# Cascaded Antithetic Integral Feedback Motifs for Robust Stability and Performance Improvement

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***Abstract*—Precise intracellular regulation and robust perfect adaptation can be achieved using biomolecular integral controllers and it holds enormous potential for synthetic biology applications. In this letter, we consider the cascaded implementation of a class of such integrator motifs. Our cascaded integrators underpin proportional-integral-derivative (PID) control structures, which we leverage to suggest ways to improve dynamic performance. Moreover, we demonstrate how our cascaded strategy can be harnessed to enhance robust stability in a class of uncertain reaction networks. We also discuss the genetic implementation of our controllers and the natural occurrence of their cascaded sequestration pairs in bacterial pathogens.**

***Index Terms*—Biomolecular feedback systems, genetic control circuits, robustness, synthetic homeostasis, synthetic biology.**

## I. Introduction

THE phenomenon of robust perfect adaptation (RPA), a hallmark of essential natural processes from the cellular to the organ level, underpins many vital life systems [1]–[4]. Achieving it through genetic devices promises advancements in synthetic biology applications [2]–[4]. Model-based designs of static feedback controllers, particularly integral feedback control (IFC), realizable through chemical reaction networks (CRNs), are promising initial steps. Recently, there has been significant interest in the characterization, design, and instantiation of biomolecular controllers and feedback systems for robust gene-expression control [5]–[21]. Antithetic integrators [8], in particular, have successfully achieved synthetic RPA in both bacteria [7], [10] and mammalian cells [21], [22].

Since its introduction in [8], the antithetic integral feedback controller (AIC) and its variants have received significant attention and have been studied theoretically from both deterministic and stochastic perspectives [23]–[35]. At its core, this motif uses a irreversible sequestration between two species to compute the integral of the tracking error. Under standard assumptions such as set-point admissibility and stability, this *core* antithetic controller tightly regulates the average concentration of a species of interest across a cell population [8].

It goes without saying that proportional-integral-derivative (PID) control has been the backbone of industrial control technology for its practical advantages [36], [37]. Using the core AIC, a rich repertoire of variant motifs have been proposed in the literature that encode PID control structures to enhance the dynamic performance [12], [24]–[27]. Recent works have also studied the stabilization of this controller when controlling certain plants [28]–[34]. Suggested stabilizing approaches hitherto include exploiting the controller constant degradation/dilution, molecular buffering [32], low-gain actuation, and the rein mechanism [30].

Building upon the core AIC motif [8], this letter introduces a higher-dimensional implementation of it—a controller motif we call "cascaded" AIC. In Section II, we first present a generalized formulation of this cascaded motif, followed by a representative minimal controller. The minimal cascaded controller motif remains the main focus of our study. We also discuss how the cascaded sequestration reactions, necessary components for our designs, naturally occur in certain bacteria.

The contributions of this paper are to explore our proposed biocontrollers and their control theoretic features. Previous works [26], [27] have shown that the core AIC follows a filtered I (PI) control structure when actuating via the reference (sensor) controller species. In comparison, our cascaded structure encodes a filtered PI (PID) mechanism when the actuation arrangement is the same. These underlying filtered PI and PID control structures are, therefore, intrinsic to our controller architecture itself. In Section III, we demonstrate how exploiting this novel architecture offers practical means and flexibility to improve nominal control performance.

Cellular systems are known for their inherent noise and complexity [38]. Considering large parametric/structural uncertainties in their nominal deterministic models is thus relevant, if not imperative. Ideally, the closed-loop system should be insensitive to these uncertainties. The risk of instability, however, is a known drawback of feedback systems [36], exacerbated when large uncertainties are present. Section IV delves into the analysis of stability robustness, characterizing a wide class of uncertain reaction networks that can be stabilized by our cascaded controller. Our approach leverages the tuning of certain constant inflow (zeroth-order) rates to lower the integrator gain. Remarkably, this method offers a flexible tuning knob for achieving robust stability in a biologically convenient manner, potentially minimizing the need for complex protein engineering that might otherwise be required if, e.g., design parameters associated to first or second-order rates were to be tuned. Before concluding this letter, we also discuss potential biomolecular realizations of our introduced controllers.

## II. Cascaded Antithetic Integrators

### A. Generalized Cascaded AIC Motifs

Let us assume that an uncertain, poorly-known process network of interest, called $\mathcal{X}$, is given. The control objective

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is a regulation task: designing a controller that steers the concentration of a target species, $\mathbf{X_n}$, within $\mathcal{X}$ to a command set-point, provided proper sensing and actuation. This controller acts on a known input species, $\mathbf{X_1}$. The regulation must be robust to process variations, and the set-point value should be inducible by the controller. A schematic illustration of our proposed controller, named cascaded AIC and capable of achieving such regulation (RPA), is provided in Fig. 1. For the $q$th-order cascaded AIC, we cascade $q-1$ different annihilation modules through $q$ controller species, $\mathbf{Z_1}$ to $\mathbf{Z_q}$. Following the generic reaction $\mathbf{Z_i} + \mathbf{Z_{i+1}} \xrightarrow{\eta_{i-1}} \varnothing$ for $i \in \{1, \cdots, q-1\}$, each controller species $\mathbf{Z_i}$ forms a sequestration pair with $\mathbf{Z_{i+1}}$, undergoing co-degradation at a rate $\eta_{i-1}$. The sensor species $\mathbf{Z_2}$ measures the current value of $\mathbf{X_n}$, which is used for computing the integral error and offsetting it. Except for $\mathbf{Z_2}$ and $\mathbf{Z_1}$, every controller species $\mathbf{Z_i}$ undergoes constant inflow production at a rate $\mu_{i-2}$. Follow this from Fig. 1A.

We do not specify functional roles for the resulting sequestration complexes, which may be active or inactive depending on the context. Nonetheless, their inclusion would not change the overall capacity of our controller to achieve RPA. By defining the integral variable $z = \sum_{i=1}^{i=q}(-1)^{i-1}z_i$, it is seen that $x_n^* = (\mu_0 + \sum_{i=1}^{i=q-2}(-1)^{i-1}\mu_i)/\rho$. Throughout, we use lowercase letters to represent the concentrations of the corresponding species denoted by bold, capital letters, and superscript asterisks to denote steady-state values. A closed-loop dynamical model for this generalized cascaded antithetic controller, including a list of necessary conditions for set-point admissibility, is reported in Supplementary Material, Section S1. In providing our dynamic models, we assume deterministic models, the law of mass action, and negligible effects of constant controller degradation/dilution.

### B. The Minimal Representation and Its Dynamical Model

The minimal cascaded AIC we obtain from the general motif by setting $q = 3$. Depicted in Fig. 1, this motif consists of three species and two intertwined annihilation modules. It has come to our attention that a recent preprint studies the noise reduction properties of this motif [35], without focusing on its control performance, which is the topic we investigate. The augmentation of the additional annihilation module changes the control structure. Later sections will discuss how this enables robustness and performance improvement. The resulting closed-loop dynamical behavior can be described by

$$\begin{cases} \dot{x} = \mathcal{F}(x) + e_1\Theta(z_1, z_2, z_3), & \text{(1a)} \\ \dot{z}_1 = \mu_0 - \eta_0 z_1 z_2, & \text{(1b)} \\ \dot{z}_2 = \rho x_n - \eta_0 z_1 z_2 - \eta_1 z_2 z_3, & \text{(1c)} \\ \dot{z}_3 = \mu_1 - \eta_1 z_2 z_3. & \text{(1d)} \end{cases}$$

Compared to the core AIC in [8], a new species, $\mathbf{Z_3}$, is added to the controller side, forming a sequestration pair with $\mathbf{Z_2}$. The dynamical behavior of $\mathcal{X}$ (i.e., the open-loop circuit) is described by the smooth vector function $\mathcal{F}(x) := [f_1, ..., f_n]^T : \mathbb{R}^n \to \mathbb{R}^n$, which includes $n \geq 1$ distinct species $\mathbf{X_1}$ to $\mathbf{X_n}$. Within the controller network, two separate annihilation reactions occur with potentially different propensities, $\eta_0$ and $\eta_1$. The smooth function $\Theta$ represents the

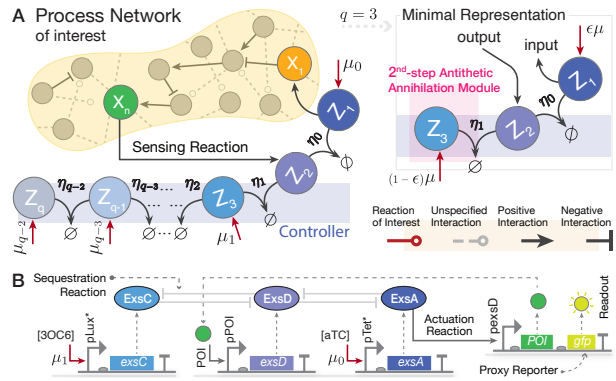


Fig. 1. Cascaded antithetic integral feedback control topology. (A) The general depiction of the motif is on the left panel, with the minimal representation featuring three active controller species on the right. The controller closes the loop by sensing from and actuating on an uncertain, poorly-known process network. (B) An example circuit to realize the minimal cascaded AIC motif, based on the regulatory genes involved in the T3SS of *P. aeruginosa* bacteria [39]. The controller is employed to regulate a one-species process comprising the protein of interest (POI). The POI acts as an activator for $\mathbf{Z_2}$. It is inserted in an operon including a fluorescent protein as a proxy for readout, whose expression level is proportional to that of the POI. pLux* and pTet* control the expressions of $\mathbf{Z_3}$ and $\mathbf{Z_1}$, respectively. The inducers 3OC6 and aTC are used to tune the activity of these two promoters. By adjusting their availability in the culture medium, one can tune both inflow rates $\mu_0$ and $\mu_1$. The mapping between the biological species and species $\mathbf{Z_1}$ to $\mathbf{Z_3}$ is provided in Table I.

cumulative effect of the control input applied to $\mathbf{X_1}$. For now, we allow the controller species $\mathbf{Z_1}$ to $\mathbf{Z_3}$ to freely act on the process input $\mathbf{X_1}$, though their structure may need to conform to certain specifications in later sections. By introducing the positive constants $\epsilon < 1$ and $\mu$, we parametrize the inflow rates $\mu_i$ as follows and hold it throughout this letter

$$\mu_0 = \epsilon\mu \quad \text{and} \quad \mu_1 = (1-\epsilon)\mu. \tag{2}$$

Under the assumptions of set-point admissibility and closed-loop stability, the output of interest regulates to the value $\mu/\rho$ at steady state. The former assumption, which corresponds to the feasibility of having a positive equilibrium point reachable from its neighborhood, remains an assumption throughout this letter. However, Section IV will provide guarantees of closed-loop stability for a class of process networks.

### C. Cascades of Sequestration Naturally Appearing in Bacteria

Reported in Table I, we observe that the sequestration pairs match up in cascaded forms among native proteins found in various bacterial pathogens [39]–[41], mainly in their type-III secretion system (T3SS) that enables them to infect human cells. For example, in *P. aeruginosa* bacteria, we observe cascaded sequestrations up to the fourth order between the proteins ExsA, ExsD, ExsC, and ExsE. The natural occurrence of the sequestration pairs required for our control topologies in bacterial genomes can simplify their incorporation. This emphasizes that higher-order complex control designs may not necessarily come with additional barriers to implementation.

## III. Exploiting the Underlying PI and PID Control Structures to Improve Nominal Performance

In this section, we focus on the closed-loop dynamic performance and explore the added flexibility in tuning offered by the minimal cascaded AIC motif. Specifically, we

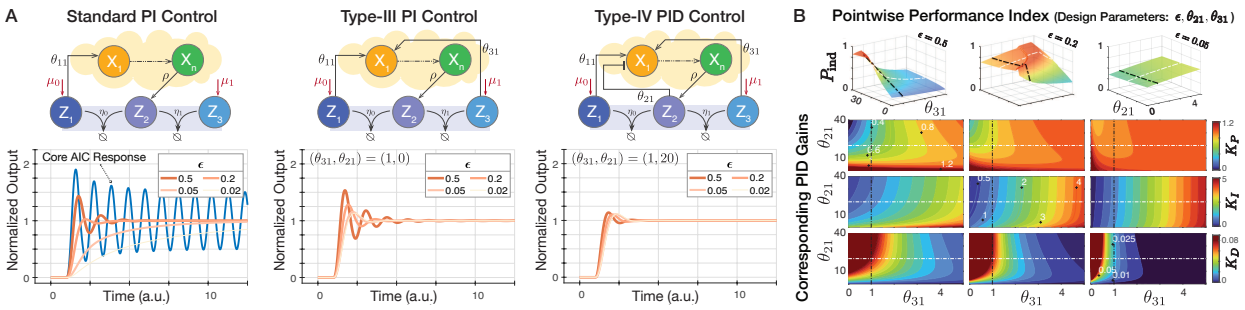


Fig. 2. Exploiting the filtered PI and PID control structures brought about by the cascaded implementation of the antithetic IFC provides a practical means for improving nominal control performance. The linear model of our cascaded AIC follows a filtered PI (PID) control structure when actuating only by $\mathbf{Z_1}$ ($\mathbf{Z_2}$). Different actuation rearrangements can lead to higher degrees of tunability, resulting in different types of PI and PID mechanisms. See Supplementary Material, Section 2B for further details. (A) For four different arrangements of the design parameter $\epsilon$, we illustrate the performance improvement for two PI and one PID type controllers, comparing them with each other and the core AIC. The core antithetic response, which corresponds to $\epsilon = 1$, shows instability. Here, $\Theta := \theta_{31} z_3 + \theta_{11} z_1/(1 + \theta_{21} z_2)$, $\theta_{11} = 6$, and the process parameters are fixed. (B) The pointwise performance index for variations in the design parameters $\theta_{21}$ and $\theta_{31}$ for three choices of $\epsilon$. The 2D contour plots indicate the proportional ($K_P$), integral ($K_I$), and derivative ($K_D$) gains corresponding to the design parameters. The transient performance is indexed by a measure $P_{\text{ind}}$, which is a weighted quantity of calculated overshoot ($Y_{\text{ovr}}$), settling time ($T_{\text{set}}$), and rise time ($T_{\text{ris}}$) from time trajectory data. This measure calculates the following value: $P_{\text{ind}} := 1/(1 + w_1 T_{\text{set}} + w_2 T_{\text{ris}} + w_3 Y_{\text{ovr}})$. The best (worst) achievable $P_{\text{ind}}$ is unity (zero). The regions within which we obtain $P_{\text{ind}}$ are chosen such that they are locally stable. From the performance index plots for the above control circuits, one can pick an optimal pair for $\theta_{21}$ and $\theta_{31}$. The weights were set to $w_1 = 1$, $w_2 = 1$, $w_3 = 10$.

TABLE I
CASCADED FORMS OF SEQUESTRATION IN BACTERIAL PATHOGENS

| Mappings to the Motif in Fig. 1 | | | Regulatory Function in[d] | Bacterial Strain | Ref. |
|---|---|---|---|---|---|
| $\mathbf{Z_3}$[a] | $\mathbf{Z_2}$[b] | $\mathbf{Z_1}$[c] | | | |
| ExsC | ExsD | ExsA | T3SS | *P. aeruginosa* | [40] |
| RsfA/B | UsfX | SigF | SACMM | *M. tuberculosis* | [42] |
| HrpG | HrpV | HrpR-HrpS | T3SS | *P. syringae* | [43] |
| InvE | SipC | InvF-SicA | T3SS | *S. typhimurium* | [44] |

[a] ExsC, RsfA/B, HrpG, and InvE: anti-anti-activators; ExsC has a secondary function as a chaperone; InvE is an invasion protein in Salmonella.
[b] ExsD, UsfX, HrpV, and SipC: anti-activators.
[c] ExsA: AraC-type transcription factor; SigF: alternate sigma factor; HrpR, HrpS, and InvF: transcriptional activators; SicA: type-III secretion chaperone.
[d] SACMM: Stress adaptation and cell membrane modification.

investigate whether the structural difference from the core AIC, brought about by the addition of the extra annihilation module, provides this flexibility. To do this, we analyze the controller transfer function standalone. We allow each $\mathbf{Z_i}$ to be involved in $\Theta$, which expands our design flexibility. Recall that $\Theta(t)$ represents the applied actuation signal. Our small-signal analysis uncovers that, enabled by certain arrangements of $\Theta$, the minimal cascaded AIC encodes PI and PID compensatory structures. The Laplace domain transformation of the control input $\Theta$ linearized about the fixed point follows

$$K_I \tilde{E}(s)/s + (K_{F_0} + K_{F_D} s)\tilde{\mu}(s) - (K_P + K_D s)\tilde{X}_n(s)$$

filtered in series through a second-order low-pass filter. Here, $K_P$, $K_I$, $K_D$, and $K_{F_\bullet}$ are the proportional, integral, derivative, and feedforward gains. $\tilde{\mu}$, $\tilde{X}_n$, and $\tilde{E}$ are the reference, output, and tracking error signals, respectively.

The above gains are available in Supplementary Material, Section S2B in terms of biomolecular parameters. By rearranging the actuation terms and based on our cascaded mechanism, we report therein seven different variant motifs that encode PI or PID control structures (Supplementary Fig. S2). These motifs neither require satisfying parametric conditions to maintain positive PID gains nor introduce additional nonminimum-phase zeros into the closed-loop system. Three of them follow filtered PI and the rest PID mechanisms. Interestingly, with only a single $\mathbf{Z_i}$ actuating—specifically, a negative (positive) actuation from $\mathbf{Z_2}$ ($\mathbf{Z_1}$ or $\mathbf{Z_3}$)—one can realize a filtered PID (PI) control structure. See Supplementary Material for details.

Specific designs for $\Theta$, e.g., those involving Hill kinetics, may offer greater flexibility in tuning the gains and cutoff frequencies, thereby enhancing the tunability of both disturbance rejection and tracking performances. Process variations, however, influence the gains, as these gains depend on both design parameters and steady-state values. This inseparability of gains is inherent to local analysis and is a known issue in nonlinear PID realizations through CRNs [26]. Despite this, we may still be able to exploit the extra degrees of freedom that the encoded PI and PID architectures offer, to significantly improve the transient dynamics of the *nominal* system. Fig. 2 confirms this for a sample $\Theta$ across three of the motifs. For our simulation case studies throughout this letter, we consider an $n$-species unimolecular activation cascade as the controlled process (Fig. 3A), characterized by a lower-triangular Metzler state matrix. Numerical values and details regarding all figures are available in Supplementary Material, Section S3.

## IV. ROBUST STABILITY ANALYSIS

The nominal performance improvement discussed in the previous section may come at the cost of robust stability attenuation when uncertainties are taken into account. This can lead to trade-offs between stability robustness and transient performance [19], [36]. Such hard limits are often imposed by the introduction of feedback, particularly relevant in IFC systems due to Bode's integral theorem [28], [36]. Simulations provided in Fig. 3 illustrate this trade-off for a set of nominal parameters and bounded uncertainties. As shown, although tuning the design parameters—here $\epsilon$, which parametrizes the induced set-point distributed among $\mathbf{Z_1}$ and $\mathbf{Z_3}$ through the inflows rates, as in (2)—can achieve the desired level of overall performance, the optimal (nominal and average) performance index may coincide with a compromised robust stability index.

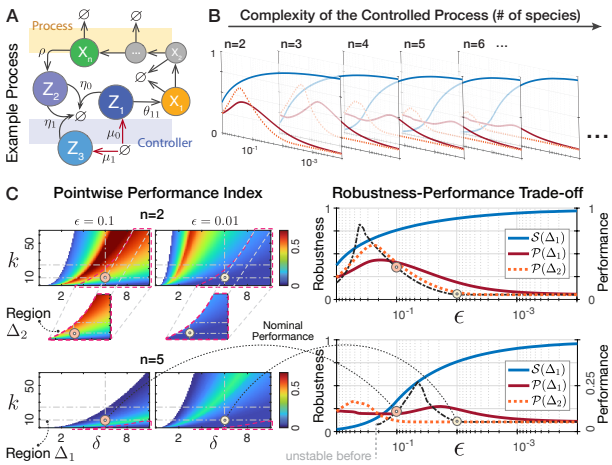


Fig. 3. The trade-off between stability robustness to process variations and control performance is illustrated using two metrics, $\mathcal{S}(\Delta)$ and $\mathcal{P}(\Delta)$. The argument $\Delta$ identifies the uncertain parametric region for computation. Both metrics operate on the pointwise results of the stability status and performance index over $\Delta$, while keeping the other parameters fixed at their nominal values. The metric $\mathcal{S} \in [0,1]$ computes the stable fraction within $\Delta$, whereas $\mathcal{P} \in [0,1]$ averages the pointwise performance indices ($P_{\text{ind}}$ defined in Fig. 2) over all stable points within $\Delta$. The dash-dotted black lines indicate the nominal performance index. The region $\Delta_1$ represents the entire rectangle within which the process parameters $k$ and $\delta$ vary. $\Delta_2$ is the common stable region across the considered variations of $\epsilon$. It is a subset of $\Delta_1$ in which every point is locally stable given all uncertain regions of $k$, $\delta$, and $\epsilon$.

This section discusses tuning strategies to improve robust stability. We are mainly interested in achieving this through tuning the controller inflow rates $\mu_i$, which can significantly simplify design constraints and facilitate experimental implementation. We carry a linear perturbation analysis local to an equilibrium point $q$ and prove its stability for sufficiently small choices of $\epsilon$. Let us denote the transfer function associated with the open-loop $\boldsymbol{\mathcal{X}}$ by $P(s) := N(s)/D(s)$, with $N$ and $D$ being polynomials in $s \in \mathbb{C}$. It follows $P(s) = e_n^{\mathrm{T}} (sI - A_{\mathrm{p}})^{-1} e_1$, where $A_{\mathrm{p}} := \partial \mathcal{F}|_q$ and $I$ is the identity matrix.

**Assumptions.** *The following hold for the closed-loop (1):*

*(A1) The set-point $\frac{\mu}{\rho}$ is admissible, implying the existence of an isolated fixed point $q = [x_1^*, \cdots, \frac{\mu}{\rho}, z_1^*, z_2^*, z_3^*] \in \mathbb{R}_{\geq 0}^{n+3}$.*

*(A2) The function $\Theta$ is independent of $z_2$ and $z_3$.*

**Theorem 1.** *Hold (A1)-(A2). Define $\sigma_{11} := \partial\Theta/\partial z_1|_q$ and $N'(s) := \mathrm{d}N(s)/\mathrm{d}s$. Adopt the $\epsilon$-parametrized formalism in (2) and fix every system parameter except $\epsilon$. If the open-loop $\boldsymbol{\mathcal{X}}$ satisfies the following conditions, then there exists a sufficiently small $\epsilon^*$ such that for every $0 < \epsilon \leq \epsilon^*$ the equilibrium point $q$ of the closed-loop system (1) is locally asymptotically stable.*

*(C1) The given open-loop network $\boldsymbol{\mathcal{X}}$ is stable*

*(C2) $\sigma_{11}\epsilon\rho\mu\eta_1 N(0) > 0$*

*(C3) $\sigma_{11}\rho\eta_0 z_1^* N(0) + \sigma_{11}\epsilon\rho\mu\eta_1 N'(0) \geq 0$*

*Proof.* The standalone controller subsystem involves an integrator, which is unstable, suggesting that the small-gain theorem may not be applicable to the closed-loop interconnection. We rely instead on an argument based on the root locus rules and perturbation of complex-valued functions to provide our proof. From the form of the corresponding linearized closed-loop system's characteristic polynomial, denoted by $p(s)$ and reported in Supplementary Material, Section S2A, it can be found that $n+2$ of its roots approach to those of the dominant term $s^2 D(s)$ as $\epsilon \to 0$. Thus, $n$ of them are stable by (C1) as they are the roots of $D(s)$. The root locus rules entail that the negative real axis is an asymptote, so there exists a stable root approaching $-\infty$ in the limit $\epsilon \to 0$. Now consider an infinitesimally small disc $\mathscr{D} = \{s \in \mathbb{C} : |s| \leq r^*\}$ with $0 < r^* \ll 1$ about the origin of the complex plane. Then, corresponding to every small $r^*$ there exists a sufficiently small $\epsilon^*$ such that for every $\epsilon \leq \epsilon^*$ two roots of $p(s)$ lie on $\mathscr{D}$, while the remaining roots have all negative real parts. The proof will be complete if we find conditions that enforce these two roots both lie in the open left-hand half of $\mathscr{D}$.

We calculate the value of $p(s)$ at $s = 0$ as $p(s)\big|_{s=0} = \sigma_{11}\epsilon\rho\mu\eta_1 N(0)$. (A2) implies $\partial z_1^*/\partial\epsilon = 0$. The derivative of $p(s)$ w.r.t. $s$, which we know exists since it is a holomorphic function, is $\partial p(s)/\partial s = (2sD(s) + s^2 D'(s))[s + \epsilon\frac{\mu}{z_1^*}(1 + \frac{\eta_1}{\eta_0}) + \eta_0 z_1^*/\epsilon] + (D(s) + sD'(s))[\mu\eta_0(1-\epsilon) + \epsilon\mu\eta_1 + \epsilon^2\frac{\eta_1}{\eta_0}(\frac{\mu}{z_1^*})^2] + s^2 D(s) + \sigma_{11}\rho\eta_0 z_1^*(sN'(s) + N(s)) + \sigma_{11}\epsilon\rho\mu\eta_1 N'(s)$, where $D'$ represents $\mathrm{d}D(s)/\mathrm{d}s$. These are polynomials of $s$ with real coefficients. Note, $D(0)$ is real and positive. Moreover, the term $\mu\eta_0(1-\epsilon) + \epsilon\mu\eta_1 + \epsilon^2\eta_1(\mu/z_1^*)^2/\eta_0$ is always positive for $\epsilon < 1$. It is easy to observe that $p(0)$ is strictly positive for any $\epsilon > 0$ if (C2) is met. Also, for any $\epsilon$, $\Re(\frac{\partial p}{\partial s}\big|_{s=0})$ is a positive number if (C3) is met, in which case $\Im(\frac{\partial p}{\partial s}\big|_{s=0})$ is equal to zero. Equivalently, the phase of $p(s)$ does not change local to the origin as $s \to 0$ in any arbitrary direction. Therefore, as $r^*$ goes to zero, $p(s)$ maps the points existing in the right-hand half of $\mathscr{D}$ to the right of the vertical line $\Re(p(0))$. These points, thus, cannot be roots of $p(s)$, as $\Re(p(0)) > 0$. Taken together, meeting (C1)-(C3) guarantees that the two roots in the interior of $\mathscr{D}$ have always negative real parts as $r^* \to 0$. □

**Remark 1.** *For small enough $\epsilon$s and when actuating positively (negatively) by $\mathbf{Z_1}$, or equivalently when $\sigma_{11}$ is positive (negative), the process-dependent conditions (C2)-(C3) are always fulfilled regardless of $N'(0)$, provided $N(0) > 0$ ($N(0) < 0$).*

**Remark 2.** *Assume that (A1)-(A2) and (C1)-(C3) hold. As $\epsilon \to 0$, two closed-loop poles approach the origin (from the stable side) and the rest go to the open-loop poles and $-\infty$.*

Considering positive actuation from $\mathbf{Z_1}$, the conditions that the open-loop has to satisfy are that it (i) needs to be stable and (ii) must have a positive DC gain (none or an even number of unstable zeros), Remark 1 says. Now, assume that the process parameters or dynamics are uncertain and vary within a bounded $\mathscr{C}^1$ set $\Delta P$. Similarly, assume that the controller parameters, including the actuation gains, are uncertain within the bounded set $\Delta C$. According to Theorem 1, there exists a sufficiently small $\epsilon$ which makes the entire uncertain closed-loop system stable as long as $\Delta P$ and $\Delta C$ do not violate (A1)-(A2) and $\Delta P$ does not make the open-loop $\boldsymbol{\mathcal{X}}$ unstable or its DC gain negative. Of note, structurally stable open-loop circuits with positive DC gain fulfill these conditions by structure, regardless of their exact parameter values. These include uncertain activation cascades of any size, which are good models of known intracellular processes, e.g., gene expression (with maturation stages) activated through signaling pathways.

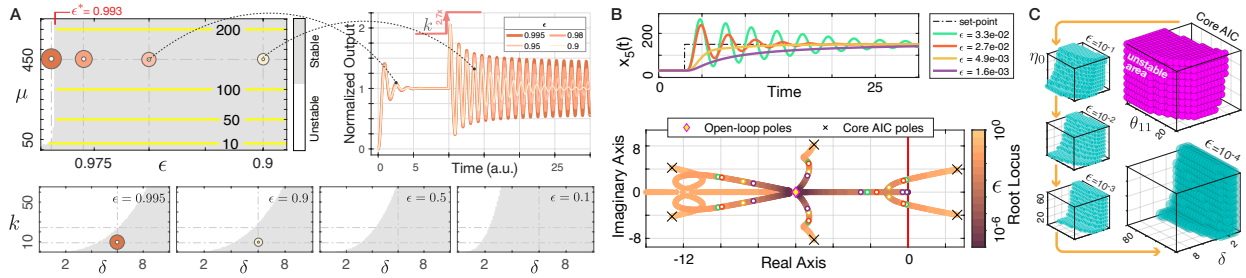


Fig. 4. Cascaded antithetic integrators can be harnessed to improve stability robustness against uncertainties by adjusting the controller constant inflow production rates. We numerically evaluate the local stability of the closed-loop dynamic model from (1), where the actuation function $\Theta$ follows $\Theta = \theta_{11} z_1$. (A) Compared to the core AIC motif (when $\epsilon = 1$), decreasing $\epsilon$ improves the closed-loop relative stability for variations in the set-point and process parametric uncertainties ($n$ is set to two). Contour lines in yellow indicate the corresponding set-point. The 2D stability plots show the stability status over an uncertain 2D parametric region. (B) Root-locus plot of the closed-loop system with $n = 5$ for variations in $\epsilon$. Corresponding set-point tracking responses for four sample $\epsilon$s are shown in the top row. (C) The 3D stability plot for simultaneous bounded parametric uncertainties in both controller and process dynamics. The three§ parameters $\delta$, $\theta_{11}$, and $\eta_0$ are varied ($n = 5$). $\delta$ represents the constant degradation of every process species, which are set to the same value.

Our numerical results provided in Fig. 4 support the findings above. According to Theorem 1 and Remark 2, two closed-loop poles approach the origin as we improve the robustness by pushing $\epsilon$ toward zero. This means that the time response of the system becomes slower in the limit $\epsilon \to 0$, resulting in an overdamped response with no overshoot that is highly robust to uncertainties. This is consistent with the robustness-performance trade-off discussed earlier. We note, however, that one may still achieve better robustness and also performance results compared to the core AIC regulator (see Fig. 3).

## V. Biomolecular Realizations using Genetic Parts

This section explores four different realizations of the control circuits considered in the previous sections using synthetic genetic parts. Three of these realizations utilize inteins—short amino acid sequences capable of protein splicing [22]—to implement the sequestration mechanisms. The fourth relies on non-coding small RNAs and a protein engineered to sequester them, complementing the cascade. These are depicted in Fig. 5. The minimal cascaded AIC motif, which actuates only through $\mathbf{Z_1}$ and follows the standard PI structure reported in Fig. 2, can be realized by either circuit in Fig. 5A or B. The type-III PI and PID mechanisms visited in Fig. 2, can be realized by the circuits in Fig. 5C and D, respectively. Upon proper protein folding, the split intein pairs dimerize and undergo protein *trans* splicing, which shuffles the sequences linked to them. This process covalently links the peptide upstream of $\text{Int}^{\text{N}}$ to the peptide downstream of $\text{Int}^{\text{C}}$, with the remaining sequences remaining as a separate heterodimer. This mode of action enables irreversible sequestration between the two protein complexes to which the intein pairs are fused, resulting in functional/inactive sequestration complexes. For more details, see [11], [22] and references therein. In intein-mediated realizations, we have intentionally fused the split DNA binding domains (DBDs) in locations such that the resulting sequestration complexes are transcriptionally inactive. Note that the species with two complementary split DBDs fused to them can act as a repressor for a downstream gene if not attached to an activation domain (AD) or as an activator if attached to an AD. We have used this flexibility in the shown circuits to wire the actuation channels, thereby spanning the design to different PI and PID mechanisms discussed earlier.

## VI. Conclusion

Promising for synthetic biology, precise regulation and synthetic adaptation can be achieved using biomolecular integral controllers. In this letter, we considered cascaded AICs. Compared to the core AIC, our proposed integrators encode various PI and PID control structures, which we showed can be exploited to improve dynamic performance. Our numerical results verified this across a range of parameters (Fig. 2). As with other PI and PID mechanisms reported in the literature, the performance improvements via these structures are highly specific to the process under control and sensitive to parameter choices. Yet, these PI and PID structures offer a powerful tool to tune the performance of the nominal system. Considering uncertainties in the nominal system, our mathematical analysis showed that the proposed cascaded integral control structure provides accessible means to improve robust stability in controlling a wide class of uncertain CRNs. This could be further leveraged to shape the governing robustness-performance trade-off (Fig. 3). Lastly, we discussed potential genetic implementations of our introduced motifs within synthetic circuits.

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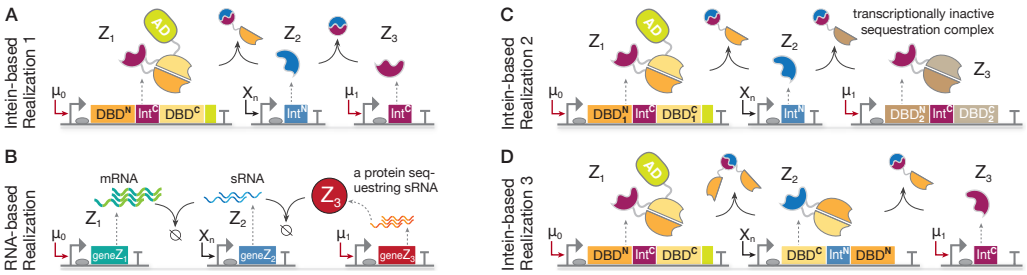


Fig. 5. Biomolecular realizations of the minimal cascaded antithetic integrators through synthetic genes. (A) Realizing the controller through protein-protein sequestration reactions mediated by split inteins. We utilize an intein-mediated framework, introduced in [22] to realize the core AIC motif, to suggest cascading several annihilation modules. Among the controller species, only $\mathbf{Z_1}$ is transcriptionally active. By fusing more activation domains or changing the activation strength, one can tune its affinity for acting on a downstream gene. $\mathbf{Z_1}$ can serve as a transcription factor for the promoter of an upstream gene that regulates $\mathbf{X_1}$, for instance, in order to establish an intermediary positive actuation on the process. (B) A specific mRNA may be sequestered by a cognate sRNA, which is simultaneously being sequestered by an RNA-binding protein. The resulting protein from the translation of this mRNA may act as a transcriptional activator or repressor for other genes, establishing the actuation in the same way as in (A). (C) and (D) Continuation of the genetic realizations of the minimal cascaded AIC motif, now with $\mathbf{Z_2}$ and $\mathbf{Z_3}$ being transcriptionally active complexes. This enables the realization of the type-III PI and the type-IV PID mechanisms considered in Fig. 2. Note that the species $\mathbf{Z_3}$ in (C) can act directly as a repressor for $\mathbf{X_1}$'s promoter or may establish an intermediary positive interaction with it, instead. As such, it may be engineered to repress an intermediate gene that is a repressor for $\mathbf{X_1}$.


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