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Frequency domain analysis of small non-coding RNAs shows summing junction-like behaviour

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***Abstract*— Small non-coding RNAs (sRNA) are a key bacterial regulatory mechanism that has yet to be fully exploited in synthetic gene regulatory networks. In this paper a linear design methodology for gene regulatory networks presented previously is extended for application to sRNAs. Standard models of both sRNA inhibition and activation are presented, linearised and transformed into the frequency domain. We demonstrate how these mechanisms can emulate subtraction and minimum comparator functions in specific parameter regimes. Finally, the design of a genetic feedback circuit is included, illustrating that sRNAs can be used to improve the performance of a range of synthetic biological systems.**

I. Introduction

Synthetic biology is an emerging discipline concerned with the rational engineering of biological organisms to address problems in fields ranging from manufacturing to healthcare [1]. This is typically achieved via the re-purposing of natural biological mechanisms, though this can prove challenging in many circumstances: synthetic systems are easily perturbed by fluctuations in the cellular environment [2], and may have unforeseen interactions with other cellular processes [3]. To address these challenges, in recent years much work has focused on the development of synthetic biological feedback control systems [4], analogues of which are utilised extensively in control engineering to overcome problems associated with noise and uncertainty [5].

Inspired by the feedback architectures found in natural biological systems, synthetic biologists have taken a range of approaches to implementing control structures to improve the reliability of their designs: Feedback systems have been built using networks of transcription factors [6], [7], interacting nucleic acid sequences [8], *in silico* control systems that measure and regulate cellular behaviour [9], and via interaction with existing cellular processes [10], [11]. Many of these designs have demonstrated favourable properties, such as robustness to both cellular fluctuations and the variability in the behavioural properties of their component parts [4], [12], [13]. Theoretical treatments have provided some guidelines for the design of such systems, outlining architectural motifs for which components must then be selected for implementation [14], [15]. As synthetic biological feedback systems mature, and their range of potential applications grows, it will be increasingly necessary for such constructs to utilise application-specific components that can optimally interface with a given system [16]. This motivates our current work, which develops a theoretical grounding for a small non-coding RNAs (sRNA) implementation of subtraction and minimum comparator junctions. These junctions are particularly useful for feedback controllers, which require a subtraction junction in order to compute the feedback error. This has motivated the development of a range of architectures for their implementation [17], [18], [19], though realising a two-sided junction (one that can produce both positive and negative outputs depending on the difference of inputs) remains challenging [20].

sRNAs find widespread use as a natural regulatory mechanism in bacteria [21]. They utilise the complementary base pairing between mRNA molecules to perform a range of functions; they can alter mRNA secondary structures to activate expression of a protein, or cover (thereby preventing the recognition of) parts of an mRNA sequence to repress expression [22], [23]. sRNAs lend themselves to application in synthetic biological circuits because of the ease with which they can be designed and implemented; the interactions between complementary RNA sequences can be predicted and tuned, and due to the variety of possible sequences many orthogonal sRNA regulatory mechanisms can operate within a cell simultaneously [24], [25], [26]. The sRNAs studied in this work can be broken down into two major parts, a target binding region, and a Host-Factor Bacteriophage QB (Hfq) protein binding region (with an overlapping terminator region) [27]. The first region, which is responsible for binding the target mRNA, can be designed using a range of binding strategies [25], [28] and tailored to avoid off-target effects [29]. The second region recruits the Hfq chaperone (whose presence slows the degradation of sRNA [30]), which is responsible for catalysing the sRNA's pairing with its target mRNA [27]. Design of the second region is more restricted, as (in the sRNAs we consider) it must recruit and bind the Hfq protein, and so it is generally adapted from natural scaffolds [25].

In this paper we design two-input sRNA/mRNA regulatory systems, which are assumed to work in a regime where catalysis by the Hfq chaperone is efficient. The system inputs are transcription-factor proteins which regulate expression rates of sRNA/mRNA respectively. In each case the targeted mRNA produces a protein (the system's output), which could be chosen as a fluorescent indicator for testing purposes, or a

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transcription factor to interface with downstream processes. We extend the linear design framework described in [31] to analyse systems that employ sRNA in both an inhibitory and activatory role, and demonstrate that these can be used to approximate subtraction and minimum comparator junctions.

In Section II we develop a four-state mathematical model for an sRNA repression system, and demonstrate its use as a subtraction junction. We examine the steady state behaviour of our model, and then linearise it to derive transfer functions between each input and an output protein's concentration. We provide a block-diagram framework for this system, and perform model-reduction to derive a simplified two-state model which demonstrates a similar dynamic response. In Section III a similar analysis of an sRNA activation system is performed, demonstrating its use as a minimum comparator. Section IV provides an example of our system's implementation, describing a closed-loop feedback architecture that utilises an activating sRNA to regulate a gene's expression. Finally, Section V concludes the paper.

II. sRNA Repression

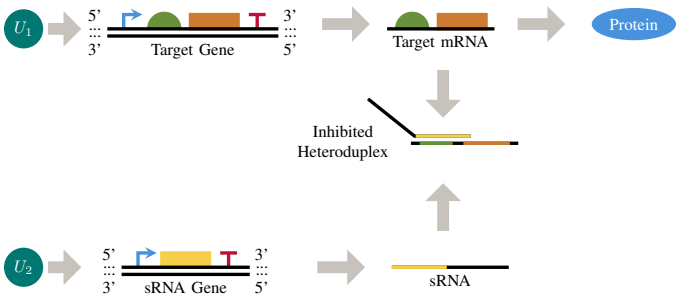

Fig. 1: The sRNA inhibition mechanism (Hfq not shown). The bottom gene is the inhibitory sRNA gene which is transcribed into its active RNA form. Its target is the top operon, encoding a protein, which is first transcribed and then translated into protein. The active sRNA blocks translation by binding the target mRNA (covering the RBS and start codon), forming an inactive heteroduplex.

We consider a two-input system that uses sRNA to bind a target mRNA, thereby controlling expression of a protein (Fig. 1). The system's inputs are transcription factors which regulate the production rates of sRNA and mRNA separately, and its output is the concentration of the protein produced by the target mRNA. A differential equation model of this system takes the form:

$$\dot{m} = \Gamma_1 - \delta_m m - kmr, \tag{1a}$$
$$\dot{r} = \Gamma_2 - \delta_r r - kmr, \tag{1b}$$
$$\dot{m}_r = kmr - \delta_{m_r} m_r, \tag{1c}$$
$$\dot{p} = \alpha_p m - \delta_p p, \tag{1d}$$

where p is the concentration of produced protein, m is the target mRNA concentration, r is sRNA concentration, and m_r is the concentration of repressed heteroduplex formed by the target mRNA and sRNA. α_p is the translation rate of protein from the un-repressed mRNA, and $\delta_p/\delta_m/\delta_r/\delta_{m_r}$ are the degradation rates of protein/mRNA/sRNA/repressed heteroduplex respectively. Finally, k is the rate with which mRNA and sRNA combine to form the inhibited heteroduplex (which we assume to be irreversible, so that degradation occurs more quickly than dissociation), and Γ_i are activating transcription factor-dependent Hill functions given by:

$$\Gamma_i = \frac{\beta_i [u_i]^{n_i}}{K_i^{n_i} + [u_i]^{n_i}} \tag{2}$$

where β_i are scaling factors for the maximal transcription rate, n_i are hill coefficients, and K_i are the dissociation constants of the activating transcription factors u_i. The derivative of (2) evaluated at a given u_i^* is:

$$\gamma_i^* = \beta_i \left. \frac{n_i K_i^{n_i} u_i^{n_i - 1}}{(K_i^{n_i} + u_i^{n_i})^2} \right|_{u_i = u_i^*} \tag{3}$$

If a repressing transcription factor is used instead, then the form of (2) changes slightly (in the numerator, u_i is replaced by K_i), and the expression for γ_i^* in (3) is multiplied by -1. For the remainder of Section 2 we disregard (1c) since m_r has no impact on the system's output, p.

A. Parameter Selection

Values for the parameters in (1) and (2) are chosen in line with those used in [32]. The transcription rate for the output protein P is set as $\alpha_p = 0.3\ nM\ min^{-1}$. The scaling factors for transcription in the Hill functions (2) are set equal as $\beta_{1,2} = 1\ nM\ min^{-1}$. We assume the system operates with an abundance of Hfq protein, such that free sRNA is guarded from degradation by the Hfq chaperone [30]. We thus set the degradation rate of sRNA equal to that of the protein (which we assume is primarily removed by dilution due to cell growth), with a value $\delta_p = \delta_r = 0.03\ min^{-1}$ (corresponding to a cell doubling time of $\sim 21\ min$ [32]). We assume that the degradation rates of free mRNA and the mRNA-sRNA complex are equal, since we do not expect that the degradation rate of mRNA will be substantially altered by the binding of sRNA (with appropriately chosen Hfq-binding sRNA scaffold), and so set $\delta_m = \delta_{m_r} = 0.14\ min^{-1}$. Finally, the sRNA-mRNA binding rate constant is set as $k = 1\ nM^{-1} min^{-1}$, the Hill function coefficient is set as $n_{1,2} = 2$ in line with that found for many common transcription factors [33], and an intermediate value [34] for the transcription-factor dissociation constant is chosen, $K_{1,2} = 10\ nM$.

B. Steady-state Response

For an input combination $u^* = [u_1^*, u_2^*]$ the system in (1) has a steady-state equilibrium $x^* = [m^*, r^*, p^*]$ given by:

$$m^* = \frac{-\delta_m \delta_r + k(\Gamma_1^* - \Gamma_2^*) + \sqrt{\omega}}{2\delta_m k}, \tag{4a}$$
$$r^* = \frac{\Gamma_2^*}{\delta_r + km^*}, \tag{4b}$$
$$p^* = \frac{\alpha_p}{\delta_p} m^*, \tag{4c}$$

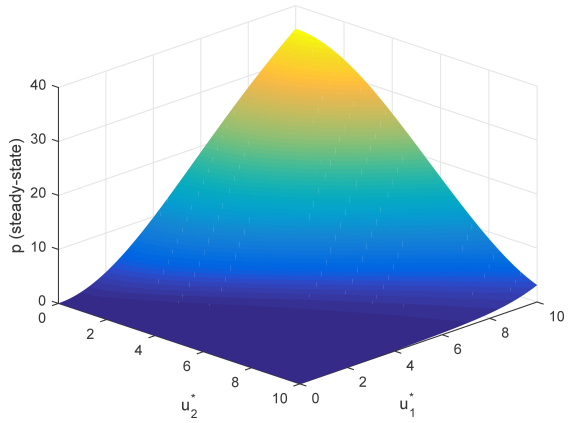

Fig. 2: Steady state output (p^* in (4c)) for the full model (1) of sRNA repression. This system functions as a subtraction junction for the two inputs u_1 and u_2.

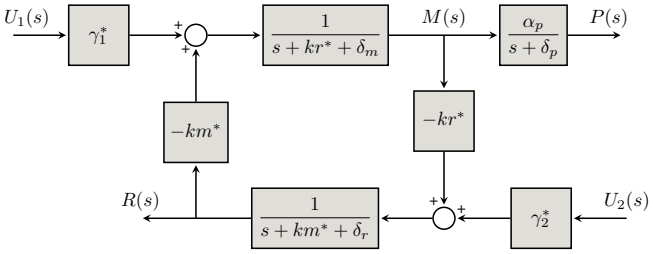

Fig. 3: Block diagram for the sRNA inhibition mechanism

where $\omega = (\delta_m\delta_r + k(\Gamma_2^* - \Gamma_1^*))^2 + 4k\delta_m\delta_r\Gamma_1^*$. Here the system output (p^*) depends on the difference between induced mRNA and sRNA production rates ($\Gamma_1^* - \Gamma_2^*$), which provides subtraction junction-like behaviour. However, due to the non-linear dependence of Γ_i on u_i, a direct subtraction of transcription-factor concentrations is not achieved. The system's steady-state output (p) response for varying inducer concentrations is shown in Fig. 2, demonstrating slight deviation from a linear subtraction $u_1 - u_2$.

C. Frequency Response

We can re-define (1) in terms of perturbations around the equilibrium values $\tilde{x} = x - x^*$ and $\tilde{u} = u - u^*$ to derive a linear approximation to our system:

$$\dot{\tilde{m}} = \gamma_1^*\tilde{u_1} - (kr^* + \delta_m)\tilde{m} - km^*\tilde{r}, \tag{5a}$$
$$\dot{\tilde{r}} = \gamma_2^*\tilde{u_2} - (km^* + \delta_r)\tilde{r} - k\tilde{m}r^*, \tag{5b}$$
$$\dot{\tilde{p}} = \alpha_p\tilde{m} - \delta_p\tilde{p}, \tag{5c}$$

We apply Laplace transforms to (5) to re-cast this system of equations in the frequency domain, for which transfer functions are expressed illustrated in Fig. 3. These expressions can be manipulated to find the transfer function between each of the inputs and the output, giving:

$$\begin{aligned} G_1(s) &= \frac{P(s)}{U_1(s)} = \frac{\alpha_p\gamma_1^*(s+\delta_r+km^*)}{(s+\delta_p)(s+s_+)(s+s_-)}, \\ G_2(s) &= \frac{P(s)}{U_2(s)} = \frac{-km^*\alpha_p\gamma_2^*}{(s+\delta_p)(s+s_+)(s+s_-)}, \\ s_\pm &= \frac{1}{2}\left(\delta_m + kr^* + \delta_r + km^*\right) \pm \frac{1}{2}\sqrt{d}, \\ d &= (\delta_m + kr^* - \delta_r - km^*)^2 + 4k^2m^*r^*, \end{aligned} \tag{6}$$

We observe that these two transfer functions (from U_1 and U_2 to the output P) have opposite sign, indicating that this system can be used as a subtraction junction. In both cases the $(s + \delta_p)$ term presents the dominant pole (since for the parameter and input values chosen $\delta_p \ll s_\pm$) and will hence dictate the approximate response time for our system. For this pair of transfer functions we observe minimal response ($G_i(0) \sim 0$) in the regime in which mRNA induction level is small compared to sRNA ($u_1^* < u_2^*$), since all mRNA is bound by sRNA (and so no output protein is produced). The subtraction junction is thus one-sided [20], though this could be overcome via architectures that implement a second similar system within the same cell that produces a "negative" output [18] (for example, two systems could produce proteins that activate or repress a downstream protein's expression respectively). sRNA (compared to other biochemical implementations) is ideal for achieving this, since orthogonal sRNA repressors with similar behavioural properties can be created. A major biological limitation of our system is that due to the functional form with which inputs are applied (the Hill function in (2)), the sensitivity to an input signal (U_i) varies according to a non-linear function of the given input's steady-state value (u_i^*). Ideally, we desire a subtraction junction where the DC gain is balanced for signals at either input. Equating the expressions for $G_1(0)$ and $G_2(0)$, and recalling that that $km^* \gg \delta_r$, we find this is true if $\gamma_1^* \approx \gamma_2^*$. Thus, in order to tune the weighting of the two inputs to this subtraction junction the parameters β_i in (2) can be adjusted (via modification of the transcription factor's promoter binding region) to achieve some parity in the values γ_i^* if substantially differing input levels (u_i^*) are to be combined.

D. Non-linear Analysis

To simplify our system (thereby reducing the number of free parameters, and aiding its utilisation as part of larger models) we can non-dimensionalise (1) and reduce the number of states by making assumptions about relative parameter values. Following the assumptions made in Section II-A we set $\delta_p = \delta_r$, and since degradation rates of mRNA are substantially greater than that of protein we have that $\frac{\delta_p}{\delta_m} \ll 1$. Setting $p = \psi\hat{p}$, $m = \phi\hat{m}$, $r = \eta\hat{r}$, $t = \theta\hat{t}$ we have from (1):

$$\epsilon\dot{\hat{m}} = 1 - \hat{m} - K_m\hat{m}\hat{r}, \tag{7a}$$
$$\dot{\hat{r}} = 1 - \hat{r} - K_r\hat{m}\hat{r}, \tag{7b}$$
$$\dot{\hat{p}} = \hat{m} - \hat{p}, \tag{7c}$$

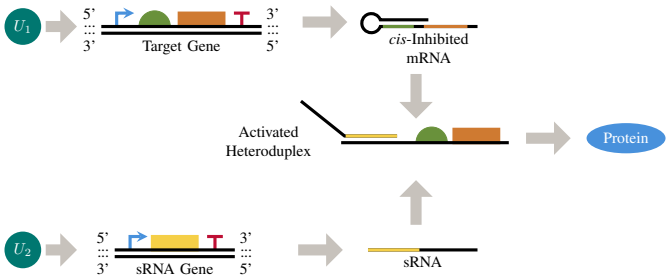

Fig. 4: The sRNA activation mechanism (Hfq not shown). Here the target gene produces a *cis*-repressed mRNA that folds upon itself, forming a hairpin that blocks access to its RBS and/or Start Codon and thereby inhibiting translation. When expressed, an activating sRNA opens up the hairpin in the target mRNA allowing access by the ribosome and translation of the gene encoded in the mRNA.

$$\psi = \frac{\alpha_p \Gamma_1}{\delta_m \delta_p}, \quad \phi = \frac{\Gamma_1}{\delta_m}, \quad \theta = \delta_p^{-1}, \quad \eta = \frac{\Gamma_2}{\delta_p},$$
$$K_r = \frac{k\Gamma_1}{\delta_p \delta_m}, \quad K_m = \frac{k\Gamma_2}{\delta_p \delta_m}, \quad \epsilon = \frac{\delta_p}{\delta_m}. \tag{8}$$

In the limit $\epsilon \to 0$ (7a) dictates that $\hat{m}$ is in quasi-equilibrium. This is expected for a system operating on two time-scales [35], where the mRNA dynamics (fast time-scale) quickly adjust to changes in the protein dynamics (slow time-scale). Thus (7) can be expressed in two ODEs by using (7a) to eliminate $\hat{m}$, which when transformed back into the original variables gives:

$$\dot{r} = \Gamma_2 - \frac{\Gamma_1 k r}{\delta_m + kr} - \delta_p r, \tag{9a}$$
$$\dot{p} = \frac{\alpha_p \Gamma_1}{\delta_m + kr} - \delta_p p, \tag{9b}$$

III. sRNA Activation

We now consider a two-input system that uses sRNA to bind a target mRNA, which by disrupting its secondary structure and revealing a RBS forms an activated heteroduplex (Fig. 4). As before the system takes two transcription factors as input, and its output is the concentration of protein produced by the activated heteroduplex mRNA. We can model this system's dynamics using a set of differential equations of the form:

$$\dot{m} = \Gamma_1 - \delta_m m - Kmr, \tag{10a}$$
$$\dot{r} = \Gamma_2 - \delta_r r - Kmr, \tag{10b}$$
$$\dot{m}_r = Kmr - \delta_{m_r} m_r, \tag{10c}$$
$$\dot{p} = \alpha_{p_2} m_r - \delta_p p, \tag{10d}$$

where variables are as defined in (1), and α_{p_2} is the translation rate of protein from the activated heteroduplex which we will set equal to α_p.

A. Steady-state Response

For an input combination $u^* = [u_1^*, u_2^*]$ the system (10) has a steady-state equilibrium $x^* = [m^*, r^*, m_r^*, p^*]$ given by:

$$m^* = \frac{-\delta_m \delta_r + k(\Gamma_1^* - \Gamma_2^*) + \sqrt{\omega}}{2\delta_m k}, \tag{11a}$$
$$r^* = \frac{\Gamma_2^*}{\delta_r + km^*}, \tag{11b}$$
$$m_r^* = \frac{\delta_m \delta_r + k(\Gamma_1^* + \Gamma_2^*) - \sqrt{\omega}}{2\delta_{m_r} k}, \tag{11c}$$
$$p^* = \frac{\alpha_{p_2}}{\delta_p} m_r^*, \tag{11d}$$

where $\omega = (\delta_m \delta_r + k(\Gamma_2^* - \Gamma_1^*))^2 + 4k\delta_m \delta_r \Gamma_1^*$. The system's steady-state output (p) response for varying inducer concentrations is shown in Fig. 5, demonstrating that the system's protein output is (approximately) linearly proportional to whichever input is smaller. The system's output thus approximates a minimum comparator (the function $\min(u_1, u_2)$), though this is difficult to intuit from (11).

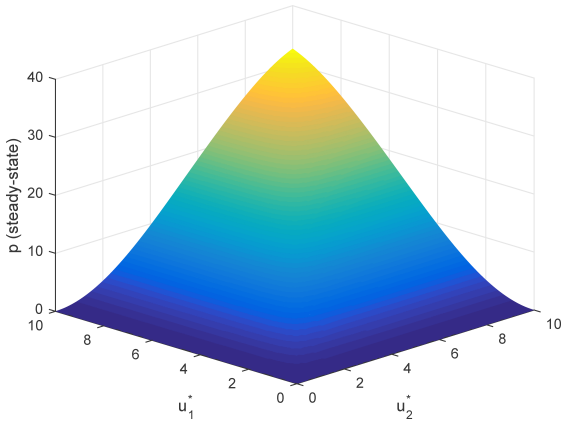

Fig. 5: Steady state output (p^* in (11d)) for the full model (10) of sRNA activation. This system functions as a minimum comparator; its output approximates the function $\min(u_1, u_2)$.

B. Frequency Response

We can re-define (10) in terms of perturbations around the equilibrium values $\tilde{x} = x - x^*$ and $\tilde{u} = u - u^*$ to derive a linear approximation to our system:

$$\dot{\tilde{m}} = \gamma_1^* \tilde{u_1} - (kr^* + \delta_m)\tilde{m} - km^* \tilde{r}, \tag{12a}$$
$$\dot{\tilde{r}} = \gamma_2^* \tilde{u_2} - (km^* + \delta_r)\tilde{r} - k\tilde{m}r^*, \tag{12b}$$
$$\dot{\tilde{m}_r} = k\tilde{m}r^* + km^*\tilde{r} - \delta_{m_r}\tilde{m_r}, \tag{12c}$$
$$\dot{\tilde{p}} = \alpha_{p_2}\tilde{m_r} - \delta_p \tilde{p}. \tag{12d}$$

We apply Laplace transforms to (12) to re-cast this system of equations in the frequency domain, for which transfer functions are expressed as a block diagram in Fig. 6. These expressions can be manipulated to find the transfer function between each of the inputs and the output, giving:

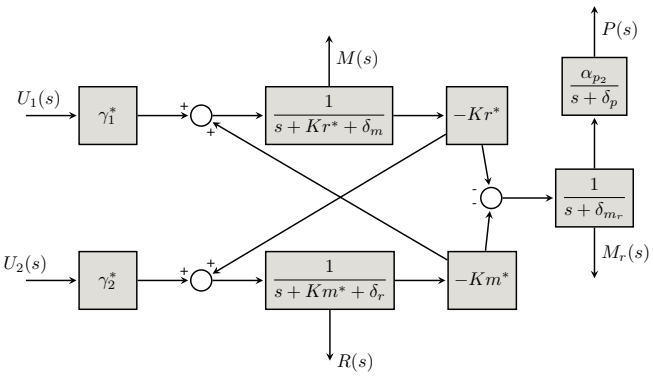

Fig. 6: Block diagram for the sRNA activation mechanism

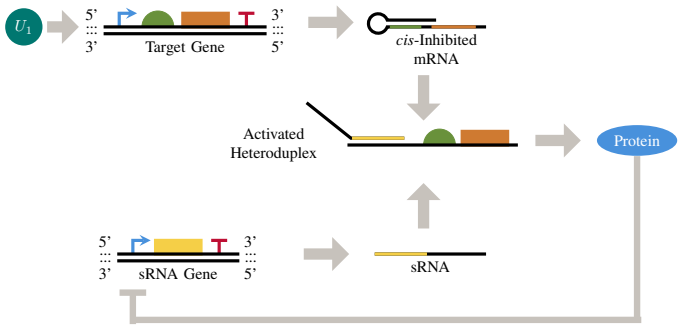

Fig. 7: An activating sRNA negative feedback architecture, which "closes the loop" around the two-input system in Fig. 4 by producing a protein that represses the expression of sRNA.

$$G_1(s) = \frac{P(s)}{U_1(s)} = \frac{\alpha_{p_2}\gamma_1^* k r^*(s+\delta_r)}{(s+\delta_{m_r})(s+\delta_p)(s+s_+)(s+s_-)},$$
$$G_2(s) = \frac{P(s)}{U_2(s)} = \frac{\alpha_{p_2}\gamma_2^* k m^*(s+\delta_m)}{(s+\delta_{m_r})(s+\delta_p)(s+s_+)(s+s_-)}, \tag{13}$$

with $s_\pm$ defined as in (6). In this case we find that either transfer function (from U_1 and U_2 to the output) is maximised at steady state when the alternate input is large. For example, $G_1(s) > G_2(s)$ when $m^* < r^*$ (which corresponds to $u_1^* < u_2^*$).

C. Non-linear Analysis

To reduce our system's order, in addition to the assumptions made in Section II-D we now set $\delta_m = \delta_{m_r}$ (as discussed in Section II-A). Setting $p = \psi\hat{p}$, $, m = \phi\hat{m}$, $r = \eta\hat{r}$, $m_r = \beta\hat{m}_r$, $t = \theta\hat{t}$ we have from (10):

$$\epsilon\dot{\hat{m}} = 1 - \hat{m} - K_m\hat{m}\hat{r}, \tag{14a}$$
$$\dot{\hat{r}} = 1 - \hat{r} - K_r\hat{m}\hat{r}, \tag{14b}$$
$$\epsilon\dot{\hat{m}}_r = \hat{m}\hat{r} - \hat{m}_r, \tag{14c}$$
$$\dot{\hat{p}} = \hat{m}_r - \hat{p}. \tag{14d}$$

where

$$\psi = \frac{\alpha_{p_2}k\Gamma_1\Gamma_2}{\delta_m^2\delta_p^2}, \quad \beta = \frac{k\Gamma_1\Gamma_2}{\delta_m^2\delta_p} \quad \phi = \frac{\Gamma_1}{\delta_m}, \quad \eta = \frac{\Gamma_2}{\delta_p},$$
$$K_r = \frac{k\Gamma_1}{\delta_p\delta_m}, \quad K_m = \frac{k\Gamma_2}{\delta_p\delta_m}, \quad \theta = \delta_p^{-1}, \quad \epsilon = \frac{\delta_p}{\delta_m}, \tag{15}$$

In the limit $\epsilon \to 0$ both m and m_r are in quasi-equilibrium, allowing (14a) and (14c) to be used to eliminate $\hat{m}$ and $\hat{m}_r$, which after transforming back into the original variables gives:

$$\dot{p} = \frac{\alpha_{p_2}}{\delta_m}\frac{\Gamma_1 kr}{\delta_m + kr} - \delta_p p, \tag{16a}$$
$$\dot{r} = \Gamma_2 - \frac{\Gamma_1 kr}{\delta_m + kr} - \delta_p r, \tag{16b}$$

IV. Example: sRNA Activation Feedback

To demonstrate the utility of our proposed sRNA regulatory architectures we present an example of a feedback system based on the sRNA activation system described in Section III. To "close the loop" we choose the output protein of our system to be a repressing transcription factor that interacts with the promoter of an activating sRNA, thus taking the place of input u_2 (Fig. 7). This system retains a single input, which regulates production of the target mRNA and through that the output protein level. For a practical implementation of this system the target mRNA may be designed to be polycistronic (or employ a fusion protein), producing both the transcription factor protein that regulates sRNA production, as well as a secondary protein of interest whose production is to be regulated. We simulate this example sRNA feedback system using the models described in Section III by setting $u_2 = p$. The closed-loop (CL) feedback architecture (Fig. 7) demonstrates a number of benefits when compared to the open-loop (OL) system (Fig. 4), including reduced sensitivity to fluctuations in model parameters (Fig. 8a), and a faster response (Fig. 8b).

V. Conclusion

In this paper we have described subtraction and minimum comparator junctions (which are a key component in many common control architectures) based upon sRNA-mRNA interactions that repress or activate a specific mRNA's translation. By analysing differential-equation models of these systems we have identified parameter regimes suitable for their operation, and we have derived reduced models that are valid for typical experimental conditions. Finally, we presented an example of the sRNA activation system's implementation, demonstrating that negative feedback can provide a range of performance improvements for synthetic biological systems.

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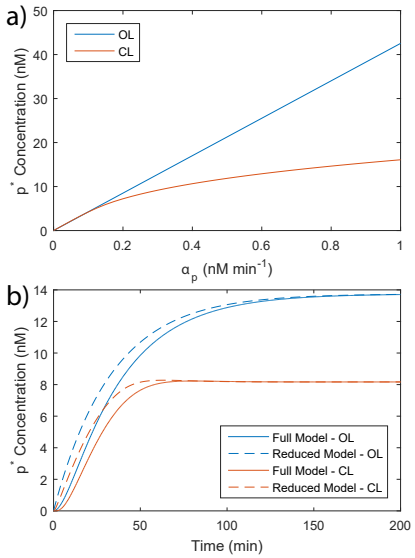

Fig. 8: Simulations of the open-loop (OL) system ($u_2^* = 2$ nM) as in Fig. 4, and the closed-loop system ($u_2 = p$) as in Fig. 7. Parameters are as described in Section II-A with the exception of $\beta_2 = 0.2$ nM min^{-1}. Repression by the output protein is modelled by adjusting (2) as described in Section II. a) The steady-state output protein concentration demonstrates reduced sensitivity to model parameter values, in this case the mRNA translation rate (α_p). b) The dynamic response of each system following $u_1 = 5$ nM induction demonstrates decreased response time in the CL system. Because parameter values satisfy the assumptions made in model reduction, we observe and that the reduced model (16) is able to replicate the behaviour of the full model (10).

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