Coordinated Spatial Pattern Formation in Biomolecular Communication Networks

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Abstract

This paper proposes a control theoretic framework to model and analyze the self-organized pattern formation of molecular concentrations in biomolecular communication networks, where bio-nanomachines, or biological cells, communicate each other using cell-to-cell communication mechanism mediated by a diffusible signaling molecule. We first introduce a feedback model representation of the reaction-diffusion dynamics of biomolecular communication networks. A systematic local stability/instability analysis tool is then provided based on the root locus of the feedback system. Using the instability analysis, we analytically derive the conditions for the self-organized spatial pattern formation, or Turing pattern formation, of the bio-nanomachines. The theoretical results are demonstrated on a novel biochemical circuit called activator-repressor-diffuser system, and the Turing pattern formation is numerically confirmed. Finally, we show that the activator-repressor-diffuser system is a minimum biochemical circuit that admits self-organized patterns in biomolecular communication networks.

1 Introduction

Designing the coordinated dynamical behavior of cell populations is one of the important challenges in synthetic biology. The study of cell population control is expected to provide not only the better understanding of biology but also the ability to build biomimetic nanomachines, or bio-nanomachines, for bioengineering applications such as tissue engineering and targeted drug delivery

In the last decade, researchers designed a variety of biochemical circuits that perform population-level behavior such as synchronized oscillations and population control, and multicellular computation. In these works, intercellular coordination was enabled by a cell-to-cell communication mechanism called quorum sensing, where cells communicate each other by releasing and receiving diffusible signaling molecules. From an engineering point of view, self-organized pattern formation is preferable since it

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does not require extra design steps of external inputs and spatial allocation of cells. Moreover, the study of self-organization is important in developmental biology to understand morphogenesis [11,12].

Recently, Hsia et al. [13] introduced a dynamical model of biomolecular communication networks using reaction-diffusion equations, where diffusion-based cell-to-cell communication was modeled based on the Fick’s laws of diffusion [14]. It was then shown that a biochemical communication network can form a self-organized concentration gradient of chemical species over cell populations despite the averaging effect of molecular diffusion. Such self-organized spatio-temporal pattern is known as Turing pattern [15].

Although the theory of Turing pattern formation was extensively studied over the last 60 years [14,16], the classical works of Turing pattern formation are not directly applicable to today’s engineering applications in biomolecular communication networks because of the difference of the number of diffusible molecules. Specifically, in biomolecular communication networks, only a single molecular species, or a small signaling molecule, can diffuse between cells and most proteins and mRNAs are localized in a cell, while the classical works assume that all molecules can diffuse in the medium. Hence, it is desirable to develop a novel theoretical framework that specifically targets the systematic modeling and analysis of the spatio-temporal dynamics of biomolecular communication networks with a single diffusible molecule.

Motivated by this situation, this paper proposes an engineering oriented modeling and analysis framework for biomolecular communication networks using a control theoretic approach. The main contributions of this paper are twofold: (i) We develop a graphical analysis method that systematically verifies the local stability/instability of biomolecular communication networks based on the root locus. (ii) Using the stability analysis, we derive analytic conditions for the formation of Turing pattern over the cell population. In particular, we propose a novel biochemical circuit structure called activator-repressor-diffusor motif and show that it is a minimum biomolecular circuit that admits Turing instability in biomolecular communication networks.

The organization of this paper is as follows. In Section 2, we provide a control theoretic modeling framework for biomolecular communication networks and introduce a useful decomposition that simplifies the stability analysis of the system. Then, in Section 3, we provide a systematic stability/instability analysis tool based on the root locus method [17] and demonstrate on a novel activator-repressor-diffusor biomolecular circuit. Section 4 is devoted to deriving the condition for Turing pattern formation. Then, in Section 5, the class of reaction networks that can admit Turing patterns is characterized. Finally, Section 6 concludes the paper.

The authors’ conference papers [18,19] provided part of the ideas of Turing instability analysis shown in this paper without the details of technical proofs. In this paper, we present complete proofs of theoretical results with illustrative biological examples. In particular, we here propose a novel activator-repressor-diffusor biochemical circuit and extensively discuss the properties of the system in Section 5.

The following notations are used throughout this paper: $\mathbb{N}_0 := \{0, 1, 2, \cdots \}$. $\mathbb{R}_{0+} := \{ r \in \mathbb{R} \mid r \geq 0 \}$. $\mathbb{C}_+ := \{ c \in \mathbb{C} \mid \text{Re}[c] > 0 \}$. $\mathbb{C}_{0+} := \{ c \in \mathbb{C} \mid \text{Re}[c] \geq 0 \}$. $\mathbb{C}_- := \{ c \in \mathbb{C} \mid \text{Re}[c] < 0 \}$. $I_n$ is an $n$ by $n$ identity matrix.

# 2 Modeling of Biomolecular Communication Networks

## 2.1 Model description

We consider molecular communication networks in a two dimensional fluidic medium $\Omega := \Xi_1 \times \Xi_2 = [0, L_1] \times [0, L_2]$ where a large number of cells communicate using a single dif-

Account for the dynamics of individual spatial modes, which will be a key result in developing a control theoretic model representation of the biomolecular communication network system. We show that the infinite dimensional system (2) can be decomposed into finite dimensional single-input single-output (SISO) subsystems that are, for example, transcription factors and associated mRNAs. In order for the cells to communicate, cells produce a transmitter molecule $M$ and release to the medium. An example of such transmitter molecule is N-Acyl homoserine lactone (AHL), which is a small molecule that can go through cell membrane and is used for quorum sensing of gram-negative bacteria. It should be noted that, in engineering applications, one could use engineered bio-nanomachines such as vesicles instead of living cells.

When the cells are densely populated in the medium, the chemical levels in the cells can be approximately modeled as the continuous gradient of the concentrations. Let $x_i(\xi, t)$ denote the concentration of $M_i$ ($i = 1, 2, \ldots, n$) at position $\xi := [\xi_1, \xi_2]^T \in \Omega$ and at time $t$, and define $x(\xi, t) := [x_1(\xi, t), x_2(\xi, t), \ldots, x_n(\xi, t)]^T$ The dynamics of the concentrations of $M_1, M_2, \ldots, M_n$ are then modeled by

$$\frac{\partial x(\xi, t)}{\partial t} = f(x(\xi, t)) + D \left( \frac{\partial^2 x(\xi, t)}{\partial \xi_1^2} + \frac{\partial^2 x(\xi, t)}{\partial \xi_2^2} \right),$$

where $f(\cdot)$ is a Lipschitz continuous function representing the rate of production and degradation of the molecules. The matrix $D$ is defined as $D := \text{diag}(0, \ldots, 0, \mu) \in \mathbb{R}^{n \times n}$ with a diffusion coefficient $\mu$. It should be noted that only the $(n, n)$-th entry is non-zero, since only the transmitter molecule $M_n$ can diffuse between cells. We assume the Neumann boundary condition $\tilde{n} \cdot \nabla x(\xi, t) = 0$ for all $\xi \in \partial \Omega$, where $\tilde{n}$ is a vector normal to the boundary $\partial \Omega$.

Let $\bar{x} \in \mathbb{R}^n_{>0}$ denote an equilibrium state of a single cell, i.e., $f(\bar{x}) = 0$. It follows that $\bar{x}$ is a spatially homogeneous equilibrium state of the system (1). Then, the local dynamics around $\bar{x}$ are modeled by the following linearized model of (1).

$$\frac{\partial \hat{x}(\xi, t)}{\partial t} = A \hat{x}(\xi, t) + D \left( \frac{\partial^2 \hat{x}(\xi, t)}{\partial \xi_1^2} + \frac{\partial^2 \hat{x}(\xi, t)}{\partial \xi_2^2} \right),$$

where $A \in \mathbb{R}^{n \times n}$ is the Jacobian of $f(\cdot)$ at $\bar{x}$, and $\hat{x}(\xi, t)$ is defined by $\hat{x}(\xi, t) := x(\xi, t) - \bar{x}$. Note that the system (2) is a finite dimensional linear time-invariant system.

In Section 3, we introduce a control theoretic model representation of the biomolecular communication network system. We show that the infinite dimensional system (2) can be decomposed into finite dimensional single-input single-output (SISO) subsystems that account for the dynamics of individual spatial modes, which will be a key result in developing a systematic stability analysis tool in the next section.

The linearized model (2) can be expressed as

$$\frac{\partial \hat{x}(\xi, t)}{\partial t} = A \hat{x}(\xi, t) + e_n u(\xi, t)$$

$$y(\xi, t) = e_n^T \hat{x}(\xi, t),$$

In what follows, we may denote $x(\xi, t)$ by $\bar{x}$ to avoid notational clutter.

2.2 Control theoretic formulation of biomolecular communication networks

In this section, we introduce a control theoretic model representation of the biomolecular communication network system. We show that the infinite dimensional system (2) can be decomposed into finite dimensional single-input single-output (SISO) subsystems that account for the dynamics of individual spatial modes, which will be a key result in developing a systematic stability analysis tool in the next section.
Figure 1: Molecular communication network

Figure 2: (Left) Feedback system representation of biomolecular communication network. (Right) Decomposed subsystem $\Sigma_{k,\ell}$.

where $u(\xi, t) := \mu \nabla^2 y(\xi, t)$ with the Laplace operator

$$
\nabla^2 := \frac{\partial^2}{\partial \xi_1^2} + \frac{\partial^2}{\partial \xi_2^2}
$$

and $e_n := [0, \cdots, 0, 1]^T \in \mathbb{R}^n$. The equations (3) and (4) imply that for each fixed position $\xi$, the dynamics of the local reactions inside a single cell can be modeled as a SISO system with the input $u(\xi, t)$ and the output $y(\xi, t)$. Note that $y(\xi, t)$ is the concentration of the transmitter molecule $M_n$ at position $\xi$. For each $\xi$, the transfer function from $u$ to $y$ is obtained as

$$
h(s) := e_n^T (s I_n - A)^{-1} e_n \ (=: n(s)/d(s)),
$$

where we define $n(s)$ and $d(s)$ as the numerator and the denominator of $h(s)$, respectively. As a result, the system can be illustrated as the feedback system shown in Fig. 2 (Left), where $I$ is an identity operator. Note that the feedback system can be viewed as a multi-agent dynamical system, where the homogeneous agents $h(s)$ communicate with the nearest neighbors by the Laplace operator $\mu \nabla^2$. A finite dimensional version of such systems is known as LTI systems with a generalized frequency variable [23].

**Assumption 1.** We assume $(A, e_n)$ is controllable, $(A, e_n^T)$ is observable, and $h(s)$ does not have zeros on the imaginary axis, that is, $n(j\omega) \neq 0$ for all $\omega \in \mathbb{R}$.

This assumption is technical but not restrictive for most practical examples of molecular communication networks.

The stability analysis of the system (2) is not necessarily straightforward due to the infinite dimensionality of the system. In what follows, we show that the infinite dimensional system can be decomposed into mathematically tractable finite dimensional subsystems. Let $\mathcal{F}$...
denote the Fourier transform operator. The Laplace operator $\nabla^2$ can be written as

$$\nabla^2 = \mathcal{I}_{\Xi_2} \otimes \frac{\partial^2}{\partial \xi_1^2} + \frac{\partial^2}{\partial \xi_2^2} \otimes \mathcal{I}_{\Xi_1},$$

(7)

with the tensor product $\otimes$ of the operators and the identity operators $\mathcal{I}_{\Xi_i}$ on $\Xi_i = [0, L_i]$ ($i = 1, 2$). It then follows that the Laplace operator $\nabla^2$ is diagonalized by $(\mathcal{F} \otimes \mathcal{F})\nabla^2(\mathcal{F}^{-1} \otimes \mathcal{F}^{-1}) = \text{diag}(\lambda_{0,0}, \lambda_{1,0}, \lambda_{0,1}, \cdots)$, where the eigenvalues $\lambda_{k,\ell}$ and the associated eigenfunctions $\phi_{k,\ell}(\xi)$ of $-\mu \nabla^2$ are

$$\lambda_{k,\ell} := \mu \left( \left( \frac{k\pi}{L_1} \right)^2 + \left( \frac{\ell\pi}{L_2} \right)^2 \right)$$

(8)

$$\phi_{k,\ell}(\xi) := \cos \left( \frac{k\pi \xi_1}{L_1} \right) \cos \left( \frac{\ell\pi \xi_2}{L_2} \right)$$

(9)

with $k = 0, 1, 2, \cdots$ and $\ell = 0, 1, 2, \cdots$. Since $(\mathcal{F}^{-1} \otimes \mathcal{F}^{-1})((\mathcal{I}_{\Xi_2} \otimes h(s) + h(s) \otimes \mathcal{I}_{\Xi_1}) (\mathcal{F} \otimes \mathcal{F})) = \mathcal{I}_{\Xi_2} \otimes h(s) + h(s) \otimes \mathcal{I}_{\Xi_1}$, the feedback system in Fig. 2 (Left) can be decomposed into subsystems $\Sigma_{k,\ell}$ ($k = 0, 1, 2, \cdots, \ell = 0, 1, 2, \cdots$) depicted in Fig. 2 (Right).

It should be noted that this corresponds to the spatial modal decomposition of the molecular communication network system. Thus, the dynamics of each subsystem $\Sigma_{k,\ell}$ represent those of each spatial mode $\phi_{k,\ell}(\xi)$, and it follows that

$$\hat{x}(\xi, t) = \sum_{k=0}^{\infty} \sum_{\ell=0}^{\infty} \hat{x}_{k,\ell}(t) \phi_{k,\ell}(\xi),$$

(10)

where $\hat{x}_{k,\ell}$ is the state of the subsystem $\Sigma_{k,\ell}$.

This intuitively suggests that the concentrations perturbed around the homogeneous equilibrium $\bar{x}$ eventually come back to $\bar{x}$, when the growth rates of all the spatial modes are negative. On the other hand, if the growth rate of a non-zero spatial mode, $\phi_{k,\ell}(\xi)$ with $k + \ell \geq 1$, is positive, we expect the formation of a spatially periodic pattern of concentration gradient, or a Turing pattern. Therefore, the stability analysis of the molecular communication network is reduced to checking the stability of the finite-order subsystems $\Sigma_{k,\ell}$.

3 Stability Analysis

3.1 Graphical stability/instability analysis based on root locus

In this section, we provide a systematic stability/instability analysis method for molecular communication networks using a control theoretic tool. We then demonstrate the stability/instability analysis on a novel activator-repressor-diffuser system.

Let a polynomial $p_\lambda(s)$ be defined by

$$p_\lambda(s) := d(s) + \lambda n(s)$$

(11)

with $d(s)$ and $n(s)$ in [4]. The characteristic polynomial of each subsystem $\Sigma_{k,\ell}$ is then written as $p_{\lambda_{k,\ell}}(s) = d(s) + \lambda_{k,\ell} n(s)$. It follows that the subsystem $\Sigma_{k,\ell}$ is asymptotically stable, if and only if all the roots of $p_{\lambda_{k,\ell}}(s) = 0$ lie in the open left-half complex plane, or Hurwitz. Since each subsystem represents the dynamics of each spatial mode as shown in the previous section, the stability of the molecular communication network [2] is guaranteed, if and only if all of the finite dimensional subsystems $\Sigma_{k,\ell}$ ($k = 0, 1, 2, \cdots, \ell = 0, 1, 2, \cdots$) are stable.
Proposition 1. Suppose Assumption 1 holds. The molecular communication network is exponentially stable if and only if \( p_{\lambda k,\ell}(s) \) is Hurwitz for all \( k, \ell \in \mathbb{N}_0 \).

Proof. Each subsystem \( \Sigma_{k,\ell} \), which is a finite dimensional linear time-invariant system, is exponentially stable and \( \hat{x}_{k,\ell}(t) \to 0 \) as \( t \to \infty \) if and only if \( p_{\lambda k,\ell}(s) \) is Hurwitz. It follows that, in the limit of \( k, \ell \to \infty \), \( p_{\lambda k,\ell}(s) \) converges to the roots of \( n(s) = 0 \), and \( n(s) = 0 \) does not have roots on the imaginary axis from Assumption 1. Hence, all modes \( \hat{x}_{k,\ell}(t) \) decay to zero exponentially as \( t \to \infty \), if and only if \( p_{\lambda k,\ell}(s) \) is Hurwitz for all \( k, \ell \in \mathbb{N}_0 \).

Proposition 1 allows us to analyze the stability/instability of the infinite dimensional system using the finite dimensional subsystems \( \Sigma_{k,\ell} \) \( (k = 0, 1, 2, \cdots, \ell = 0, 1, 2, \cdots) \). In what follows, we show that the root locus method \(^{17}\) is useful for systematically analyzing the stability of the subsystems \( \Sigma_{k,\ell} \).

It follows that the roots of \( p_{\lambda k,\ell}(s) = 0 \) \( (k = 0, 1, 2, \cdots, \ell = 0, 1, 2, \cdots) \) lie on the root locus of the polynomial \( p_{\lambda}(s) = d(s) + \lambda n(s) \), which is the characteristic polynomial of a feedback system composed of \( h(s) \) and a feedback gain \( \lambda \) (see Fig. 2 (Right)). Moreover, the definition \(^{8}\) implies that the feedback gain \( \lambda = \lambda_{k,\ell} \) monotonically increases in terms of \( k \) and \( \ell \). Thus, the root locus starts from the point where \( \lambda = \lambda_{0,0} = 0 \) and converges to the point where \( \lambda_{k,\ell} \) at \( k, \ell \to \infty \). This observation leads to the following graphical algorithm for stability analysis.

Algorithm 1. Given the dynamics of a single cell \( h(s) \), we draw the root locus \( \mathcal{R} \) of a negative feedback system composed of \( h(s) \) and a constant feedback gain \( \lambda \) with varying \( \lambda \in [0, \infty] \). If the root locus \( \mathcal{R} \) lies inside the open left-half complex plane \( \mathbb{C}_- \), the molecular communication network is stable.

This algorithm allows us to check the local stability of the molecular communication network system \(^2\) using the transfer function of a single cell \( h(s) \) and its root locus. Thus, the stability analysis of the infinite dimensional system is greatly simplified.

Remark 1. The reason that the root locus gives only a sufficient condition for stability is because the root locus is continuous in terms of the feedback gain \( \lambda \), while the poles of the subsystems \( \Sigma_{k,\ell} \) \( (k = 0, 1, 2, \cdots, \ell = 0, 1, 2, \cdots) \) are discrete. In practice, however the length of the spatial domain \( L_1 \) and \( L_2 \) is sufficiently large such that the discrete feedback gains \( \lambda_{k,\ell} = \mu ((k\pi/L_1)^2 + (\ell\pi/L_2)^2) \) \( (k = 0, 1, 2, \cdots, \ell = 0, 1, 2, \cdots) \) are close to each other as shown in Section 3.2 and the above algorithm can be practically considered as necessary and sufficient.

It is worth noting that the root locus can be systematically drawn based on a set of known rules \(^{17}\). In particular, the root locus \( \mathcal{R} \) starts from the poles of \( h(s) \) and converges to the zeros of \( h(s) \).

Suppose the internal circuit in a cell is composed of \( n \geq 2 \) molecules, and let the matrix \( A \in \mathbb{R}^{n \times n} \) be partitioned as

\[
A = \begin{bmatrix}
A_{\text{circ}} & A_{\text{cd}}^T \\
A_{\text{dc}} & A_{\text{diff}}
\end{bmatrix}
\]

(12)

with \( A_{\text{circ}} \in \mathbb{R}^{(n-1) \times (n-1)}, A_{\text{cd}} \in \mathbb{R}^{n-1}, A_{\text{dc}} \in \mathbb{R}^{1 \times (n-1)} \) and \( A_{\text{diff}} < 0 \). Then, the matrix \( A_{\text{circ}} \) represents the structure of the internal circuit, and \( A_{\text{diff}} < 0 \) is the degradation rate of the single diffusible molecule. The vectors \( A_{\text{cd}} \) and \( A_{\text{dc}} \) correspond to the interactions between the internal circuit molecules \( \mathcal{M}_1, \mathcal{M}_2, \cdots, \mathcal{M}_{n-1} \) and the diffuser \( \mathcal{M}_n \). When \( n = 1 \), we
define $A = a_{\text{diff}}$. The following proposition provides a physical interpretation of the poles and zeros of $h(s)$.

**Proposition 2.** Consider the molecular communication network system (2). Then, the characteristic polynomial $p_\lambda(s)$ of the system can be written as

$$p_\lambda(s) = d(s) + \lambda n(s) = |sI_n - A| + \lambda |sI_{n-1} - A_{\text{circ}}|,$$

where we define $|sI_0 - A_{\text{circ}}| = 1$ when $n = 1$. In particular, the root locus $R$ starts from $\text{spec}(A)$ and converge to $\text{spec}(A_{\text{circ}})$ when $n \geq 2$.

The proof can be found in Appendix A. Proposition 2 states that the zeros of $h(s)$ are determined from the dynamics of the internal circuit specified by $A_{\text{circ}}$, while the poles are determined from the overall reaction dynamics including the diffuser $M_n$ (see Fig. 1). This implies that the start and the terminal point of the root locus $R$ can be characterized by the dynamics of the entire circuit and the internal circuit, respectively.

### 3.2 Example: activator-repressor-diffuser system

We illustrate the graphical stability/instability analysis using a hypothetical biochemical network shown in Fig. 3. The system is composed of an activator-repressor internal circuit and a single diffuser molecule that allows cell-to-cell communication. In Fig. 3, $A, R$ and $D$ stand for activator, repressor and diffuser, respectively.

Let $a(\xi,t), r(\xi,t)$ and $d(\xi,t)$ denote the concentration of activator, repressor and diffuser, respectively. The dynamics of the molecular communication network system are then modeled by

$$\dot{a} = -\delta_a a + \gamma_a \frac{\alpha^2}{1 + \alpha^2} \frac{1}{1 + r^2},$$

$$\dot{r} = -\delta_r r + \gamma_r \frac{\alpha^2}{1 + \alpha^2} \frac{1}{1 + d^2},$$

$$\dot{d} = -\delta_d d + \gamma_d \frac{1}{1 + r^2} + \mu \nabla^2 d,$$

where $\delta_*(\# = a, r, d)$ and $\gamma_*(\# = a, r, d)$ denote the degradation rates and the production rates of each molecule, respectively.

**Case A. (Stable case):** Suppose $\delta_a = 0.7, \delta_r = 0.25, \delta_d = 0.1, \gamma_a = 5.0, \gamma_r = 3.3, \gamma_d = 0.6$ and $\mu = 0.006$. A spatially homogeneous equilibrium point of the system is then obtained as
\[[a_*, r_*, d_*]^T = [0.763, 1.564, 1.452]^T.\] The Jacobian matrix \( A \) around this equilibrium point is

\[
A = \begin{bmatrix}
0.0487 & -0.5867 & 0 \\
0.4589 & -0.2500 & -0.3514 \\
-0.7 & -0.15 & -0.1
\end{bmatrix}.
\] (17)

The transfer function of a single cell \( h(s) \) is then calculated from (13) as

\[
h(s) = \frac{s^2 + 0.2013s + 0.2571}{s^3 + 0.3013s^2 + 0.2241s + 0.0285}.
\] (18)

Figure 4 (Right) illustrates the root locus \( \mathcal{R} \) drawn by Algorithm 1. The eigenvalues of \( A \) and \( A_{\text{circ}} \) are \( \text{spec}(A) = \{-0.0804 \pm 0.4416j, -0.1405\} \) and \( \text{spec}(A_{\text{circ}}) = \{-0.1007 \pm 0.4970j\} \), and we can see that these eigenvalues correspond to the initial and the terminal points of the root locus \( \mathcal{R} \). Since the root locus \( \mathcal{R} \) stays in the left-half complex plane, the equilibrium point of the molecular communication network is locally stable.

Figure 4 (Left) is the concentration gradient of the activator \( a(\xi, t) \) at steady state \((t = 1996)\). As expected from the graphical stability analysis result, the concentration converged to the spatially homogeneous equilibrium point. In the simulation, we used \( L_1 = 3 \) and \( L_2 = 2 \), and the initial values \( a(\xi, 0), r(\xi, 0) \) and \( d(\xi, 0) \) were set as \( x_*(1 + 0.01 \sum_{k=1}^{5} \sum_{\ell=1}^{5} \psi_{k, \ell}(\xi_1, \xi_2)) \), where \( x_* \) is the equilibrium point of \( a(\xi, 0), r(\xi, 0) \) and \( (\xi, 0) \), respectively, and

\[
\psi_{k, \ell}(\xi_1, \xi_2) := \cos \left( \frac{k\pi}{L_1} \left( \xi_1 - \frac{L_1}{2} \right) \right) \cos \left( \frac{\ell\pi}{L_2} \left( \xi_2 - \frac{L_2}{2} \right) \right).
\]

The time axis of the simulation, \( \tau \), was scaled by \( \tau = 0.005t \). The full temporal dynamics is available in a movie format (see ancillary file). We used COMSOL® Multiphysics 4.4 for the simulation.

**Case B. (Unstable case):** Let \( \gamma_d = 0.6 \) and the rest of the parameters being kept the same as Case A. The equilibrium point is then \( [a_*, r_*, d_*]^T = [0.933, 1.600, 1.684]^T. \) The eigenvalues of \( A \) and \( A_{\text{circ}} \) are calculated as \( \text{spec}(A) = \{-0.0034 \pm 0.4744j, -0.1585\} \) and \( \text{spec}(A_{\text{circ}}) = \{-0.0326 \pm 0.5164j\} \). As shown in Fig. 4 (Right), the root locus lies in the open right-half complex plane. In fact, multiple roots of \( p_A(s) = 0 \) lie in the right-half complex plane, implying that the corresponding subsystems, or spatial modes, are unstable.

Figure 5 (Left) illustrates the concentration gradient of the activator \( a(\xi, t) \) at steady state \((t = 1996)\). Since the equilibrium point is not stable, the concentration does not converge to the spatially homogeneous equilibrium. In fact, a coordinated spatial pattern appeared in this example. The time axis of the simulation, \( \tau \), was scaled by \( \tau = 0.005t \). The full temporal dynamics is also available (see ancillary file). We used COMSOL® Multiphysics 4.4 for the simulation.

As Turing [15] pointed, this coordinated pattern formation can be explained by the fact that the subsystems that are destabilized. In the next section, we provide more rigorous argument of why and when the pattern formation can be expected.

# 4 Conditions for Turing Pattern Formation

## 4.1 Finite mode Turing instability

As seen in the previous example, molecular communication networks can exhibit coordinated spatial patterns based on the passive communication mechanism, or diffusion. This
is because non-zero finite spatial modes are destabilized and the growth rate of $\tilde{x}_{k,\ell}$ in (10) is positive.

The instability of the non-zero spatial mode caused by diffusion is called Turing instability named after Alan Turing, who first pointed out the mathematical basis of the emergence of inhomogeneous spatial patterns in reaction-diffusion equations [15]. In what follows, we introduce a formal definition of finite mode Turing instability for biomolecular communication networks, then we investigate conditions for the Turing instability.

**Definition 1. (Finite mode Turing instability)** A biomolecular communication network (1) is finite mode Turing unstable around an equilibrium $\bar{x}$, if

1. $p_{\lambda_{0},s}(s) = p_{0}(s) = d(s) \neq 0 \forall s \in C_{0+}$, and
2. $p_{\lambda}(\gamma + s) = 0; \exists \lambda \in (0, \infty)$ and $\exists s \in C_{+}$, where $\gamma = \max(\text{Re}[s_{0}], 0)$ and $s_{0}$ is a root of $n(s) = 0$ with the largest real part.

The condition (i) implies that the dynamics of each cell $h(s)$ is stable around an equilibrium point $\bar{x}$ when there is no diffusion. The condition (ii) guarantees that the root locus of $h(s)$ goes into the open right-half plane of the complex plane for some feedback gain $\lambda > 0$ (see Fig. 2 (Right)). In particular, the roots of $n(s) = 0$ are the terminal points of the root locus (Proposition 2), thus the condition (ii) also implies that the right-most roots of $p_{\lambda}(s) = 0$ are given when $\lambda$ is a non-zero and finite value. This guarantees that the dominant unstable spatial mode is finite and the resulting spatial pattern is of a non-zero finite spatial mode.

Note that it is important to guarantee that the dominant spatial mode is finite, since it is physically and biologically implausible to achieve coordinated spatial patterns of infinite spatial mode at steady state. The following toy example shows the case where the right-most roots of $p_{\lambda}(s) = 0$ is given in the limit of $\lambda \to \infty$. 

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Figure 4: Case A: (Left) Concentration gradient of the activator $A$ over cell population at steady state ($t = 1996$). (Right) Root locus $R$ of the single cell dynamics $h(s)$.

Figure 5: Case B: (Left) Concentration gradient of the activator $A$ over cell population at steady state ($t = 1996$). (Right) Root locus $R$ of the single cell dynamics $h(s)$. 

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Example: Suppose

\[
A = \begin{bmatrix}
1.954 & -3.114 & 0 \\ 1.0 & -0.9540 & i(1.223) \\ 0 & 1.0 & -2.0 \\
\end{bmatrix},
\]

and \( \mu = 0.006, L_1 = 3 \) and \( L_2 = 2 \). We can calculate the dynamics of a single cell \( h(s) \) using \( A \) and the sub-matrix \( A_{\text{circ}} \) and obtain the root locus \( R \) of \( h(s) \). Figure 6 (Right) shows the right-most pole of the root locus \( R \). As \( k, \ell \to \infty \), or equivalently \( \lambda \to \infty \), the root of \( p_\lambda(s) = 0 \) converges to the root of \( n(s) = 0 \), or \( s_0 \). This implies that the growth rate of the state \( \tilde{x} \) is the largest for the infinite spatial mode, which is not plausible in real physical and biological systems. In fact, the simulation result in Fig. 6 (Left) shows spatial patterns with non-realistic high spatial frequency. The time axis of the simulation, \( \tau \), was scaled by \( \tau = 0.005t \). The full temporal dynamics is available in a movie format (see ancillary file).

We used COMSOL® Multiphysics 4.4 for the simulation.

It should be noted that the importance of the finite mode instability was not actively discussed in many classical works [14,24] where the systems are composed of \( n = 2 \) molecules and both can diffuse in the spatial domain. This is because the dominant spatial mode is always guaranteed to be finite mode, no matter how the reaction rates are varied.

### 4.2 Condition for finite mode Turing instability

In this section, we show that at least three molecules, \( M_1, M_2 \) and \( M_3 \), are necessary to achieve finite mode Turing instability in molecular communication networks. This implies that the well-known activator-repressor model [14] fails to make spatial patterns when there is only one diffusible molecule in the system.

The following lemma provides the necessary and sufficient condition for finite mode Turing instability.

**Lemma 1.** The biomolecular communication network system (1) is finite mode Turing unstable around an equilibrium \( \tilde{x} \), if and only if (i), and (ii-a) or (ii-b) hold.

(i) \( A \) is Hurwitz.

(ii-a) \( A_{\text{circ}} \) is Hurwitz, and \( \Re[p_\lambda(j\omega)] = 0 \), \( \Im[p_\lambda(j\omega)] = 0 \) for some \( \lambda > 0 \) and some \( \omega \in \mathbb{R} \).

(ii-b) \( A_{\text{circ}} \) is not Hurwitz, and \( \Re[p_\lambda(\gamma + j\omega)] = 0 \), \( \Im[p_\lambda(\gamma + j\omega)] = 0 \) for some \( \lambda > 0 \) and some \( \omega \in \mathbb{R} \), \( \gamma := \max_{\nu \in \text{spec}(A_{\text{circ}})} \Re[\nu] \).
This lemma is a direct consequence of Definition 1. The condition (i) of Lemma 1 corresponds to (i) of Definition 1, and the conditions (ii-a) and (ii-b) are equivalent to (ii) of Definition 1 with $\gamma = \text{Re}[s_0]$ and $\gamma = 0$, respectively.

Using this lemma, we can derive the following theorem showing that at least three molecules are necessary to achieve finite mode Turing instability.

**Theorem 1.** Biomolecular communication networks composed of $n = 1$ and $n = 2$ molecules cannot be finite mode Turing unstable.

The proof of this theorem can be found in Appendix B. This theorem implies that the activator-repressor-diffuser system illustrated in Section 3.2 is one of the minimal biomolecular communication networks that can exhibit finite mode Turing instability with $n = 3$.

Moreover, the following theorem shows that finite Turing instability is always caused by Hopf-Turing bifurcation, implying that not only coordinated spatial patterns but also temporal oscillations of concentration gradients are expected at steady state.

**Theorem 2.** Suppose a molecular communication network is finite mode Turing unstable. Then, the right-most pole of the characteristic polynomial $p_\lambda(s) = 0$ is a pair of complex conjugate.

**Proof.** We prove by contradiction. Suppose the right-most root of $p_\lambda(s) = 0$ is a real number $\sigma$, and it is given when $\lambda = \lambda_0$. That is, $p_{\lambda_0}(\sigma) = 0$. It follows from (i) of Definition 1 that all of the poles of $h(s)$ lie in $\mathbb{C}_-$. Moreover, (ii) of Definition 1 implies that $\sigma > \max(\text{Re}[s_0], 0) \geq 0$, where $s_0$ is a root of $n(s) = 0$ with the largest real part. Therefore, $\sigma$ is greater than any real poles and zeros of $h(s)$. This, however, contradicts with the property of the root locus that the locus exists on real axis to the left of an odd number of poles and zeros of $h(s)$ [17].

In the next section, we turn our attention to the minimal molecular communication networks with $n = 3$ molecules and investigate their properties in detail.

5 Finite Mode Turing Instability in Three-Component Circuits

In this section, we consider molecular communication networks consisting of two internal circuit molecules, $M_1$ and $M_2$, and one diffuser $M_3$. For this class of systems, we first derive an analytic condition for finite mode Turing instability. Using the analytic condition, we characterize a structural property of biomolecular communication networks that admit Turing instability.

Let $\alpha_i$ and $\beta_i$ denote the coefficients of the following characteristic polynomials.

\[
|sI_3 - A| = s^3 + \alpha_2 s^2 + \alpha_1 s + \alpha_0, \\
|sI_2 - A_\text{circ}| = s^2 + \beta_1 s + \beta_0.
\]  

(20)  

(21)

Then, a necessary and sufficient condition for finite mode Turing instability is given by the following theorem.

**Theorem 3.** Consider the molecular communication network system composed of $n = 3$ molecules. The system is finite mode Turing unstable around an equilibrium $\bar{x}$, if and only
if the following (I), (II-A) and (II-B) are satisfied.

(I) \( \alpha_2 > 0 \), \( \alpha_1 \alpha_2 - \alpha_0 > 0 \), \( \alpha_0 > 0 \),
(II-A) \( \beta_1 > 0 \), \( \beta_0 > 0 \),
\[ \alpha_1 + \beta_1 \alpha_2 - \beta_0 \leq -2\sqrt{\beta_1 (\alpha_1 \alpha_2 - \alpha_0)} , \]
(II-B) \( \beta_1 \leq 0 \), \( \beta_1^2 - 4\beta_0 < 0 \),
\[ -\beta_1^2 + \beta_0 + \beta_1 \alpha_2 - \alpha_1 > 0 \).

The conditions (I), (II-A) and (II-B) in Theorem 3 are equivalent to (i), (ii-a) and (ii-b) of Lemma 1, respectively. The complete proof is shown in Appendix C. It should be noticed that \( \beta_i \) \( (i = 0, 1) \) are determined from the parameters of the internal circuit \( A_{\text{circ}} \), while \( \alpha_i \) \( (i = 0, 1, 2) \) depend on the entire system including the diffuser. Theorem 3 allows us to further investigate the properties of three-component systems. In what follows, we show that a molecular communication network needs to have a certain structural property to admit finite mode Turing instability.

We consider a class of cascaded three-component circuits illustrated in Fig. 7. The matrix \( A \) is then defined by

\[
A = \begin{bmatrix}
  a_{11} & a_{12} & 0 \\
  a_{21} & a_{22} & a_{23} \\
  0 & a_{32} & a_{\text{diff}}
\end{bmatrix}.
\]  (22)

The activator-repressor-diffuser network in Section 3.2 is a class of the cascaded circuit with \( a_{12} < 0 \), \( a_{21} > 0 \), \( a_{23} < 0 \) and \( a_{32} < 0 \). Note that the signs of \( a_{12}, a_{21}, a_{23} \) and \( a_{32} \) depend on whether the molecule \( M_1, M_2 \) and \( M_3 \) are activator or repressor. The following corollary specifies the combinations of activator and repressors that admit finite mode Turing instability.

**Corollary 1.** Consider the molecular communication networks with the cascaded structure shown in Fig. 7. Let \( r_1 := a_{12} a_{21} \) and \( r_2 := a_{23} a_{32} \). Then, the network admits finite mode Turing instability only if \( r_1 < 0 \) and \( r_2 > 0 \).

The proof can be found in Appendix D. This corollary narrows down the design space of biomolecular communication networks. In other words, the combination of the molecules \( M_1, M_2 \) and \( M_3 \) needs to be either of (activator, repressor, repressor) or (repressor, activator, activator) in order for the system to achieve finite mode Turing instability. It should be noticed that the example shown in Section 3.2 is an example of (activator, repressor, repressor) configuration.
6 Conclusion

In this paper, we have proposed a control theoretic modeling and analysis framework for biomolecular communication networks modeled by reaction-diffusion equations with a single diffusible molecule. We have first shown that the analysis of the infinite dimensional system can be simplified by decomposing it into finite dimensional subsystems. We have then provided a graphical stability/instability analysis tool using the root locus approach and demonstrated on the novel activator-repressor-diffuser system, which allows for the formation of inhomogeneous concentration gradient over the cell population. The latter half of the paper has been devoted to analyzing the conditions for finite mode Turing instability. Using the analytic condition of Turing instability, we have revealed the structural property of biomolecular communication networks that admit Turing instability.

References

[1] I. F. Akyildiz, F. Fekri, R. Sivakumar, C. R. Forest, and B. K. Hammer, “MONACO: Fundamentals of molecular nano-communication networks,” IEEE Wireless Communications, vol. 19, no. 5, pp. 12–18, 2012.

[2] T. Nakano, T. Suda, Y. Okaie, M. J. Moore, and A. V. Vasilakos, “Molecular communication among biological nanomachines: a layered architecture and research issue,” IEEE Transactions on NanoBioscience, vol. 13, no. 3, pp. 169–197, 2014.

[3] T. Danino, O. Mondragón-Palomino, L. Tsimring, and J. Hasty, “A synchronized quorum of genetic clocks,” Nature, vol. 463, no. 7279, pp. 326–330, 2010.

[4] L. You, R. S. C. III, R. Weiss, and F. H. Arnold, “Programmed population control by cell-cell communication and regulated killing,” Nature, vol. 428, no. 6985, pp. 868–871, 2004.

[5] F. K. Balagaddé, H. Song, J. Ozaki, C. H. Collins, M. Barnet, F. H. Arnold, S. R. Quake, and L. You, “A synthetic Escherichia coli predatorprey ecosystem,” Molecular Systems Biology, vol. 4, no. 1, 2008.

[6] J. J. Tabor, H. Salis, Z. B. Simpson, A. A. Chevalier, A. Levenskaya, E. M. Marcotte, C. A. Voigt, and A. D. Ellington, “A synthetic genetic edge detection program,” Cell, vol. 137, no. 7, pp. 1272–1281, 2009.

[7] A. Tamsir, J. J. Tabor, and C. A. Voigt, “Robust multicellular computing using genetically encoded nor gates and chemical wires,” Nature, vol. 469, no. 7329, pp. 212–215, 2011.

[8] S. Regot, J. Macia, N. Conde, K. Furukawa, J. Kjellén, T. Peeters, S. Hohmann, E. de Nadal, F. Posas, and R. Solé, “Distributed biological computation with multicellular engineered networks,” Nature, vol. 469, no. 7329, pp. 207–211, 2011.

[9] S. Basu, Y. Gerchman, C. H. Collins, F. H. Arnold, and R. Weiss, “A synthetic multicellular system for programmed pattern formation,” Nature, vol. 434, no. 7037, pp. 1130–1134, 2005.

[10] C. Liu, X. Fu, L. Liu, X. Ren, C. K. Chau, S. Li, L. Xiang, H. Zeng, G. Chen, L.-H. Tang, P. Lenz, X. Cui, W. Huang, T. Hwa, and J.-D. Huang, “Sequential establishment of stripe patterns in an expanding cell population,” Science, vol. 334, no. 6053, pp. 238–241, 2011.
A. Koch and H. Meinhardt, “Biological pattern formation: from basic mechanisms to complex structures,” *Reviews of Modern Physics*, vol. 66, no. 4, pp. 1481–1507, 1994.

S. Kondo and T. Miura, “Reaction-diffusion model as a framework for understanding biological pattern formation,” *Science*, vol. 329, no. 5999, pp. 1616–1620, 2010.

J. Hsia, W. J. Holtz, D. C. Huang, M. Arcak, and M. M. Maharbiz, “A feedback quenched oscillator produces Turing patterning with one diffuser,” *PLOS Computational Biology*, vol. 8, no. 1, 2012, e1002331.

L. Edelstein-Keshet, *Mathematical models in biology*. SIAM, 2005.

A. M. Turing, “The chemical basis of morphogenesis,” *Philosophical Transactions of the Royal Society of London B*, vol. 237, no. 641, pp. 37–72, 1952.

A. Gierer and H. Meinhardt, “A theory of biological pattern formation,” *Kybernetik*, vol. 12, no. 1, pp. 30–39, 1972.

W. S. Levine, *The Control Handbook*. CRC Press, 1996.

H. Miyazako, Y. Hori, and S. Hara, “Turing instability in reaction-diffusion systems with a single diffuser: characterization based on root locus,” in *Proceedings of IEEE Conference on Decision and Control*, 2013, pp. 2671–2676.

Y. Hori, S. Kumagai, and S. Hara, “Network structure for Turing instabilizability in reaction-diffusion systems with one diffuser: a case study for three-gene networks,” in *Proceedings of SICE Annual Conference*, 2014, pp. 892–895.

T. S. Gardner, C. R. Cantor, and J. J. Collins, “Construction of a genetic toggle switch in escherichia coli,” *Nature*, vol. 403, no. 6767, pp. 339–342, 2000.

M. B. Elowitz and S. Leibler, “A synthetic oscillatory network of transcriptional regulators,” *Nature*, vol. 403, no. 6767, pp. 335–338, 2000.

T.-S. Moon, C. Lou, A. Tamsir, B. C. Stanton, and C. A. Voigt, “Genetic programs constructed from layered logic gates in single cells,” *Nature*, vol. 491, no. 7423, pp. 249–253, 2012.

S. Hara, T. Hayakawa, and H. Sugata, “LTI systems with generalized frequency variables: A unified framework for homogeneous multi-agent dynamical systems,” *SICE Journal of Control, Measurement and System Integration*, vol. 2, no. 5, pp. 299–306, 2009.

J. Murray, *Mathematical Biology II*, 3rd ed. Springer, 2003.

**A  Proof of Proposition 2**

We use the fact that the root locus $\mathcal{R}$ starts at the poles of $h(s)$ and converges to the zeros of $h(s)$ [17]. Using the definition [6], we can write $h(s)$ as

$$h(s) = \frac{e_n^T \text{adj}(sI_n - A)e_n}{|sI_n - A|},$$

where $\text{adj}(X)$ denotes the adjugate matrix of $X$. It follows from the definition of the adjugate matrix that the $(n,n)$-th entry of $\text{adj}(sI_n - A)$ is $|sI_{n-1} - A_{\text{circ}}|$, and thus the numerator of $h(s)$ can be written as $e_n^T \text{adj}(sI_n - A)e_n = |sI_{n-1} - A_{\text{circ}}|$. Therefore, the root locus $\mathcal{R}$ starts from the roots of $|sI_n - A| = 0$ and converges to the roots of $|sI_{n-1} - A_{\text{circ}}|$. 

[100x118]14
B  Proof of Theorem 1

For \( n = 1 \), it follows that the molecular communication network is finite mode Turing unstable if and only if the characteristic polynomial \( p_\lambda(s) = s - a_{\text{diff}} + \lambda \) satisfies (ii-a) of Lemma 1. We can easily see that \( \text{Re}[p_\lambda(j\omega)] = 0 \) cannot be satisfied for all \( \lambda > 0 \) and \( \omega \in \mathbb{R} \), since \( a_{\text{diff}} < 0 \).

For \( n = 2 \), we define coefficient \( \alpha_0, \alpha_1, \beta_0 \) by \( |sI_2 - A| = s^2 + \alpha_1 s + \alpha_0 \) and \( |sI_2 - A_{\text{circ}}| = s + \beta_0 \). In what follows, the proof will be given based on Lemma 1. Suppose \( A \) is Hurwitz, which corresponds to the condition (i) in Lemma 3. We show that both (ii-a) and (ii-b) cannot be satisfied.

Case 1: \( A_{\text{circ}} \) is Hurwitz: It follows that \( \text{Im}[p_\lambda(j\omega)] = \omega(\alpha_1 + \lambda) \) for all \( \lambda > 0 \) and \( \omega \neq 0 \), since \( \alpha_1 > 0 \) holds when \( A \) is Hurwitz. For \( \omega = 0 \), \( \text{Re}[p_\lambda(0)] = \alpha_0 + \lambda\beta_0 \neq 0 \) holds for all \( \lambda > 0 \) because of \( \alpha_0 > 0 \) and \( \beta_0 > 0 \). Thus, the condition (ii-a) in Lemma 1 cannot be satisfied.

Case 2: \( A_{\text{circ}} \) is not Hurwitz: It follows that \( \text{Im}[p_\lambda(j\omega)] = \omega(2\gamma + \alpha_1 + \lambda) \), where \( \gamma = \beta_0(\geq 0) \) from the definition. Thus, \( \text{Im}[(\lambda, \gamma + j\omega)] \neq 0 \) for all \( \lambda > 0 \) and \( \omega \neq 0 \), since \( \alpha_1 > 0 \) and \( \gamma \geq 0 \). When \( \omega = 0 \), \( \text{Re}[p_\lambda(\gamma)] = \gamma^2 + \gamma(\alpha_1 + \lambda) + \alpha_0 + \lambda\beta_0 > 0 \) hold, and \( \text{Re}[p_\lambda(\gamma)] \neq 0 \) hold for all \( \lambda > 0 \). This completes the proof of the proposition.

C  Proof of Theorem 3

We prove the theorem by calculating conditions (i), (ii-a) and (ii-b) of Lemma 1 for \( n = 3 \).

It can be verified that \( A \) is Hurwitz if and only if

\[
\alpha_2 > 0, \quad \alpha_1 \alpha_2 - \alpha_0 > 0, \quad \alpha_0 > 0, \tag{24}
\]

which is equivalent to the condition (i) in Lemma 1.

In what follows, we derive the conditions (II-A) and (II-B) by considering the case where \( A_{\text{circ}} \) is Hurwitz and not Hurwitz, respectively. We can easily verify that neither (ii-a) nor (ii-b) of Lemma 3 can be satisfied when \( \omega = 0 \). Hence, we hereafter show the proof for the case of \( \omega \neq 0 \).

Case 1: \( A_{\text{circ}} \) is Hurwitz: A necessary condition for \( A_{\text{circ}} \) being Hurwitz is

\[
\beta_1 > 0 \text{ and } \beta_0 > 0. \tag{25}
\]

Moreover, \( p_\lambda(j\omega) = 0 \) implies that

\[
\text{Re}[p_\lambda(j\omega)] = -\omega^2 + \alpha_0 + \lambda(-\omega^2 + \beta_0) = 0 \tag{26}
\]

\[
\text{Im}[p_\lambda(j\omega)] = -\omega\{\omega^2 - (\alpha_1 + \lambda\beta_1)\} = 0. \tag{27}
\]

It follows from (27) that \( \omega^2 = \alpha_1 + \lambda\beta_1(> 0) \), since \( \omega \neq 0 \). We can then eliminate \( \omega^2 \) from (26), and we have the following quadratic equation of \( \lambda \).

\[
\beta_1\lambda^2 + (\alpha_1 + \beta_1\alpha_2 - \beta_0)\lambda + \alpha_1\alpha_2 - \alpha_0 = 0. \tag{28}
\]

Since \( \beta_1 > 0 \) and \( \alpha_1\alpha_2 - \alpha_0 > 0 \) hold from (24) and (25), a necessary and sufficient condition for (28) having a positive real solution is given by the following (C1) and (C2).

(C1) The determinant of (28) is non-negative. That is,

\[
((\alpha_1 + \beta_1)\alpha_2 - \beta_0^2)^2 - 4\beta_1(\alpha_1\alpha_2 - \alpha_0) \geq 0. \tag{29}
\]
The coefficient of $\lambda$ in (28) is negative. That is,
\[
\alpha_1 + \beta_1 \alpha_2 - \beta_0 < 0. 
\] (30)
Summarizing (29) and (30), we have
\[
\alpha_1 + \beta_1 \alpha_2 - \beta_0 \leq -2 \sqrt{\beta_1 (\alpha_1 \alpha_2 - \alpha_0)}.
\] (31)
The condition (II-A) is obtained from (25) and (31).

**Case 2**: $A_{\text{circ}}$ is not Hurwitz

A necessary condition for $A_{\text{circ}}$ not being Hurwitz is
\[
\beta_1 \leq 0 \text{ or } \beta_0 \leq 0. 
\] (32)
Let $\gamma \pm j\eta$ ($\gamma, \eta \geq 0$) denote a pair of eigenvalues of $A_{\text{circ}}$ with the largest real part. Then, $| (\gamma + j\eta)I_2 - A_{\text{circ}} | = 0$ implies
\[
\gamma^2 + \beta_1 \gamma + \beta_0 - \eta^2 = 0 
\] (33)
\[
(2\gamma + \beta_1)\eta = 0. 
\] (34)

In what follows, we first show that $p_\lambda(\gamma + j\eta) \neq 0$ for all $\lambda > 0$ and $\omega$ when $\eta = 0$. It follows from $\text{Im}[p_\lambda(\gamma + j\omega)] = 0$ that
\[
\omega^2 = 3\gamma^2 + 2\alpha_2 \beta + \alpha_1 + \lambda(2\gamma + \beta_1). 
\] (35)
Substituting this into $\text{Re}[p_\lambda(\gamma + j\omega)] = 0$ yields the following equation of $\lambda$.
\[
(2\gamma + \beta_1)\lambda^2 + \{9\gamma^2 + (4\alpha_2 + 3\beta_1)\gamma + \alpha_1 + \beta_1 \alpha_2 \} \lambda 
\] 
\[
+ \{8\gamma^3 + 8\alpha_2 \gamma^2 + 2(\alpha_1 + \alpha_2^2)\gamma + (\alpha_1 \alpha_2 - \alpha_0) \} = 0. 
\] (36)
It should be noted that the coefficient of $\lambda^2$ is non-negative, or $2\gamma + \beta_1 \geq 0$, since $\gamma$ is the largest real root of $s^2 + \beta_1 s + \beta_0 = 0$ from the definition. Moreover, (24) and (32) imply that the coefficient of $\lambda$ and the constant terms of (36) are positive as well. Thus, $p_\lambda(\gamma + j\eta) \neq 0$ for all $\lambda > 0$ and $\omega$ when $\eta = 0$.

When $\eta \neq 0$, the determinant of $| sI_2 - A_{\text{circ}} | = 0$ is negative, thus we have
\[
\beta_1^2 - 4\beta_0 < 0. 
\] (37)
Moreover, $\text{Im}[p_\lambda(\gamma + j\omega)] = 0$ implies
\[
\omega^2 = 3\gamma^2 + 2\alpha_2 \beta + \alpha_1. 
\] (38)
Substituting this into $\text{Re}[p_\lambda(\gamma + j\omega)] = 0$ yields
\[
\{\gamma^2 - (3\beta_1^2 + 2\alpha_2 \beta + \alpha_1) \} \lambda - \{8\gamma^3 + 8\alpha_2 \gamma^2 + 2(\alpha_1 + \alpha_2^2) \gamma + (\alpha_1 \alpha_2 - \alpha_0) \} = 0. 
\] (39)
Note that the constant term of (39) is negative because of (24) and $\gamma > 0$. Thus, (39) has a positive real root if and only if
\[
\eta^2 - (3\gamma^2 + 2\alpha_2 \gamma + \alpha_1) > 0. 
\] (40)
The condition (II-B) is obtained from (32), (33), (34), (37) and (40).
D Proof of Corollary 1

We first show $r_1 < 0$.

Suppose (I) and (II-A) of Theorem 3 hold. It follows from $\beta_1 = -(a_{11} + a_{22}) > 0$ of (II-A) that either or both of $a_{11}$ and $a_{22}$ need to be negative. It clearly follows that $r_1 = a_{12}a_{21} < 0$ when $a_{11}$ and $a_{22}$ have opposite signs, hence we consider the case of $a_{11} < 0$ and $a_{22} < 0$ in the following.

We first note that the condition $\alpha_1\alpha_2 - \alpha_0 > 0$ of (I) can be equivalently written as

$$(a_{11} + a_{22})r_1 + (a_{22} + a_{\text{diff}})r_2 - (a_{11} + a_{22})(a_{\text{diff}} + a_{11}) > 0.$$  \hspace{1cm} (41)

Dividing (41) by $a_{11} + a_{22}(<0)$, we have

$$r_1 < (a_{22} + a_{\text{diff}})(a_{\text{diff}} + a_{11}) - \frac{a_{22} + a_{\text{diff}}}{a_{11} + a_{22}}r_2.$$  \hspace{1cm} (42)

On the other hand, $r_2$ satisfies the following inequality from

$$\alpha_1 + \beta_1\alpha_2 - \beta_0 \leq -2\sqrt{\beta_1(\alpha_1\alpha_2 - \alpha_0)} \leq 0$$  \hspace{1cm} (43)

of (II-A).

$$(a_{11} + a_{22})(a_{11} + a_{22} + 2a_{\text{diff}}) - r_2 \leq 0.$$  \hspace{1cm} (44)

Since $a_{11} < 0, a_{22} < 0$ and $a_{\text{diff}} < 0$, the second term on the right side of (42) satisfies

$$-\frac{a_{22} + a_{\text{diff}}}{a_{11} + a_{22}}r_2 < -(a_{22} + a_{\text{diff}})(a_{11} + a_{22} + 2a_{\text{diff}}).$$  \hspace{1cm} (45)

Summerizing (42) and (45), we have

$$r_1 < (a_{22} + a_{\text{diff}})(a_{\text{diff}} + a_{11}) - \frac{a_{22} + a_{\text{diff}}}{a_{11} + a_{22}}r_2$$

$$< (a_{22} + a_{\text{diff}})(a_{\text{diff}} + a_{11})$$

$$- (a_{22} + a_{\text{diff}})(a_{11} + a_{22} + 2a_{\text{diff}})$$

$$= -(a_{22} + a_{\text{diff}})^2 < 0.$$  \hspace{1cm} (46)

Suppose (I) and (II-B) of Theorem 3 hold. It then follows from $\beta_1^2 - 4\beta_0 < 0$ of (II-B) that

$$(a_{11} + a_{22})^2 - 4(a_{11}a_{22} - r_1) = (a_{11} - a_{22})^2 + 4r_1 < 0.$$  

This leads to $r_1 < 0$.

Next, we show that $r_2 > 0$ is necessary. Suppose (II-B) of Theorem 3 holds. Then, the direct calculation leads to $-\beta_1^2 + \beta_0 + \beta_1\alpha_2 - \alpha_1 = r_2 > 0$. In what follows, we consider the case where (II-A) of Theorem 3 holds.

It follows from $a_{\text{diff}} < 0$ and $\beta_1 = -(a_1 + a_2) > 0$ of (II-A) that $(a_1 + a_2 + 2a_3) < 0$. Then, $\alpha_1 + \beta_1\alpha_2 - \beta_0 \leq -2\sqrt{\beta_1(\alpha_1\alpha_2 - \alpha_0)} \leq 0$ of (II-A) implies (44). Thus, $r_2 > (a_1 + a_2)(a_1 + a_2 + 2a_3) > 0$. 

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