig. S4a–c). Even under an increase of 10,000 times, noise RPA was preserved, though with slower convergence to the target Fano factor level (Fig. S4d). This finding suggests that NC can maintain noise RPA across a wide range of perturbation magnitudes. Nonetheless, a rigorous theoretical bound on the admissible perturbation size is an important direction for future work. We have included these points in the revised manuscript.

Results:

...

To test this, we simulated the output noise level of the original network combined with MC and NC before and after the perturbation in the system. A simulation result shows that combining both MC and NC preserves both the mean (Fig. 1e) and noise (Fig. 1f) at its original level, *across a wide range of perturbation magnitudes (Fig. S4).*

Discussion:

...For example, ergodicity is required for noise RPA, yet the precise conditions of the open-loop structure and the parameters for ergodicity, *including the admissible perturbation size*, are not fully understood.

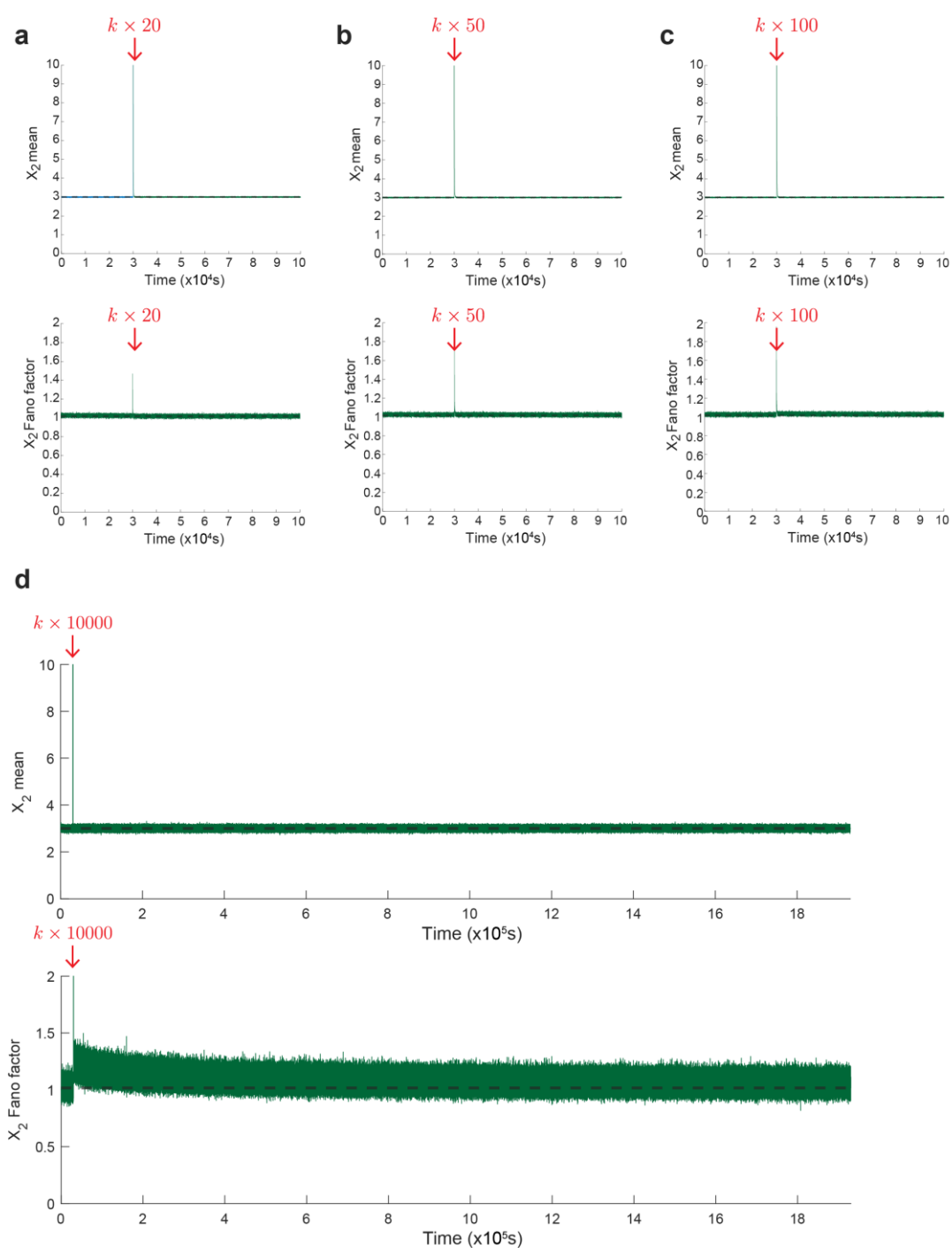


Fig. S4 MC and NC preserve the output mean and noise even after large perturbations. (a-d) The mean (top) and Fano factor (bottom) of 20,000 trajectories (1,000 trajectories for (d)) of X_2 simulated with the reaction networks and the parameters of Fig. 1d-f with a perturbation magnitude increased to 20-fold (a), 50-fold (b), 100-fold (c), and 10000-fold (d). Even under higher magnitudes of perturbations, MC and NC preserve the output mean and noise, although larger perturbations result in a longer convergence time to the desired Fano factor level (dotted line).

3. All parameter values have no unit. For example, η_1 and η_2 in Figure 3 are set to 50. What unit is this 50. Also, isn't 50 very large for a sequestration rate? Please include relevant unit to those parameters. Also, are those parameters taken from literature or arbitrary assigned. If it is the later, are those parameters biologically realistic? If not, why not do the analysis from the start with values that are biologically feasible?

All parameters in this study were taken from the previous simulation studies of MC (ref. 6 and 14 in the revised manuscript), without explicit specification of unit or biological feasibility. However, Aoki et al. (ref. 20 in the revised manuscript), the noteworthy study that confirmed the biological feasibility of MC, suggested the biologically feasible range of the relevant parameters as follows:

$$\eta_1: 3.66 \times 10^{-3} - 5.40 \times 10^{-2} \text{ nM}^{-1} \cdot \text{min}^{-1}$$

$$\mu_1: 10^0 - 10^4 \text{ nM} \cdot \text{min}^{-1}$$

$$\theta_1: 10^{-2} - 10^2 \text{ min}^{-1}$$

$$k_1: 10^{-2} - 10^2 \text{ min}^{-1}$$

Compared with these biologically feasible ranges, all of the parameter values used in our simulations fall inside the relevant range except η_1 and η_2 . To address this, we also performed simulations of an example network in Fig.1 using parameter values within the feasible ranges ($\mu_1 = 3, \theta_1 = 1, \eta_1 = 0.05, k_1 = 1, \eta_2 = 0.05$), and the results are as follows.

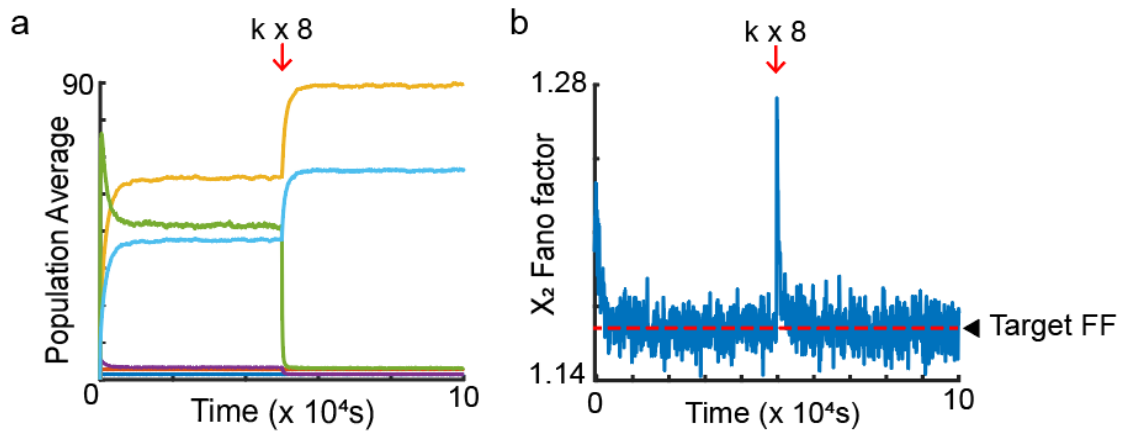


Fig. R2. The noise controller achieves noise RPA with biologically relevant parameter values. (a) Population average of example network in Fig. 1. Using biologically feasible parameter values, the system reaches steady-state, even after the parameter perturbation ($t = 50,000$). (b) Fano factor of X_2 of example network in Fig. 1. NC still achieves its target Fano factor and maintains noise RPA after parameter perturbation ($t = 50,000$).

NC could still successfully achieve the target Fano factor and noise RPA. This demonstrates that the NC functions effectively with biologically feasible parameter values.

4. Fano factor less than 1 showing Z_1 and Z_4 diverge. Any insight on why these two species diverge?

Thank you for raising this important point. Adjusting the NC parameters to set the target Fano factor below 1 causes the average production rate of Z_4 to exceed that of Z_3 (see Text S2 for details). Consequently, newly produced Z_3 is rapidly sequestered by excess Z_4 , driving the Z_3 level toward zero (Fig. S3). Once Z_3 is depleted, $Z_3 - Z_4$ sequestration—the sole degradation pathway for Z_4 —halts, so Z_4 accumulates without bound. The resulting excess Z_4 degrades X_1 and reduces X_2 , hindering the precise output mean control. To preserve the output mean, the MC compensates by driving Z_1 upward, causing both Z_1 and Z_4 to diverge. We have added this explanation to the revised manuscript.

Results:

...This occurs because some controller species (Z_1 in Fig. 1a and Z_4 in Fig. 1d) diverged when the target Fano factor was set below 1 (Fig. 2d), failing to function properly as controllers in contrast to the case when the target Fano factor was greater than 1 (Fig. 2d). *A detailed explanation of why Z_1 and Z_4 diverge is provided in Fig. S3 and Text S2.*

Supplementary Information:

Text. S2 Z_1 and Z_4 diverges when the target Fano factor is below 1

Here, we explain why Z_1 and Z_4 diverge when the target Fano factor is set below 1. Reducing the target Fano factor (i.e., $1 - \frac{\mu_1}{\theta_1} + \frac{\theta_1 \mu_2}{\theta_2 \mu_1}$) to 1 requires $\frac{\mu_2}{\theta_2} < \left(\frac{\mu_1}{\theta_1}\right)^2$, which is equivalent to $\mu_2 < \theta_2 \left(\frac{\mu_1}{\theta_1}\right)^2$. In this inequality, the left-hand side is the production rate of Z_3 (see (1) in Methods), whereas the right-hand side equals $\theta_2 \left(\frac{\mu_1}{\theta_1}\right)^2 = \theta_2 (\mathbb{E}[X_2])^2 = \theta_2 \mathbb{E}[X_2(X_2 - 1)]$, because the Fano factor of X_2 converges to 1 (i.e., $\mathbb{E}[X_2^2] - (\mathbb{E}[X_2])^2 = \mathbb{E}[X_2]$) when the target Fano factor is set below 1 (Fig. 2b). As $\theta_2 \mathbb{E}[X_2(X_2 - 1)]$ is the average production rate of Z_4 , which is generated by dimerization of X_2 (see (1) in Methods), $\mu_2 < \theta_2 \left(\frac{\mu_1}{\theta_1}\right)^2$ indicates that the average production rate of Z_4 becomes bigger than that of Z_3 . As a result, any newly produced Z_3 is rapidly sequestered by the excess Z_4 , making Z_3 converges to 0 (Fig. S3). Once Z_3 disappears, sequestration between Z_3 and Z_4 , the sole degradation pathway of Z_4 ceases, so Z_4 accumulates without bound. The resulting excess of Z_4 degrades the X_1 and reduces X_2 , hindering the precise output mean control. To preserve the output mean, MC compensates the reduction of X_2 by driving Z_1 upward, leading Z_1 and Z_4 to diverge together.

In contrast, if only MC exists without NC, then Z_1 and Z_2 are produced at the same rate; Z_1 is produced at rate μ_1 , and Z_2 is produced at rate $\theta_1 \mathbb{E}[X_2] = \theta_1 \cdot \left(\frac{\mu_1}{\theta_1}\right) = \mu_1$. This enables MC to control the mean over a wide range of target mean values.

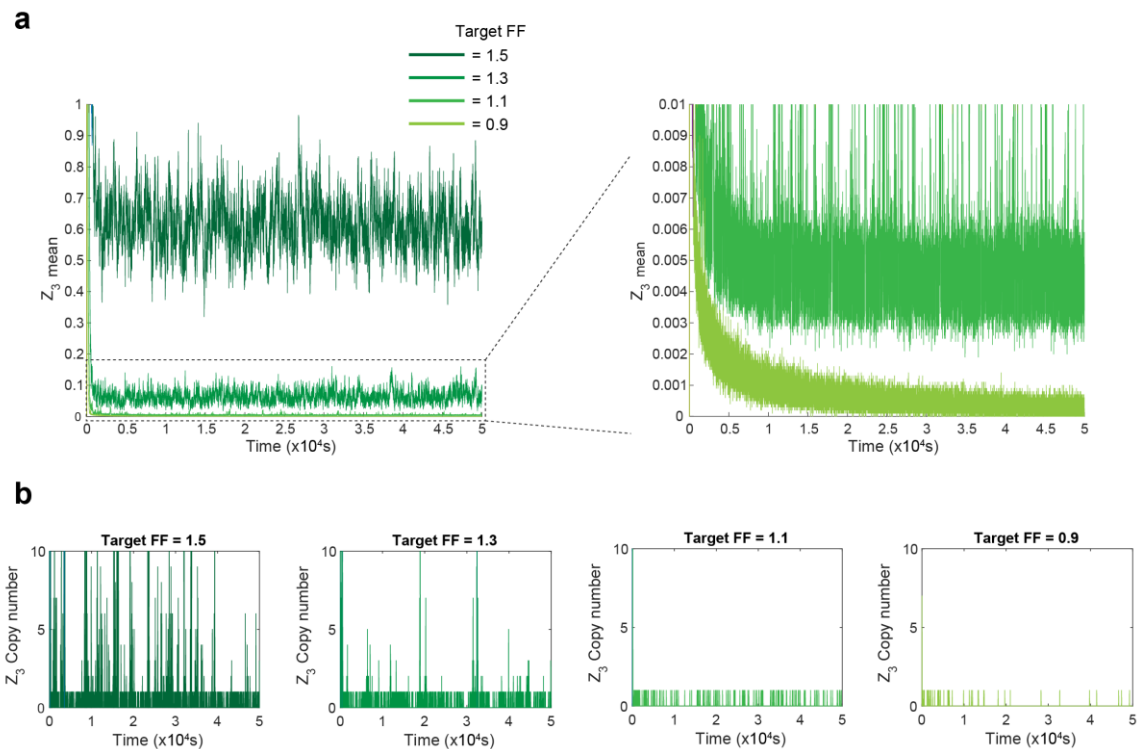


Fig. S3 Z_3 converges to zero when the target Fano factor is below 1. (a) The mean of 10,000 trajectories of Z_3 simulated with the reaction networks and the parameters of Fig. 2b (left) and with a magnified version near zero (right). The Z_3 mean converges to 0 when the target Fano factor (Target FF) is set below 1. (b) One representative single-cell trajectory of Z_3 simulated under the same conditions as in (a). The trajectory exhibits prolonged intervals at $Z_3 = 0$, indicating an increased probability mass at zero.

5. In line 225. Shouldn't (Fig. 4c, d Red) be (Fig. 4c, d, Blue)? And similarly in line 227 shouldn't (Fig. 4c, d Blue) be (Fig. 4c, d Red)?

We have revised this typo.

Results:

... The group where the feedback loop is activated displays a cell death rate of 5% (Fig. 5c, d. blue), which is lower than the 15% cell death rate in the other group where the positive feedback loop fails to initiate (Fig. 5c, d. red).

6. Figure 5. In the NC structure (Fig. 1d), there is an autoregulation of Z4, but this is not accounted for in Figure 5 (at least it's not obvious to me). Any comment on this?
7. Equation (2). If cross referencing to Figure 1a, why is that the degradation involving k1 is not included in the ODE?
8. Equation (4). If cross referencing to Figure 1d, why is the autoregulation of Z4 and the arrow with no variable entering into Z4 is not included in the ODE?

As Comments 6–8 all arise from confusion caused by Fig. 1a and 1d, we address them together here. In response to Comments 6 and 8, the arrow leaving and returning to Z_4 does not represent auto-regulation; rather, it was drawn to indicate that Z_4 is not consumed in the reaction labeled d_2 . Accordingly, neither Fig. 5 (i.e., Fig. 6 in the revised manuscript) nor Eq. (4) includes Z_4 auto-regulation. In response to Comment 7, the k_1 reaction does not denote degradation of Z_1 but instead indicates that Z_1 promotes the production of X_1 . Thus, the only degradation of Z_1 occurs through sequestration with Z_2 , as described in Eq. (2). To eliminate such ambiguity, we have revised Fig. 1a and 1d accordingly.

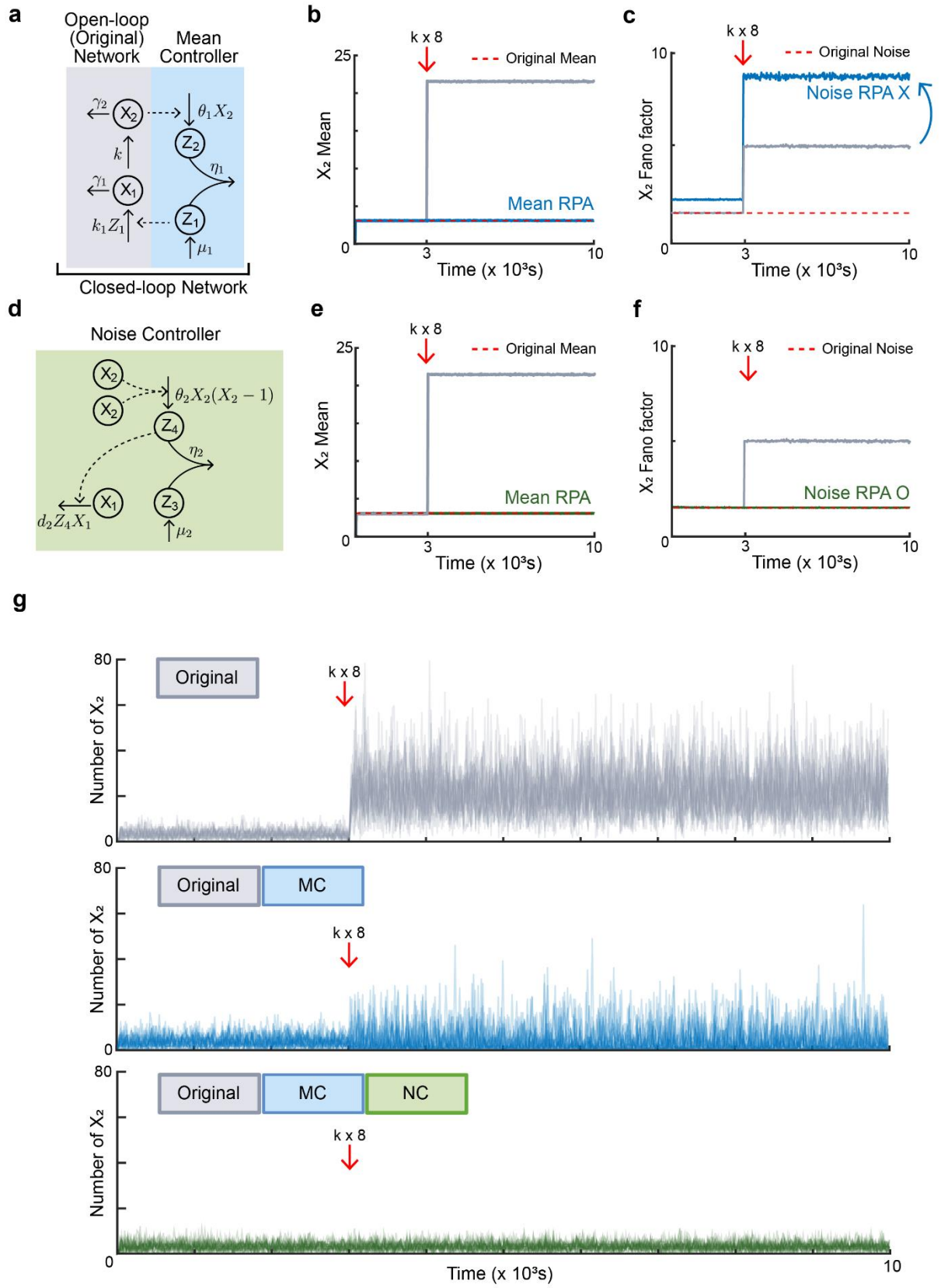
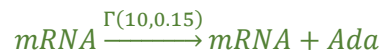


Figure 1. The noise controller achieves noise RPA.

9. The chemical reactions shown from lines 453-460. For the second reaction, instead of writing Gamma, why not use the symbol Γ ?

We have revised this typo.

Methods:



10. References. Ensure consistency. There are mixture of using title case, journal not abbreviated, etc. Specifically, Refs. 1, 6, 31 uses title case. Refs. 13 uses uppercase for the journal title, Refs. 20 and 28, journals are not abbreviated. Ref. 25, the alignment is more to the left compared to other references.

We have revised the references to ensure consistency in their formatting.

11. Data availability. I would highly recommend making the code available instead of the statement made in the manuscript for transparency and reproducibility.

We have provided 'noiseRPA', which includes the MATLAB code for reproducing the main figures in the revised manuscript.

Data and Code Availability:

The codes underlying this work are provided in the Github repository (<https://github.com/Mathbiomed/noiseRPA.git>)

We thank the reviewers for reviewing our response and acknowledging the improvement of revised manuscript. In light of additional comments, we have made revisions to our manuscript.

Below, you will find a point-by-point response to each comment. Our responses are indicated in blue, while the revised portions of the manuscript are highlighted in green (with italic font indicating the modified sections).

Reviewer #1 (Remarks to the Author):

The authors have done a commendable and thorough job in addressing the concerns raised in my previous review, providing substantial additional analysis and insightful discussion. Although certain theoretical aspects I mentioned earlier, such as checks for ergodicity, consistency with existing noise suppression limits etc., have only been partially explored, the manuscript nevertheless offers valuable perspectives and identifies promising directions for future research. Overall, I consider this an important and well-executed paper, and I find it acceptable for publication in Nature Communications in its current form.

We sincerely appreciate the reviewer's positive feedback on our revised manuscript.

Minor comment:

In the analysis of saturation effects presented in Supplementary Text S5, the authors state that as $K \rightarrow \infty$, the propensity $f_{Hill}(X_2)$ approaches $2\theta_2 X_2^2$. This does not appear to be correct, as the limit seems to approach zero unless V_{max} increases proportionally with K^2 to keep θ_2 constant. I recommend that the authors clarify this point.

As the reviewer pointed out, to make $f_{Hill}(X_2) = V_{max} \frac{X_2^2}{K^2 + X_2^2}$ approaches $2\theta_2 X_2^2$, we assumed that V_{max} increases proportionally with K^2 (i.e., $\theta_2 = \frac{V_{max}}{2K^2}$). This assumption is derived from the requirement that propensity of sensing reaction, expressed in mass-action form as $f(X_2) = \theta_2 X_2^2$, passes through the inflection point of the Hill function $\left(K, \frac{V_{max}}{2}\right)$ —an approach conceptually similar to that used in a previous study [1]. Substituting this relationship into the Hill-function expression shows that $f_{Hill}(X_2) = 2\theta_2 K^2 \frac{X_2^2}{K^2 + X_2^2}$ asymptotically approaches $2\theta_2 X_2^2$ as $K \rightarrow \infty$. We have clarified this point in the revised Supplementary Information.

Reference:

[1] Stephanie K Aoki, Gabriele Lillacci, Ankit Gupta, Armin Baumschlager, David Schweingruber, and Mustafa Khammash. A universal biomolecular integral feedback controller for robust perfect adaptation. Nature, 570(7762):533–537, 2019.

Supplementary Information:

Text S5. Effect of saturation effect

...To determine θ_2 in this reaction, we require the Hill function to closely approximate mass-action kinetics. Similar to the approach of S1.2 in [6], we do that by assuming that the propensity of sensing reaction, written in mass-action kinetics as $f(X_2) = \theta_2 X_2^2$, passes through the inflection point of the Hill function, $\left(K, \frac{V_{max}}{2}\right)$. Under this assumption, θ_2 is determined by the ratio of V_{max} to K^2 :

$$\theta_2 = \frac{V_{max}}{2K^2}$$

In other words, to achieve the desired value of θ_2 , V_{max} should be proportional to K^2 . By substituting this relationship into the equation, we get

$$f_{Hill}(X_2) = 2\theta_2 K^2 \frac{X_2^2}{K^2 + X_2^2}$$

We see that as $K \rightarrow \infty$, the propensity $f_{Hill}(X_2)$ approaches $2\theta_2 X_2^2$.

...

Reviewer #2 (Remarks to the Author):

The authors have clearly and successfully addressed all my queries. I am happy for the manuscript to be accepted in the current form.

Reviewer #2 (Remarks on code availability):

The provided codes seem complete and contain readme file for user to use and reproduce.

We sincerely appreciate the reviewer's positive feedback on our revised manuscript. We are pleased that the modifications have successfully addressed the previous concerns and improved the paper's quality.