Modelling periodic oscillation of biological systems with multiple timescale networks

R. Wang, T. Zhou, Z. Jing and L. Chen

Abstract: In this paper, we aim to develop a new methodology to model and design periodic oscillators of biological networks, in particular gene regulatory networks with multiple genes, proteins and time delays, by using multiple timescale networks (MTN). Fast reactions constitute a positive feedback-loop network (PFN), while slow reactions consist of a cyclic feedback-loop network (CFN), in MTN. Multiple timescales are exploited to simplify models according to singular perturbation theory. We show that a MTN has no stable equilibrium but stable periodic orbits when certain conditions are satisfied. Specifically, we first prove the basic properties of MTNs with only one PFN, and then generalise the result to MTNs with multiple PFNs. Finally, we design a biologically plausible gene regulatory network by the *cI* and *Lac* genes, to demonstrate the theoretical results. Since there is less restriction on the network structure of a MTN, it can be expected to apply to a wide variety of areas on the modelling, analysing and designing of biological systems.

1 Introduction

Living organisms have rhythmic phenomena at all levels with periods ranging from less than a second to years [1–5]. From both theoretical and experiment viewpoints, it is a greatly challenging problem in biological science to model, analyse and further predict the periodic behaviours of bio systems. One of the best studied rhythmic phenomena so far is circadian oscillations, which are assumed to be produced by limit cycle oscillators at the molecular level [6] from the gene regulatory feedback loops. With the rapid advances in mathematics and experiments concerning the underlying regulatory mechanisms, more sophisticated theoretical models and general techniques are increasingly demanded to elucidate periodic behaviours, with the consideration of time delays that are particularly important for the eukaryotes due to time-consuming transportation or diffusion processes of molecules between the nucleus and cytoplasm in a cell.

On the other hand, in addition to the natural systems, recent progress in genetic engineering has made the design and implementation of artificial or synthetic gene networks realistic from both theoretical and experimental viewpoints [7, 8], in particular for simple organisms such as *E. coli* and *yeast*. Actually, from the theoretical predictions, several simple gene networks have been experimentally constructed, e.g. genetic toggle switch [9], repressilator [10] and other gene circuits [11, 12]. The data in these experiments agree well with the theoretical predictions, which implies that the mathematical model is a powerful tool for designing synthetic gene regulatory networks. Such simple models clearly represent a first step towards logical cellular control by manipulating and monitoring biological processes at the DNA level, and not only can be used as building blocks to synthesise the artificial biological systems, but also have great potential for biotechnological and therapeutic applications [3, 8, 13]. For synthetic switching networks, a general design procedure based on positive feedback-loop networks (PFNs) [7, 8] has been recently developed, which guarantees the stable switching states without any non-equilibrium dynamics, thereby making theoretical analysis and designing tractable even for large-scale systems with time delays. However, for synthetic oscillating networks, although the repressilator and hysteresis-based oscillators were proposed [2, 3, 14], there has not yet been a general network to theoretically ensure the existence of periodic oscillations in particular for large-scale systems with the consideration of the time delays.

Mathematically, there are a tremendous number of theoretical results [2, 3, 15–21] providing the sufficient conditions of limit cycles in the framework of functional differential equations (FDEs), but mainly with a few variables or with linear or certain special structures when time delays are considered. Generally, it is difficult to guarantee that a system converges to a limit cycle or a sustained oscillation even for a simple-structured non-linear system. Therefore, many important physiological factors such as time delays are simply ignored in order to reduce dimensionality and complexity of the systems. It is well known, however, that such factors may play important roles in the dynamics of biological systems. Recently, based on monotone dynamical systems [22, 23], Mallet-Paret and Sell [24, 25] introduced a discrete Lyapunov functional and

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successfully developed a general theory to show the existence of the omega-limit set by obtaining a Morse decomposition of the global attractor for a cyclic feedback-loop network (CFN) with time delays, which opened the door to a general inquiry into not only the topological structure but also the sufficient conditions of the existence for a specific attractor, in particular a periodic attractor. However, rather than a general network structure, a CFN is a single cyclic loop which considerably limits its application, although the original CFN has been extend to a general cyclic feedback-loop system for which the sufficient conditions to ensure the periodic orbit are also derived in [26, 27].

Explicitly considering all variables and chemical reactions in a cell is unrealistic for a gene regulatory network from a modelling, analysing and computing viewpoint. However, in a cell, many different timescales characterise the gene regulatory processes, which can be exploited to reduce the complexity of the mathematical models [2, 28, 29]. For instance, the transcription and translation processes in a gene network generally evolve on a timescale that is much slower than that of phosphorylation, dimerisation or binding reactions of proteins in a protein network. In addition, although dynamics are intertwined between the gene network and protein network or metabolic network, interactions among each network are generally more active than those between them, or they are relatively independent [30]. Such properties can be also exploited to simplify the model, provided that the simplified system is guaranteed to behave both qualitatively and quantitatively as the original one.

Specifically, this paper aims to develop a new methodology to analyse and design a biological oscillating network with time delays, by using a multiple timescale network (MTN) which is composed of a CFN and multiple PFNs. A PFN is mainly constituted by fast reactions or a protein network, whereas a CFN consists of slow reactions or a gene network. It has been shown that a general PFN has no dynamical attractors but stable equilibria [7, 8]. In contrast, a CFN has omega-limit sets composed of only periodic orbits and equilibria [24, 25]. In this paper, we prove that a MTN with certain conditions has no stable equilibria but stable periodic oscillations, depending on the total time delay of the CFN, although it has a complicated network structure including both positive and negative feedback loops. Such a property is clearly ideal for designing or modelling biological oscillators. Since there is less restriction on the network structure of a MTN, it can be expected to apply to a wide variety of areas on the modelling, analysing and designing of biological systems.

2 PFN and CFN

Next, we first describe the notation and briefly summarises recent theoretical results for PFN [7, 8] and CFN [24, 25, 26, 27], and then derive our main result for MTN which includes both PFNs and a CFN. The readers who are mainly interested in the application, are recommended to continue from Section 4.

2.1 Notation

Let $\mathbb{R}^+$ be the set of non-negative real numbers. Assume that a biological network is composed of n chemical components, which represent proteins, mRNAs, chemical complexes, different states of the same protein, and proteins at different locations in a cell. Then the network can be written by the following functional differential equations

$$\dot{z}(t) = F(z_t) \tag{1}$$

where $z(t) = (z_1(t), \ldots, z_n(t)) \in Z \subset \mathbb{R}^{+n}$ is the concentrations of all components at time $t \in \mathbb{R}$. Let $\mathbb{C}^+ \equiv \mathbb{C}([-r, 0], \mathbb{R}^{+n})$, where $\mathbb{C}([-r, 0], \mathbb{R}^{+n})$ is the space of continuous maps on $[-r, 0]$ into $\mathbb{R}^{+n}$. $z_t \in \mathbb{C}^+$ is defined by $z_t \equiv z(t+\theta), -r \le \theta \le 0$. $F = (F_1, \ldots, F_n)$: $\mathbb{C}^+ \to \mathbb{R}^{+n}$ are the reaction rates of components, continuously differentiable, and map a bounded subset of $\mathbb{C}^+$ to a bounded subset of $\mathbb{R}^{+n}$. In addition, we define $N = \{1, \ldots, n\}$. Note that the reaction rates F include both the synthesis and degradation rates of components.

For (1), a function $z(t; \phi) \in \mathbb{R}^{+n}$ is said to be a solution of (1) if it satisfies (1) for all $t \ge t_0$ with $z(t_0 + \theta; \phi) = \phi(\theta), -r \le \theta \le 0$, where $\phi \in \mathbb{C}^+$ is a given initial function. The orbit of (1) for the initial condition ϕ is $\mathbb{O}^+(\phi) \stackrel{\Delta}{=} \{z(t; \phi)\colon t \ge t_0\}$. We assume that the solution of (1) exists and is bounded, i.e. $\|z(t; \phi)\| < \infty$ for all $t \ge t_0$ and $z(t; \phi) \in Z$. To emphasise on the initial function, we define $z_t(\phi) \equiv z(t + \theta; \phi)$ with $z_{t_0}(\phi) = z(t_0 + \theta; \phi) = \phi(\theta), -r \le \theta \le 0$. Moreover, equilibria, periodic orbit, omega and alpha limit sets are defined in the following ways.

Definition 1: (Equilibria). The set of equilibria for (1) is defined by:

$$E \stackrel{\Delta}{=} \{\phi \in \mathbb{C}^+ : \phi = \hat{z} \text{ for some } z \in Z \subset \mathbb{R}^{+n} \text{ satisfying } F(\hat{z}) = 0\},$$

where $\hat{z}$ is the constant function equal to z for all values of its argument, i.e. $\hat{z}(\theta) \equiv z, -r \le \theta \le 0$.

Definition 2: (Omega and alpha limit sets). The omega limit set is defined by

$$\omega(\phi) \stackrel{\Delta}{=} \bigcap_{s \ge 0} \overline{\{z_t(\phi)\colon t \ge s\}} \tag{2}$$

whereas the alpha limit set is

$$\alpha(\phi) \stackrel{\Delta}{=} \bigcap_{s \le 0} \overline{\{z_t(\phi)\colon t \le s\}} \tag{3}$$

Definition 3: (Periodic orbit). The orbit $\mathbb{O}^+(\phi)$ is said to be a T-periodic orbit if $z_{T+t}(\phi) = z_t(\phi)$ for all t and the minimal $T > 0$.

Time delays are primarily caused by transcription, translation, translocation, and diffusion processes. For the sake of simplicity, we only consider the case of one time delay for each chemical component, although there may exist multiple direct interactions from one chemical component to another with different time delays.

Before defining PFNs and CFNs, we make several general assumptions as follows.

Assumption 1: (Monotone system). $\partial F_i(z)/\partial z_j > 0, < 0$ or $= 0$ for $i, j \in N$ but $i \ne j$, and all $z \in Z$ in (1).

Assumption 1 is a monotone assumption, which implies that for $1 \le i \le n$, the reaction rate of the ith chemical component at t, i.e. $F_i(z_t)$ monotonously increases or decreases or is unaffected, with respect to the concentration of the jth chemical component $z_j(t - \tau_{ij})$ at $t - \tau_{ij}$. Such assumption can be equivalently expressed as: all elements of the Jacobian matrix for F with respect to z have fixed signs for all $z \in Z$. The basic mechanism of chemical reactions is the *law of mass action*, which implies that the speed of a chemical reaction often has the monotonicity. In other words, assumption 1 is generally satisfied in most biological systems.

To describe network structure, we define the types of interactions.

Definition 4: (Interactions)

1. If $\partial F_i(z)/\partial z_j > 0$ (or < 0) for all $z \in Z$, then the type of the interaction from the jth chemical component to the ith chemical component is called positive (or negative), and we set $s_{ij} = 1$ (or $s_{ij} = -1$).
2. If $\partial F_i(z)/\partial z_j = 0$, then we set $s_{ij} = 0$.

Thus, $s_{ij} = 1$ (or $s_{ij} = -1$) means that the jth chemical component affects *positively* (or *negatively*) the ith chemical component with time delay τ_{ij}. For instance, $s_{ij} = 1$ for $F_i = x_j(t-\tau_{ij})/(1 + x_j(t-\tau_{ij}))$ and $s_{ij} = -1$ for $F_i = 1/(1 + x_j^2(t-\tau_{ij}))$.

Now, we describe the definition of interaction *graph* [8], which enable us to intuitively understand the relations between the components.

Definition 5: (Graph). An interaction graph, $IG(F)$, of the biological network defined by (1) is a directed graph whose nodes represent the individual chemical components of the biological network and whose edges represent the interactions between nodes. When $s_{ij} \neq 0$, the graph has an edge e_{ij}, directed from node j to node i, where $i, j \in N$.

Figure 1 is an example of an interaction graph with six nodes and eight edges, where node-2 affects positively nodes 1 and 4, i.e. $s_{12} = s_{42} = 1$, but has no effect on nodes 3, 5 and 6, i.e. $s_{32} = s_{52} = s_{62} = 0$. Next, we define the types of a feedback loop, which are qualitative characteristics of biological networks.

Definition 6: (Feedback loops). If there is a path from the ith node of an interaction graph to itself, $p(i,i) = (i = p_1 \overset{e_{p_2p_1}}{\rightarrow} p_2 \ldots p_{l-1} \overset{e_{p_lp_{l-1}}}{\rightarrow} p_l = i)$ for $l > 2$, then this path is said to be a feedback loop. In addition, this feedback loop is said to be positive (or negative) if $\Pi_{m=1}^{l-1} s_{p_{m+1}p_m} = 1$ (or -1).

In Fig. 1, there are three feedback loops, e.g. $1 \rightarrow 2 \rightarrow 4 \rightarrow 5 \rightarrow 1$ is a positive feedback loop because of $s_{21}s_{42}s_{54}s_{15} = 1$ although there are negative interaction edges in it. Notice that definition 6 requires $l > 2$. Actually, when $l = 2$, it is a self-feedback loop, and not included in the feedback loops in the paper, which means that there are may be many linear or non-linear, positive or negative self-feedback loops with no time delay in the network.

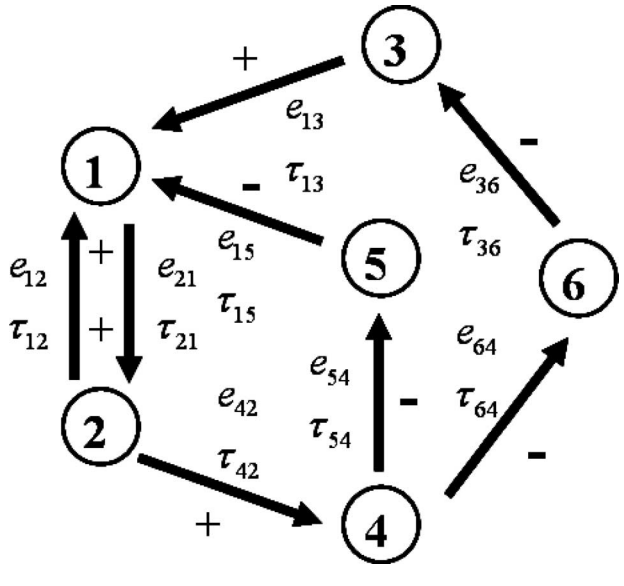

Fig. 1 *An example of PFN. Signs + and − on an edge indicate $s = 1$ and -1, respectively. There are total three loops composed of nodes (1,2), nodes (1,2,4,5) and nodes (1,2,4,6,3) respectively, which are all positive. There is no restriction on the self-feedback loops, which may be either positive or negative*

2.2 PFN

A PFN is a network with only positive feedback loops. We impose irreducibility on PFN, which means that for any two different nodes: i and j, there is at least one path from node-j to node-i.

Assumption 2: The interaction graph IG(F) defined by (1) has only positive feedback loops.

A network satisfying assumptions 1 and 2 is called a PFN. Although we require that the solution of (1) is bounded, any chemical component in a cell is finite and a biological system generally satisfies the bounded condition. However, to show the bounded solution for a system mathematically is a difficult task. As indicated in Appendix 8.2, for a PFN, we can prove that the solution of (1) is bounded if there exists two points $z_N, z_M \in R^{+n}$, such that $G(\mathbb{P}z_M) < 0$ and $G(\mathbb{P}z_N) \geq 0$ with $\mathbb{P}z_M \geq \mathbb{P}z_N$, where $G(\mathbb{P}z) = \mathbb{P}F(z)$, and $\mathbb{P}$ is a coordinate transformation, under which the original genetic network with only positive feedback loops can be transformed into an equivalent one with only positive interaction edges by $y = \mathbb{P}z$ of Appendix 8.1. An example is given in Appendix 8.2 to demonstrate the scheme.

As a simple example of PFN, we can verify that assumptions 1 and 2 and irreducibility are satisfied for the following simple example

$$\dot{x}_i(t) = \frac{1}{1 + x_j^2(t-\tau_j)} - x_i(t) \tag{4}$$

where i and j have the following two pair of values: $(i = 1, j = 2)$, $(i = 2, j = 1)$. Another example is a genetic switch by *lacI*, *tetR* and *cI* genes, which is studied in [7, 8].

Based on the monotone dynamical theory, Kobayashi *et al.* proved that a general biological network or (1) with only positive feedback loops has no dynamical attractors except stable equilibria [7, 8] and is robust to time delay variations.

Theorem 1: (Convergence to equilibria). If (1) satisfies assumptions 1 and 2, then for all most all initial conditions ϕ, $z_t(\phi)$ converges to equilibria.

Theorem 2: (Robustness on time delays). Let (5) be the associated ordinary differential equations (ODE) of (1) obtained by ignoring all time delays.

$$\dot{z}(t) = F(z(t)) \tag{5}$$

then (5) and (1) have identical equilibria. Moreover, if (1) satisfies all conditions of theorem 1, then the corresponding equilibria of (5) and (1) have identical stability.

Theorem 1 indicates that there are neither stable periodic oscillations nor other non-equilibrium attractors for PFNs, except stable equilibria. Theorem 2 means that if there exists an equilibrium that is asymptotically stable (or unstable) for ODE (5), then it is also asymptotically stable (or unstable) for FDE (1), and vice versa. In other words, time delays do not qualitatively affect the dynamics of PFNs, which are robust to time delay variations and their omega-limit sets are composed only of equilibria. Therefore, due to such properties, PFNs have been adopted to design and implement synthetic gene switches [8, 9, 12, 31]. In fact, although not proved, such properties are exploited to simplify models in many papers, such as in [28, 29]. In this paper, when PFNs are fast sub-networks mainly

representing protein networks in a MTN, we reduce PFNs to derive a simplified but dynamically equivalent MTN, by using theorems 1 and 2.

Figure 1 is an example of an interaction graph with only positive feedback loops. In this graph, there are three positive feedback loops: $1 \xrightarrow{+} 2 \xrightarrow{+} 1$, $1 \xrightarrow{+} 2 \xrightarrow{+} 4 \xrightarrow{-} 5 \xrightarrow{-} 1$ and $1 \xrightarrow{+} 2 \xrightarrow{+} 4 \xrightarrow{-} 6 \xrightarrow{-} 3 \xrightarrow{+} 1$.

2.3 CFN

Different from PFNs, whose orbits have a strong tendency to converge to equilibria, a CFN may have attracting periodic orbits, and its omega-limit sets are composed of only periodic orbits and equilibria [24–27]. The original CFN has been expanded to a general form, which has less restriction on the network structure [26, 27].

Assumption 3: For any *i*th node in the interaction graph IG(F) described by (1):

1. only the nearest neighbours, i.e. the $(i-1)$th node, the $(i+1)$th node and itself may affect the *i*th node;
2. if the interaction from the *i*th node to the $(i+1)$th node is positive (or negative), then the interaction from the $(i+1)$th node to the *i*th node is also non-negative (or non-positive);
3. $\tau_{i,i+1} = \tau_{i+1,i} = 0$ if both $s_{i,i+1}$ and $s_{i+1,i}$ are non-zero, otherwise $\tau_{i+1,i}$ can be any finite number;
4. if $i = n$, then $s_{n,1} = 0$ and $\tau_{1,n}$ can be any finite number.

For the sake of simplicity, we assume that there is only one interaction between nodes 1 and *n* in the fourth statement of assumption 3. Note that the 0th and $(n+1)$th nodes represent the *n*th and 1st nodes, respectively, in assumption 3. Assumption 3 can be mathematically expressed by

$$\begin{aligned} \dot{z}_1(t) &= F_1(z_2(t-\tau_{12}), z_1(t), z_n(t-\tau_{1n})) \\ \dot{z}_i(t) &= F_i(z_{i+1}(t-\tau_{i,i+1}), z_i(t), z_{i-1}(t-\tau_{i,i-1})) \\ & \quad 2 \le i \le n-1 \\ \dot{z}_n(t) &= F_n(z_n(t), z_{n-1}(t-\tau_{n,n-1})) \end{aligned} \tag{6}$$

where $\tau_{i+1,i} = \tau_{i,i+1} = 0$ if both $\partial F_{i+1}/\partial z_i$ and $\partial F_i/\partial z_{i+1}$ are non-zero for $1 \le i \le n-1$. Although we assume $\tau_{i+1,i} \ge 0$ for all *i* in this paper, mathematically the time delays $\tau_{i,j}$ can be of any sign in (6) [24, 25]. Notice that, for any two nodes with bi-directional interactions, the time delays between the two nodes must be zero according to statement 3 of assumption 3. In addition, assumption 3 requires

$$\frac{\partial F_i(z_{i+1}, z_i, z_{i-1})}{\partial z_{i+1}} \frac{\partial F_{i+1}(z_{i+2}, z_{i+1}, z_i)}{\partial z_i} \ge 0 \tag{7}$$

where $\partial F_{i+1}(z_{i+2}, z_{i+1}, z_i)/\partial z_i \ne 0$, for $1 \le i \le n-1$ and all $z \in Z$.

A network satisfying assumptions 1 and 3 is called a CFN. Figure 2 is an example of an interaction graph with cyclic feedback loops. Sings $+, -, +(0)$ and $-(0)$ on the edges indicate $s = 1$, -1, 1 or 0 and -1 or 0, respectively. Note that each node may have a linear or non-linear self-feedback loop that is omitted in Fig. 2. We can verify that (4) satisfies assumptions 1 and 3 when *i* and *j* have the following three pairs of values: $(i = 1, j = 2)$, $(i = 2, j = 3)$ and $(i = 3, j = 1)$, and is a CFN. Another example is Repressilator, which is studied in [3, 10, 26, 27].

Definition 7: (One-direction interaction). For any two nodes *i* and $i+1$ $(1 \le i \le n-1)$ of a CFN, if $s_{i,i+1} = 0$, then the interaction between these two nodes is said to be one-direction interaction.

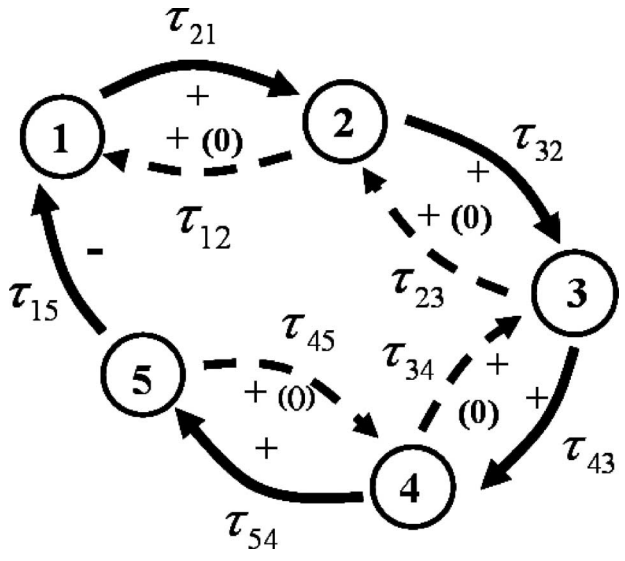

Fig. 2 *An example of a CFN. An interaction designated by a solid curve means that it cannot be zero, whereas a dashed curve means that it can be zero. Each node may have a linear or non-linear self-feedback loop that is omitted from the Figure. There is no restriction on the self-feedback loops, which may be either positive or negative*

Thus, $s_{i,i+1} = 0$ means the interaction between node-*i* and node-$(i+1)$ is a one-direction interaction. For instance, due to $s_{1,2} = 0$ for (4), the interaction between x_1 and x_2 is a one-direction interaction. In fact, statement 4 of assumption 3 is not necessary if there is at least one one-direction interaction in the system. Moreover, for one-direction interaction between any two nodes, the time delay can be any non-negative finite real number.

Based on the monotone dynamical system theory and discrete Lyapunov functional, Mallet-Paret and Sell [24, 25] obtained the Morse decomposition and derived the following Poincaré-Bendixson type theorem for the monotone cyclic feedback (6). Let the natural phase space for the (6) be $C(\mathbb{K})$, where:

$$\mathbb{K} = [0 \quad \tau_{21}] \cup [0 \quad \tau_{32}] \cup \cdots [0 \quad \tau_{n,n-1}] \cup [0 \quad \tau_{1,n}] \cup N. \tag{8}$$

Theorem 3: (Poincaré-Bendixson type theorem). Assume that assumptions 1 and 3 hold, and (6) is differentiable. Let $z(t)$ be a solution of (6) on some time interval $[t^0, \infty)$. Let $\omega(z) \subseteq C(\mathbb{K})$ denote the omega-limit set of this solution in the phase space $C(\mathbb{K})$. Then either:

1. $\omega(z)$ is a single non-constant periodic orbit; or else,
2. for each solution $u(t)$ of (6) in $\omega(z)$, i.e. for solutions with $u_t \in \omega(z)$ for all $t \in \mathbb{R}$, we have

$$\alpha(u) \cup \omega(u) \subseteq E \tag{9}$$

where $\alpha(u)$ and $\omega(u)$ denote the alpha- and omega-limit sets, respectively, of this solution, and where $E \subseteq C(\mathbb{K})$ denotes the set of equilibria of (6).

This theorem does not provide sufficient conditions for periodic orbits, but indicates that the CFN of (6) has omega-limit sets composed only of periodic orbits and equilibria, which is a desirable property for modelling the oscillations of biological systems. A general CFN is adopted in this

paper as a main feedback loop primarily representing the slow gene network in a MTN, to generate a periodic oscillation whose sufficient conditions are also provided in [26, 27].

3 MTN and main result

In a cell, there are not only many subsystems, such as a gene network and protein network or metabolic network, but also many different timescales characterising the gene regulatory processes, which can be exploited to reduce the complexity of the mathematical models [2, 28, 29]. Generally, the dynamics of a gene network primarily including the gene regulatory reactions, such as the transcription and translation processes, evolves on a timescale that is much slower than those of a protein network mainly including protein reactions, such as phosphorylation, dimerisation or binding reactions. In addition, although dynamics are intertwined between the gene network and protein network or metabolic network, the topological structure of interactions for each network is relatively independent of each other. A MTN is constituted by exploiting such properties to transform a complicated biological model into a simplified but dynamically equivalent system.

A MTN is composed of one slow CFN and multiple fast PFNs, mainly representing the gene network and protein networks, respectively. We first analyse a basic MTN with one slow CFN and only one fast PFN, and then extend our results to a general MTN with multiple PFNs, chiefly based on the singular perturbation theory.

3.1 Basic MTN

A basic MTN consists of a fast PFN and a slow CFN. Assume that there are m fast variables $y = (y_1, \ldots, y_m) \in \mathbb{R}^{+m}$ and p slow variables $x = (x_1, \ldots, x_p) \in \mathbb{R}^{+p}$, representing the concentrations of chemical components at time $t \in \mathbb{R}$, where $p \geq 2$. Then (1) can be rewritten as

$$\dot{x}(t) = f(x_t, y_t), \tag{10}$$

$$\dot{y}(t) = \frac{1}{\epsilon} g(x_t, y_t) \tag{11}$$

where $z = (x, y)$ and $F = (f, g/\epsilon)$. ϵ is a small positive real parameter. (10) and (11) are called a *singularly perturbed system* also known as a fast-slow system with slow x and fast y. Such multiple timescale properties are found in many biochemical systems, in particular gene regulatory systems [2, 28, 29].

Assumption 4: (11) is a PFN for the fixed x_t. (10) has a CFN structure except two neighbouring variables in x_t interacting with y_t or the PFN.

Assumption 4 means that all loops in (11) are positive for the fixed x_t, and (10) has the same structure as (6) except those affecting y_t. Figure 3 is an example of a MTN. The slow sub-network is composed of p slow chemical components from the 1st node to the pth node, and has the same structure as the CFN shown in Fig. 2 when all y are eliminated. The fast sub-network is comprised of m fast chemical components from the $(p+1)$th node to the $(p+m)$th node, and is a PFN, which has the same structure as Fig. 1. Note that there may be many variables in y interacting with f or x but only two variables (i.e. x_{p-1}, x_p in Fig. 3) in x affecting g or y.

When $\epsilon = 0$, (10) and (11) degenerate to a set of p only functional differential equations, i.e. (10) with a constraint:

$$0 = g(x_t, y_t) \tag{12}$$

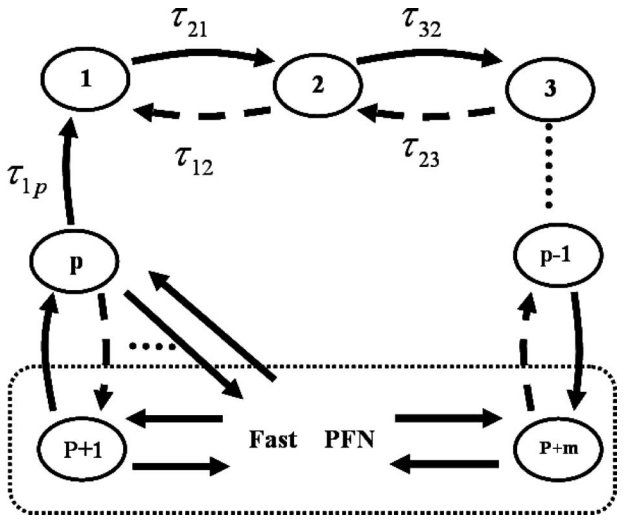

Fig. 3 *An example of a basic MTN. The slow sub-network is composed of p nodes from the 1st node to the pth node. The fast sub-network comprises m nodes from the $(p+1)$th node to the $(p+m)$th node, and is a PFN, which has the same structure as Fig. 1. The PFN structure and all self-feedback loops are omitted in the Figure*

With assumption 4 and properties of PFNs, for the fixed x_t, the fast subsystem (11) converges to a stable equilibrium $E_0 = \{y_0(x_t)\}$. Let K denote the set of solution points of (12). Since (11) is a PFN that is irreducible, $\partial g/\partial y$ is negatively definite in K, thereby $\det(\partial g/\partial y) \neq 0$ or $\operatorname{rank}(\partial g/\partial y) = m$ at the point E_0 of K. By the implicit function theorem, there exist neighbourhoods of A^o of x_{E_0} and B^o of y_{E_0}, and a unique smooth mapping $h : A^o \to B^o$ such that $g(x_t, h(x_t)) = 0$ for all $x_t \in A^o$. Therefore, locally around (x_{E_0}, y_{E_0}), the degenerate system (10) and (12) is equivalent to a p-dimensional FDE defined on the graph of the mapping h, i.e. on the set

$$S = \{(x_t, y_t) \in A^o \times B^o : y_t = h(x_t)\} \tag{13}$$

and represented by the equation

$$\dot{x}(t) = f(x_t, h(x_t)) \stackrel{\Delta}{=} \hat{f}(x_t). \tag{14}$$

This system is called *reduced system*. The main purpose here is to study the behaviour of the singular perturbed system (10) and (11) for a small (non-zero) value of ϵ, and to prove the reduced system (14) is a CFN, which has the same dynamical properties as the original (10) and (11).

Without loss of generality, for the MTN described by (10) and (11), we assume that the $(p-1)$th and the pth nodes are two neighbouring slow chemical components, which connect with the fast chemical components. To prove the reduced network defined by (14) to be a CFN, we have the following assumption.

Assumption 5: For (14),

$$\frac{\partial \hat{f}_{p-1}}{\partial x_p} \frac{\partial \hat{f}_p}{\partial x_{p-1}} \geq 0 \tag{15}$$

with $\partial \hat{f}_p / \partial x_{p-1} \neq 0$ for all $x_t \in X \subset \mathbb{C}([-r, 0], \mathbb{R}^{+p})$. Moreover, if both $\partial \hat{f}_{p-1}/\partial x_p$ and $\partial \hat{f}_p / \partial x_{p-1}$ are non-zero, then time delays for these two slow chemical components are $\tau_{p-1,p} = \tau_{p,p-1} = 0$.

In Assumption 5,

$$\frac{\partial \hat{f}_{p-1}}{\partial x_p} = \frac{\partial f_{p-1}}{\partial x_p} - \frac{\partial f_{p-1}}{\partial y} \left(\frac{\partial g}{\partial y} \right)^{-1} \frac{\partial g}{\partial x_p} \tag{16}$$

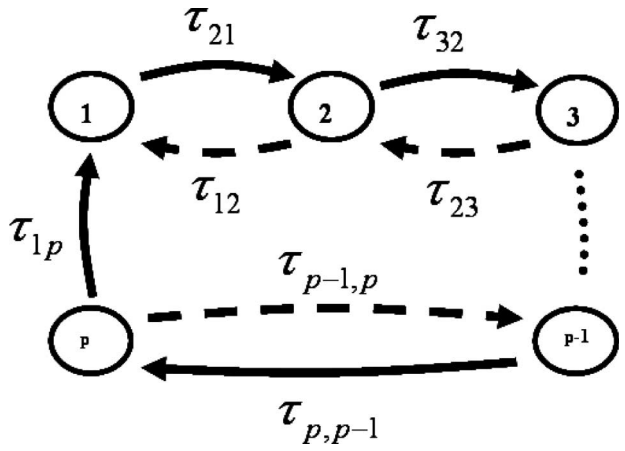

Fig. 4 *The reduced MTN of Fig. 3, which is a CFN representing slow reactions*

and

$$\frac{\partial \hat{f}_p}{\partial x_{p-1}} = \frac{\partial f_p}{\partial x_{p-1}} - \frac{\partial f_p}{\partial y}\left(\frac{\partial g}{\partial y}\right)^{-1}\frac{\partial g}{\partial x_{p-1}} \tag{17}$$

When assumptions 1, 4 and 5 are satisfied, the network described by (14) is a CFN, which means that theorem 3 holds for (14). When assumptions 4 and 5 hold and ϵ is sufficiently small, we next show that the reduced system (14) has the same dynamics as the original (10) and (11). The omega limit sets of a CFN have only periodic orbits when certain conditions are satisfied. Instead of analysing the complicated MTN (10) and (11), such properties for CFNs can drastically simplify modelling and make theoretical analysis and design of biological oscillators tractable even for a large system.

The reduced MTN from Fig. 3 is shown in Fig. 4, which is a CFN representing slow reactions.

3.2 Main result for the basic MTN

Now, assume that (14) has a periodic solution:

$$x = \Phi(t) \tag{18}$$

Define a sufficiently small neighbourhood $S(M, \delta)$ of the orbit M for the periodic solution $\Phi(t)$. In parallel with (14), we consider

$$\dot{x}(t) = \hat{f}(x_t) + r(t, x_t) \tag{19}$$

where $r(t, x_t)$ is a vector function with $x_t \in S(M, \delta), t \in [0, +\infty)$, which is real and continuous throughout its domain.

Definition 8: The periodic solution $\Phi(t)$ to (14) is called stable under persistent perturbations if, for each $\bar{\epsilon} > 0$, it is possible to select two numbers $\epsilon_1 > 0$ and $\epsilon_2 > 0$ such that any integral curve $x = x(t_0, \phi)$ of (19) starting at the initial function $\phi \in S(M, \epsilon_1)$, for $t = t_0$ continues to stay for all $t > t_0$ in the domain $S(M, \bar{\epsilon})$ under any choice of the vector functional $r(t, x_t)$ satisfying $|r(t, x_t)| < \epsilon_2$ for any $t \geq 0$, $x_t \in S(M, \delta)$.

Definition 8 describes a stably-periodic solution of (14) under perturbations. CFNs in the form of differential delay equations, i.e. (6) or (14), were extensively studied in [24–27]. One important property of such systems is that there are omega-limit sets which include only non-constant periodic orbits and equilibria. On the other hand, orbits of PFNs have a strong tendency, to equilibria [8, 23].

Next, we derive the main theorem, and show that a MTN of (10) and (11) is dynamically equivalent to the reduced MTN (14) when ϵ is sufficiently small. Since there is no limitation for the dimensionality and less restriction on the network structures, a bio-oscillator can be modelled or designed even by a large-scale system with a variety of structures.

Theorem 4: An orbitally and asymptotically stable periodic-solution $x = \Phi(t)$ of (14) is stable under persistent perturbations. Moreover, when assumptions 1 to 3 and 4 to 6 hold and for a sufficiently small ϵ, $x = \Phi(t)$ is a stable periodic solution of (10) and (11).

Theorem 4 means that the orbitally and asymptotically stable periodic solution $x = \Phi(t)$ of the reduced MTN described by (14) is still stable for the MTN described by (10) and (11), when ϵ is sufficiently small. The orbitally and asymptotically stability means that all eigenvalues but one (i.e. the zero eigenvalue corresponding to translations along the orbit) of the monodromy matrix corresponding to the periodic solution have strictly negative real parts according to Floquet's theorem, which are actually generic for stable periodic solutions. Therefore, in theorem 4, we deliberately exclude the periodic solutions without orbitally or hyperbolically stable structure. The detailed proof of theorem 4 is given in Appendix 8.3.

In theorem 4, we assume sufficient small ϵ, i.e. the dynamics of the PFN are much slower than those of the CFN. Actually, for a gene-protein network, when CFN represents the gene sub-network, a typical timescale of the CFN is minutes because the dominant reactions are transcription and translation that both generally require minutes to process. On the other hand, since the protein reactions, such as binding and multimerisation typically require less than a second, the dynamics of the PFN representing the protein sub-network evolve generally on a timescale of seconds or less. Therefore, if ϵ is less than 0.1, generally we assume that the reduced system (14) is sufficiently accurate to represent the original system (10) and (11), or theorem 4 holds.

It is generally not easy to guarantee asymptotical behaviours such as equilibria or periodic orbits even for a small network due to the non-linearity of the system. Theorem 4 implies that if the reduced MTN has a asymptotic periodic solution, then this periodic solution is still stable for the original MTN, which in turn can be used to model and design bio-oscillators in complicated systems.

According to theorem 2, a PFN is robust to time delay variations, whereas time delays in CFN may significantly affect the dynamics of the network. Such characteristics actually also hold in MTNs. As indicated in (14), different from the time delays in the slow sub-network, the time delays in the fast sub-network or PFN have no effect on the asymptotical dynamics of the original MTN or the reduced MTN. In other words, we do not need care about the time delays in the fast subsystems for analysing or designing gene oscillators, although they may influence the transient dynamics of the system.

3.3 Generalised MTN with multiple PFNs

The result for the basic MTN with one fast PFN and one slow CFN can be easily extended to a general MTN with multiple fast PFNs and a slow CFN. Provided that each PFN interacts with two neighbouring variables in x_t, theorem 4 still holds for the corresponding CFN or the reduced MTN.

Figure 5 gives an example of a MTN with multiple PFNs. There are two fast PFN's and the reduced network is a CFN, which is shown in Fig. 6. In the implementation example in the following Section, we adopt a genetic network, which has one slow CFN and two fast PFNs, to demonstrate our theoretical result.

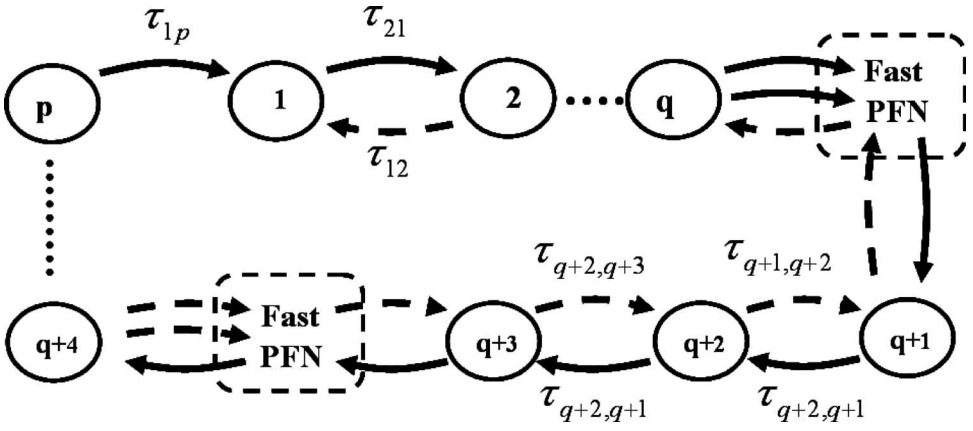

Fig. 5 *A MTN with multiple PFNs*

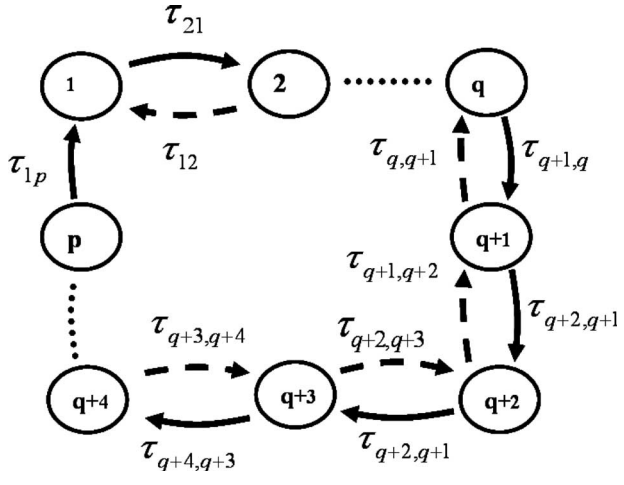

Fig. 6 *The reduced MTN of Fig. 5 which is a CFN*

3.4 Sufficient conditions for periodic orbits

Although the Poincaré-Bendixson type theorem [24, 25] shows that there are omega-limit sets of CFNs which are composed of only periodic orbits and equilibria, it does not provide sufficient conditions for periodic orbits. Here, we give sufficient conditions for periodic orbits of the reduced MTNs, and the detailed proofs of these results can be found in [26]. Note that the reduced MTN or CFN has the form of (6).

Assumption 6: The maximal feedback loop connecting all nodes for the network described by (14) is negative.

Although (14) is a CFN, clearly it is also a PFN if the maximal feedback loop is positive, which falls into the class of cooperative dynamical systems and exhibits very regular behaviour, e.g. typical solutions tend to equilibria in the case of autonomous systems [7, 22, 23]. Therefore, provided that the maximal feedback loop is negative, (14) may have a stably periodic solution.

Let $\bar{x}$ be an equilibrium of (14). Define

$$A(\lambda)=\begin{pmatrix} \hat{f}_{11} & \hat{f}_{12}e^{-\tau_{12}\lambda} & 0 & \cdots & \hat{f}_{1,p}e^{-\tau_{1,p}\lambda} \\ \hat{f}_{21}e^{-\tau_{21}\lambda} & \hat{f}_{22} & \hat{f}_{23}e^{-\tau_{23}\lambda} & \cdots & 0 \\ 0 & \hat{f}_{32}e^{-\tau_{32}\lambda} & \hat{f}_{33} & \cdots & 0 \\ \cdots & \cdots & \cdots & \cdots & \cdots \\ 0 & 0 & \cdots & \hat{f}_{p,p-1}e^{-\tau_{p,p-1}\lambda} & f_{p,p} \end{pmatrix} \tag{20}$$

where $\hat{f}_{ij}=\frac{\partial \hat{f}_i}{\partial x_j}\big|_{x=\bar{x}}$ for $1\le i,j\le p$. Then the characteristic equation of (14) is

$$\det(\lambda I-A(\lambda))=0 \tag{21}$$

and has the following form

$$b_p\lambda^p+b_{p-1}\lambda^{p-1}+\cdots+b_0+(-1)^{p+1}Be^{-\lambda\tau}=0 \tag{22}$$

where p is the node number of the reduced MTN, I is the $p\times p$ identity matrix, $b_p=(-1)^p$ and $B=\hat{f}_{1,p}\Pi_{i=1}^{p-1}\hat{f}_{i+1,i}$. b_j for $j=0,\cdots,p-1$ are functions of $\hat{f}_{ij}\hat{f}_{ji}, 1\le i,j\le p$. The total time delay is $\tau=\sum_{i=1}^{p}\tau_{i+1,i}$. Notice that b_j for $j=0,\ldots,p-1$ are functions of $\hat{f}_{k,k+1}\hat{f}_{k+1,k}$ for $1\le k\le p$, which means that all effects of interactions between nodes k and $k+1$ on b_j disappear but on B exist if $\hat{f}_{k,k+1}$ is zero.

Let $I_e=\{i:\mathrm{mod}(i,2)=0,\,1\le i\le p\}\cup\{0\}, I_o=\{i:\mathrm{mod}(i,2)=1,\,1\le i\le p\}$, the range of arccos be $[0,\pi]$, and $\bar{\nu}$ be a non-zero real root of the following equation:

$$\left(\sum_{i\in I_e}(-1)^{\frac{i}{2}}b_i\nu^i\right)^2+\left(\sum_{i\in I_o}(-1)^{\frac{i-1}{2}}b_i\nu^i\right)^2-B^2=0 \tag{23}$$

By using the characteristic (22), we derive the following sufficient conditions for nontrivial periodic orbits.

Theorem 5: (Convergence to nontrivial periodic orbits). Assume that the reduced MTN (14) satisfies assumptions 1, 3 and 6. If the feedback for total one-direction interactions is sufficiently strong, i.e. $\frac{\partial \hat{f}_1}{\partial x_p}\prod_i\frac{\partial \hat{f}_i}{\partial x_{i-1}}$ for all i with $\frac{\partial \hat{f}_{i-1}}{\partial x_i}=0$ (or $s_{i-1,i}=0$) at any equilibrium is sufficiently large, then there exists a total time delay $\bar{\tau}$

$$\bar{\tau}=\frac{1}{\bar{\nu}}\left[\arccos\left(\frac{(-1)^p\sum_{i\in I_e}(-1)^{\frac{i}{2}}b_i\nu^i}{B}\right)\right] \tag{24}$$

such that (14) converges to an orbitally and asymptotically stable periodic solution or a periodic self-oscillation [32] when $\tau>\bar{\tau}$.

Actually, we can prove that when $p=2$ or 3, only $b_0-B^2<0$ is needed for the existence of stable periodic orbits in Theorem 5 instead of sufficiently strong one-direction interactions.

Theorem 6: (Convergence to non-trivial periodic orbits). Assume that the reduced MTN (14) satisfies assumptions 1, 3 and 6. If $\det(A(0))<0$ at all equilibria, where $A(0)$ is the Jacobian matrix of (14), then for almost all initial conditions

ϕ, $x_t(\phi)$ converges to an orbitally and asymptotically stable periodic solution or a periodic self-oscillation [32].

Theorems 5 and 6 provide sufficient conditions for periodic orbits. When the conditions of theorem 5 or 6 are satisfied, the omega-limit sets of the reduced MTN (14) or the CFN (6) are composed of only attracting periodic orbits. Notice that there is no restriction on the self-feedback loops for cyclic feedback systems; the interaction from the ith chemical component directly to itself may be either non-linear or linear, and either positive or negative.

Although there are six assumptions in this paper, all of them are not hypothesis but simply stipulate the structure of the network, and are generally easily satisfied or constructed in a biological system. The essential assumption is assumption 1, which requires the monotone property of all components in the system, thereby making the theoretical analysis tractable in this paper, whereas other assumptions can actually be viewed as the constructive conditions corresponding to the particular sub-structures of the network, respectively.

4 Numerical implementation

In this Section, we demonstrate our theoretical results by designing a synthetic gene network, which is actually a MTN and consists of two fast PFNs and one slow CFN. As shown in Fig. 7, the synthetic gene regulatory network is a simple two-gene model with genes *cI* and *lac* under the control of promoters $P_L lacO1$ and P^*_{Rm}, respectively. Both genes are both well-characterised transcriptional regulators, which can be found in bacterium *E. coli* and λ phage. We assume that the designed gene network is implemented in a eukaryotic cell, e.g. in *yeast*, so as to examine the effect of time delays on the oscillation dynamics. mRNA or m_x of the gene *cI* translates the protein CI or p_x in cytoplasm, which in turn forms a homodimer p_{2x} and is transported or diffused into the nucleus in the form p'_{2x} to enhance the expression of the gene *Lac* by binding on the two operator sites of the promoter P^*_{RM}. On the other hand, mRNA or m_y of the gene *lac* translates the protein Lac or p_y, which forms a homodimer p_{2y} and further a tetramer p_{4y} in the cytoplasm. When moved to the nucleus, the tetramer p_{4y} is in the form of p'_{4y}, which represses the expression of the gene *cI* by binding on the operator site of the promoter $P_L lacO1$. The promoter $P_L lacO1$ has one binding site *OR* for the Lac tetramer, but the promoter P^*_{RM} has two binding sites OR_1 and OR_2 for the CI dimers with the affinity priority binding first on OR_1 and second on OR_2. Note that P^*_{RM} is a mutated promoter from P_{RM}, which has no binding site for the tetramer Lac.

Different from prokaryotes, there are significant time delays $(\tau_{mx}, \tau_{my}, \tau_{px}, \tau_{py})$ due to transportation or diffusion of mRNAs and transcriptional factors between the nucleus and cytoplasm, which may considerably affect the dynamics of the system. Such a circuit can be engineered on plasmids, and can then be cloned to multiple copies, e.g. by PCR. The engineered plasmids are further assumed to grow in *yeast*, by injecting into *yeast* and recombining into their genome. Furthermore, the equivalent degradation effect of the proteins Lac and cI can also been changed, e.g. by introducing small molecules IPTC (isotropyl-β-*D*-thiogalactopyranoside) and aTc (anhydrotetracycline), which bind to the tetramer Lac and prevent them from binding to operator sites [28]. In order to measure the behaviours of the genetic network, a gene for GFP (green fluorescent protein) or YFP (yellow fluorescent protein) is assumed to be incorporated in each plasmid under the control of a targeted promoter to represent the targeted gene in experiments [10].

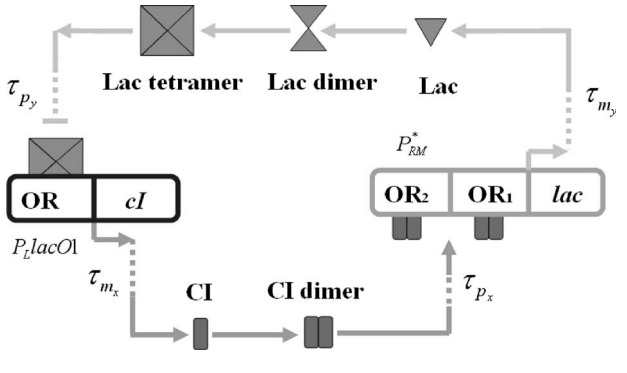

Fig. 7 *Schematic for the synthetic gene network by cI and Lac genes (τ's represent time delays)*

We define the following chemical species in terms of concentrations: m_x, mRNA CI; p_x, CI protein; p_{2x}, CI dimer in cytoplasm; p'_{2x}, CI dimer in nucleus; D_y, the free DNA binding or operator site in the promoter P^*_{RM}; $p'_{2x}D_y$, CI dimer bound to operator site OR_1 of the promoter P^*_{RM}; $p'_{2x}p'_{2x}D_y$, CI dimers bound to both OR_1 and OR_2 of the promoter P^*_{RM}; m_y, mRNA Lac; p_y, Lac protein; p_{2y}, Lac dimmer; p_{4y}, Lac tetramer in cytoplasm; p'_{4y}, Lac tetramer in nucleus; D_x, the free DNA binding site in the promoter $P_L lacO1$; $p'_{4y}D_x$, the Lac tetramer bound to the operator site OR of the promoter $P_L lacO1$.

The fast reactions are mainly multimerisation and binding reactions for the protein network. As indicated in Fig. 8, we have fast reactions for CI, which consist of a PFN:

$$p_x + p_x \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} p_{2x}$$

$$p_{2x} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} p'_{2x}$$

$$p'_{2x} + D_y \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} p'_{2x}D_y$$

$$p'_{2x} + p'_{2x}D_y \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} p'_{2x}p'_{2x}D_y$$

The fast reactions for Lac also consist of a PFN:

$$p_y + p_y \underset{k_{-5}}{\overset{k_5}{\rightleftharpoons}} p_{2y}$$

$$p_{2y} + p_{2y} \underset{k_{-6}}{\overset{k_6}{\rightleftharpoons}} p_{4y}$$

$$p_{4y} \underset{k_{-7}}{\overset{k_7}{\rightleftharpoons}} p'_{4y}$$

$$p'_{4y} + D_x \underset{k_{-8}}{\overset{k_8}{\rightleftharpoons}} p'_{4y}D_x$$

On the other hand, the slow reactions involve transcription of mRNAs and translation of proteins, and degradation of proteins and mRNAs. The slow reactions for CI are:

$$m_x \overset{k_{px}}{\longrightarrow} p_x + m_x$$

$$D_y \overset{k_{my0}}{\longrightarrow} m_y + D_y$$

$$p'_{2x}D_y \overset{k_{my1}}{\longrightarrow} m_y + p'_{2x}D_y$$

$$p'_{2x}p'_{2x}D_y \overset{k_{my2}}{\longrightarrow} m_y + p'_{2x}p'_{2x}D_y$$

$$m_x \overset{d_{mx}}{\longrightarrow} 0$$

$$p_x \overset{d_{px}}{\longrightarrow} 0$$

The slow reactions for Lac are:

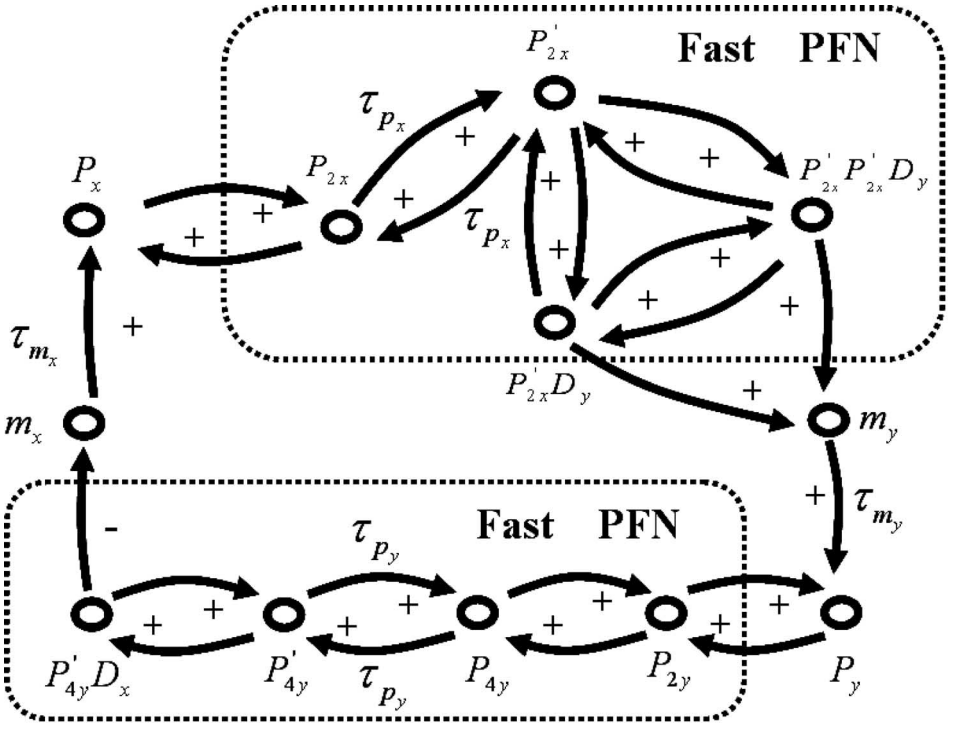

Fig. 8 *Schematic of MTN for the synthetic gene regulatory network shown in Fig. 7. Proteins p_x (CI), p_y (Lac) and mRNA m_x (cI), m_y (Lac) constitute a slow CFN. Other chemicals, such as CI dimmer, Lac dimmer, Lac tetramer, consist of two fast PFNs*

$$
\begin{aligned}
m_y &\xrightarrow{k_{py}} p_y + m_y \\
D_x &\xrightarrow{k_{mx0}} m_x + D_x \\
p'_{4y}D_x &\xrightarrow{k_{mx1}} m_x + p'_{4y}D_x \\
m_y &\xrightarrow{d_{my}} 0 \\
p_y &\xrightarrow{d_{py}} 0
\end{aligned}
$$

There are also conservation conditions for total binding sites of promoters, i.e. $D_y + p'_{2x}D_y + p'_{2x}p'_{2x}D_y = n_y$ and $D_x + p'_{4y}D_x = n_x$, where n_x, and n_y are the concentration of the genes *cI* and *lac*, respectively.

For convenience, m_x is denoted by X_1, p_x by X_2, m_y by X_3, p_y by X_4, p_{2x} by Y_1, p'_{2x} by $Y_2, p'_{2x}D_y$ by $Y_3, p'_{2x}p'_{2x}D_y$ by Y_4, p_{2y} by Y_5, p_{4y} by Y_6, p'_{4y} by Y_7 and $p'_{4y}D_x$ by Y_8. Then, the time evolution of the twelve-variable model is governed by the following functional differential equations, in which all parameters and concentrations are defined with respect to the total cell volume.

$$
\begin{aligned}
\frac{dX_1}{dt} &= k_{mx0}(n_x - Y_8) + k_{mx1}Y_8 - d_{mx}X_1, \\
\frac{dX_2}{dt} &= k_{px}X_1(t-\tau_{m_x}) + 2k_{-1}Y_1 - 2k_1X_2^2 - d_{px}X_2, \\
\frac{dX_3}{dt} &= k_{my0}(n_y - Y_3 - Y_4) + k_{my1}Y_3 + k_{my2}Y_4 - d_{my}X_3, \\
\frac{dX_4}{dt} &= k_{py}X_3(t-\tau_{m_y}) - 2k_5X_4^2 + 2k_{-5}Y_5 - d_{py}X_4, \\
\frac{dY_1}{dt} &= k_1X_2^2 + k_{-2}Y_2(t-\tau_{p_x}) - k_{-1}Y_1 - k_2Y_1, \\
\frac{dY_2}{dt} &= k_2Y_1(t-\tau_{p_x}) - k_{-2}Y_2 + k_{-3}Y_3 \\
&\quad - k_3(n_y - Y_3 - Y_4)Y_2 + k_{-4}Y_4 - k_4Y_2Y_3, \\
\frac{dY_3}{dt} &= k_3(n_y - Y_3 - Y_4)Y_2 + k_{-4}Y_4 - k_4Y_2Y_3 - k_{-3}Y_3, \\
\frac{dY_4}{dt} &= k_4Y_2Y_3 - k_{-4}Y_4, \\
\frac{dY_5}{dt} &= k_5X_4^2 - k_{-5}Y_5 - 2k_6Y_5^2 + 2k_{-6}Y_6, \\
\frac{dY_6}{dt} &= k_6Y_5^2 - k_{-6}Y_6 + k_{-7}Y_7(t-\tau_{p_y}) - k_7Y_6, \\
\frac{dY_7}{dt} &= k_7Y_6(t-\tau_{p_y}) - k_{-7}Y_7 - k_8Y_7(n_x - Y_8) + k_{-8}Y_8, \\
\frac{dY_8}{dt} &= k_8(n_x - Y_8)Y_7 - k_{-8}Y_8.
\end{aligned} \tag{25}
$$

where Y_i are fast variables, and ϵ is not explicitly expressed in (25).

It is easy to check that the two fast reaction subgroups are two PFNs for the fixed slow variables, satisfying assumption 2. Therefore, by using theorem 1, the fast reactions will converge to equilibria rapidly and all fast variables can be eliminated. To demonstrate the example in a clear way, we explicitly derive the reduced MTN although it is not necessary in general. Specifically, by $dY_i/dt = 0$ in (25) according to (12), we eliminate fast variables as follows: $Y_1 = Y_2 = K_1X_2^2, Y_3 = n_yK_3K_1X_2^2/(1+K_3K_1X_2^2+K_4K_3K_1^2X_2^4)$, $Y_4 = n_yK_4K_3K_1^2X_2^4/(1+K_3K_1X_2^2+K_4K_3K_1^2X_2^4), Y_5 = K_5X_4^2$, $Y_6 = Y_7 = K_6K_5^2X_4^4, Y_8 = n_xK_8K_6K_5^2X_4^4/(1+K_8K_6K_5^2X_4^4)$, where $K_i = k_i/k_{-i}, (i = 1, \cdots, 8)$ and $K_2 = K_7 = 1$. Then we obtain the reduced equations as follows:

$$
\begin{aligned}
\frac{dx_1}{dt'} &= \frac{1}{r_s}\left(k'_{mx1}\frac{n_xx_4^4(t)}{1+x_4^4(t)} - \frac{d_{mx}}{K_a}x_1(t) + k_{mx0}n_x\right) \\
\frac{dx_2}{dt'} &= \frac{1}{r_s}\left(\frac{k_{px}K_b}{K_a^2}x_1(t'-\tau'_{m_x}) - \frac{d_{px}}{K_a}x_2(t)\right) \\
\frac{dx_3}{dt'} &= \frac{1}{r_s}\left(\frac{k'_{my1}n_yx_2^2(t) + k'_{my2}\sigma n_yx_2^4(t)}{1+x_2^2(t)+\sigma x_2^4(t)} - \frac{d_{my}}{K_b}x_3(t) + k_{my0}n_y\right) \\
\frac{dx_4}{dt'} &= \frac{1}{r_s}\left(\frac{k_{py}}{K_b}x_3(t'-\tau'_{m_y}) - \frac{d_{py}}{K_a}x_4(t)\right).
\end{aligned} \tag{26}
$$

The dimensionless variables are scaled by $x_1 \equiv (K_8K_6K_5^2)^{1/4}X_1$, $x_2 \equiv (K_1K_3)^{1/2}X_2$, $x_3 \equiv (K_1K_3)^{1/2}X_3$, $x_4 \equiv (K_8K_6K_5^2)^{1/4}X_4$ $t' \equiv r_sK_at, \tau'_{mx} \equiv r_sK_a\tau_{mx}$ and $\tau'_{px} \equiv r_sK_a\tau_{px}$, where $r_s = n_xk_{px}rk'_{mx1}/d_{mx}$, $r = k_b/K_a, K_a = (K_8K_6K_5^2)^{1/4}$, $K_b = (K_1K_3)^{1/2}$, $\sigma = K_4/K_3, k'_{mx1} = k_{mx1} - k_{mx0}$, $k'_{my1} = k_{my1} - k_{my0}$ and $k'_{my2} = k_{my2} - k_{my0}$. The reduced network described by (26) is shown in Fig. 9. Clearly, according to assumptions 3, 5 and 6, when $k'_{mx1} < 0, k'_{my1} > 0$ and $k'_{my2} > 0$, (26) is a CFN with a negative cyclic feedback loop.

By using a functional transformation [26], i.e.

$$\begin{aligned} x_1(t' - \tau) &\rightarrow x'_1(t') \\ x_2(t' - \tau'_{m_y}) &\rightarrow x'_2(t') \\ x_3(t' - \tau'_{m_y}) &\rightarrow x'_3(t') \\ x_4(t') &\rightarrow x'_4(t') \end{aligned} \tag{27}$$

we can equivalently change all time delays into a single time delay $\tau = \tau'_{m_x} + \tau'_{m_y}$ for (26), i.e.

$$\begin{aligned} \frac{dx'_1}{dt'} &= \frac{1}{r_s}\left(k'_{mx1}\frac{n_x x'^4_4(t'-\tau)}{1 + x'^4_4(t'-\tau)} - \frac{d_{mx}}{K_a}x'_1(t') + k_{mx0}n_x\right) \\ \frac{dx'_2}{dt'} &= \frac{r}{r_s}\left(\frac{k_{px}K_b}{K_a^2}x'_1(t') - \frac{d_{px}}{K_a}x'_2(t')\right) \\ \frac{dx'_3}{dt'} &= \frac{r}{r_s}\left(\frac{k'_{my1}n_y x'^2_2(t') + k'_{my2}\sigma n_y x'^4_2(t')}{1 + x'^2_2(t') + \sigma x'^4_2(t')}\right. \\ &\quad \left. - \frac{d_{my}}{K_b}x'_3(t') + k_{my0}n_y\right) \\ \frac{dx'_4}{dt'} &= \frac{1}{r_s}\left(\frac{k_{py}}{K_b}x'_3(t') - \frac{d_{py}}{K_a}x'_4(t')\right). \end{aligned} \tag{28}$$

Notice that τ does not include τ_{px} and τ_{py}, which are eliminated in the fast PFNs.

Parameters are mainly from [28] with slight modifications, and are set as $k_{mx1} = 0.2\,\text{min}^{-1}$, $K_8 = 2 \times 10^{13}\text{m}^{-1}$, $n_x = 1\,\text{nm}, n_y = 1\,\text{nm}$, $K_6 = 10^7\text{m}^{-1}$, $K_5 = 10^8\text{m}^{-1}$, $k_{mx0} = 3\,\text{min}^{-1}$, $k_{px} = 4\,\text{min}^{-1}$, $k_{my1} = 3\,\text{min}^{-1}$, $k_{my2} = 12\,\text{min}^{-1}$, $K_1 = 5 \times 10^7\text{m}^{-1}$, $K_3 = 3 \times 10^8\text{m}^{-1}$, $d_{my} = 5\,\text{min}^{-1}$, $k_{my0} = 2\,\text{min}^{-1}$, $k_{py} = 1\,\text{min}^{-1}$, $d_{py} = 2\,\text{min}^{-1}$ and $\sigma = 2$. Other parameters are given when they are used. According to the above parameters, the variables are scaled as $X_1(\text{nm}) \sim 0.8x_1, X_2(\text{nm}) \sim 8x_2, X_3(\text{nm}) \sim 8x_3, X_4 \sim 0.8x_4$ and $t(\text{min}) \sim t'/1.37$. Note that τ is also a scaled time delay by 1.37.

Clearly (28) is a negative cyclic feedback network and satisfies assumptions 1, 3, 6 and theorem 5. Figure 10 shows a case for the sustained oscillations generated by (28) with $d_{px} = 0.5\,\text{min}^{-1}$, $d_{mx} = 1\,\text{min}^{-1}$ and $\tau = 100$, which confirms our theoretical prediction. The limit cycle as a projection of Fig. 10 onto the plane formed by the concentrations of the CI protein x'_2 and the Lac protein x'_4 is shown in Fig. 11. Because the fast reactions as perturbations do not change their period or amplitude in the long run, limit cycle oscillations represent a particularly stable mode of the periodic behaviour. Such stability holds with the robust nature of circadian clocks which have to maintain their amplitude and period in the changing environment.

The bifurcation diagram of (28) for x'_2 is shown in Fig. 12, where the control parameter is the total time delay τ. At low values of τ, the system reaches a stable steady state corresponding to some constant concentrations of the state variables. With the increase of τ, a bifurcation occurs at a critical value $\bar{\tau} = 10.65$. After $\tau > \bar{\tau}$, the steady state

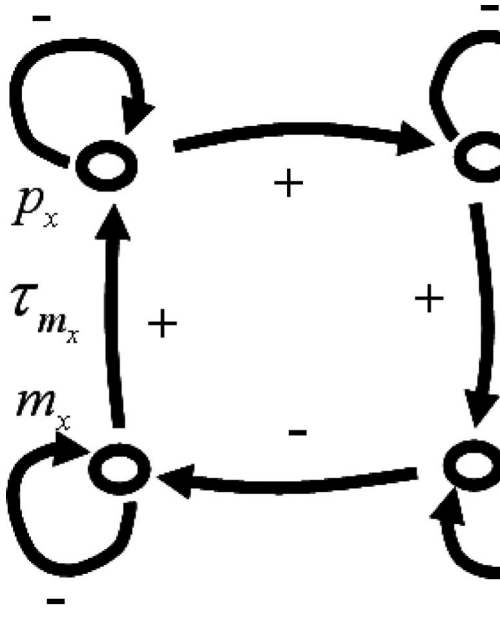

Fig. 9 *The reduced MTN with proteins $p_x(CI), p_y(Lac)$ and mRNAs $m_x(cI), m_y(Lac)$. The self-feedback loops are explicitly illustrated in the Figure*

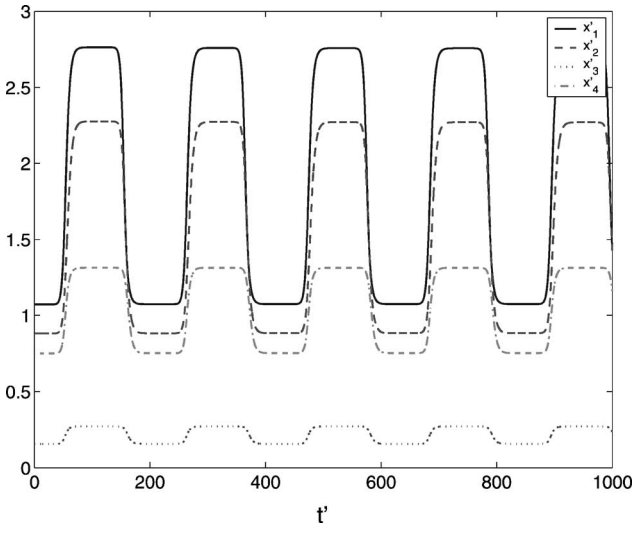

Fig. 10 *Sustained oscillations generated by the reduced MTN described by (28) at $d_{px} = 0.5\,\text{min}^{-1}, d_{mx} = 1\,\text{min}^{-1}$ and $\tau = 100$*

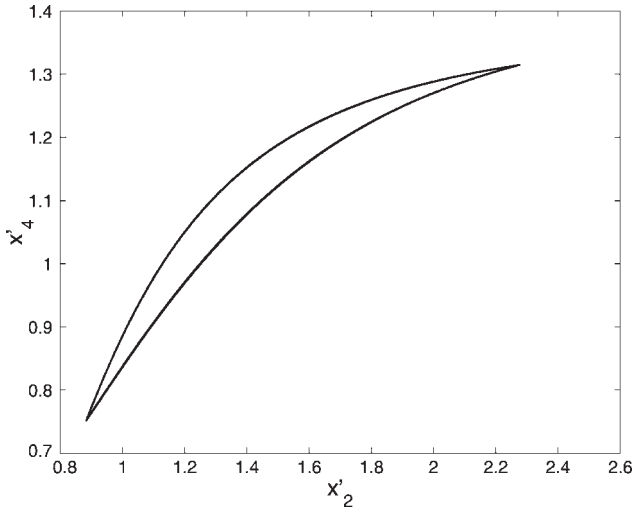

Fig. 11 *A limit cycle of (28) corresponding to the sustained oscillation shown in Fig. 10*

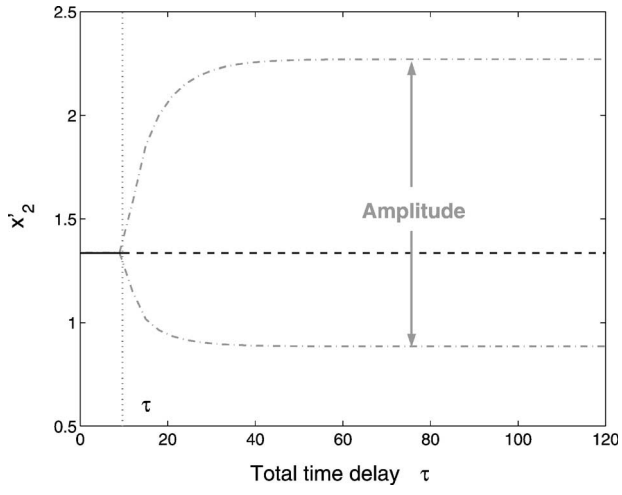

Fig. 12 *A bifurcation diagram with total time delay τ as a parameter at $d_{px} = 0.5\,min^{-1}$ and $d_{mx} = 1\,min^{-1}$. The solid line and dashed lines represent stable or unstable equilibrium, respectively. The dash-dotted lines indicate the maximum and minimum values of x_2' for the sustained oscillation. Limit cycles exist when $\tau > \bar{\tau}$ for which the equilibrium is unstable*

becomes unstable and the sustained oscillations occur. The amplitudes of the sustained oscillations are also shown in Fig. 12. When $\tau = \bar{\tau}$, we get a pair of imaginary roots $\lambda = \pm 0.20j$ for the characteristic equation, which corresponds to a Hopf bifurcation point. Moreover, from Fig. 12, the amplitudes increase with the time delay τ when τ is small, which means that the time delay can be used to control the amplitudes of the oscillations. However, when τ is large, the change of amplitudes is not sensitive to the time delay τ.

The oscillatory region (OS) and steady-state region (SS) at $\tau = 100$ are shown in Fig. 13, from which we can see that oscillations are generally enhanced with the increase of the degradation rates of the mRNA *cI*.

The analysis of the effect for total time delay τ on the oscillation period T is shown in Fig. 14. In addition to amplitudes as shown in Fig. 13, the period of oscillation, namely T, increases with the total time delay τ in an almost linear way although there are some kinks due to the nonlinearity of the system. Therefore, the total time delay τ can be viewed as a key parameter to control both the amplitude and period of an oscillation in a biological system.

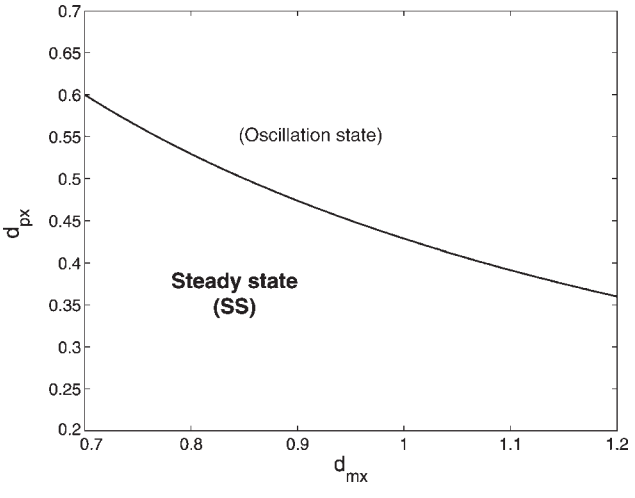

Fig. 13 *The oscillatory region (OS) and steady-state region (SS) at $\tau = 100$. The oscillatory region grows with increasing mRNA and protein degradation rates: d_{mx} and d_{px}*

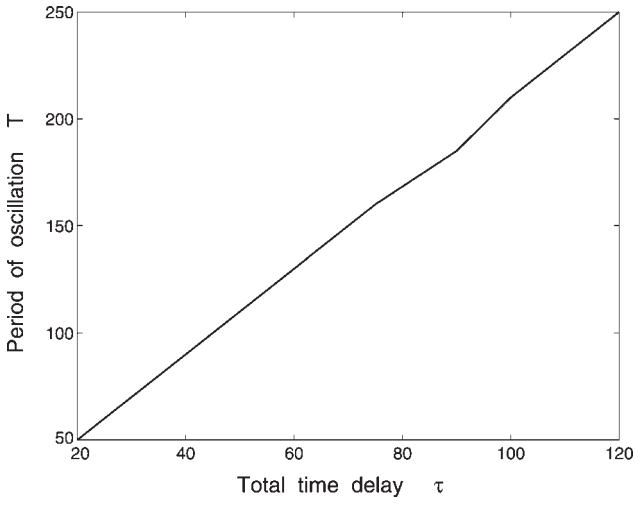

Fig. 14 *Total time delay τ and the oscillation period T*

There are mainly four delays, (τ_{mx}, τ_{my}) and (τ_{px}, τ_{py}) representing transportation or diffusion processes from nucleus to cytoplasm of mRNAs and from cytoplasm to nucleus of proteins respectively, which play different roles in dynamical behaviours of the system. Since τ_{px} and τ_{py} are in PFNs, they have no effects on the asymptotical dynamics. On the other hand, τ_{mx} and τ_{my} both qualitatively and quantitatively affect the dynamical behaviours not separately, but in the form of $\tau = \tau_{mx} + \tau_{my}$, due to the cyclic structure of the CFN.

In this example, changing the delay means adjusting the transportation and diffusion processes due to their dominant role in the time delay. Although the diffusion and transportation are very complicated processes and are not yet well understood, many factors have been found to be involved in the transportation and diffusion processes in a cell, such as enzymes in enzyme-mediated diffusion or facilitated diffusion, ATP as an exogenous source of energy in active transportation, ion gradient and temperature in both passive and active transportations or diffusions. Therefore, depending on the mechanism of the transportation and diffusion, we can control the time delay by perturbing the appropriate factors, e.g. concentrations or values.

5 Conclusion

This paper developed a new methodology to analyse and design a biological oscillating network with time delays by using a MTN, which is composed of fast PFNs and a slow CFN. We first provided sufficient conditions for the existence of limit cycles for a basic MTN with only one PFN from theoretical analysis, and then further extended the result for multiple PFNs, which enables our model to be applied to wider systems for modelling and designing bio-oscillators. As indicated in this paper, in contrast to the time delays in the slow sub-network that significantly affect the dynamics of the system, the time delays in the fast sub-network or PFN have no effect on the asymptotical dynamics of the MTN although they may play an important role in the transient dynamics. Such a property is important in designing or modelling gene oscillators when time delays are concerned.

We designed a network exerted by the genes *cI* and *Lac* to demonstrate the theoretical results. To examine the effect of time delays, we assumed that the engineered circuit was implemented in a eukaryotic cell. The simulation results showed that the total time delay can be used as an important

control parameter both quantitatively and qualitatively to change dynamics of the systems, in particular for the amplitudes and periods of periodic oscillations. Moreover, the effects of degradation rates of mRNA and protein on oscillatory and steady-state regions were also examined, which indicated that degradation rates of mRNAs or proteins can be used as other important control parameters to change the global dynamics of the system.

Although we have mainly examined effects of time delays and degradation rates of mRNA and protein on the cellular dynamics, there are also other important factors which may play crucial roles in biological processes and should be further investigated in future works from both the theoretical and experimental viewpoints, such as stochastic noise [33, 34] and the discrete nature of biological models.

6 Acknowledgment

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8 Appendix

8.1 Coordinate transformation

A genetic network with only positive feedback loops, i.e. a PFN may have positive and negative interaction edges, which make analysis complicated. Next, we consider a coordinate transformation to reduce the original PFN (1) into an equivalent one with only positive interaction edges [8, 26].

Choose a node, j arbitrarily. First, we set $\sigma_j = 1$. If $s_{ij} = 1$ (or -1) for some node i, then set $\sigma_i = 1$ (or -1). It is easy to show that σ_i is well defined. Using σ_i, we define a transformation $\mathbb{P}$ described by a matrix:

$$\mathbb{P} = \mathrm{diag}(\sigma_1, \ldots, \sigma_n) \tag{29}$$

By subsituting $y = \mathbb{P}_Z$ into (1), we have

$$\dot{y}(t) = G(y_t) = \mathbb{P}F(\mathbb{P}y_t) \tag{30}$$

where $y = \mathbb{P}z$ for (1). It is easy to prove that (30) has only positive interaction edges and each s_{ij} is always 1 [8]. Therefore, (30) is a cooperative dynamical system [23].

8.2 Bounded condition for a PFN

We next prove that the solution of a PFN (1) is bounded if there exists two points $z_N, z_M \in R^{+n}$, such that $G(y_M) < 0$ and $G(y_N) \geq 0$ with $y_M > y_N$, where G is defined by (30) and $\mathbb{P}$ is defined by (29), $y_M = \mathbb{P}z_M$ and $y_N = \mathbb{P}z_N$. Notice that z_M, z_N or y_M, y_N are constant with respect to t.

By the coordinate transformation $y = \mathbb{P}z$ of Appendix 8.1, a PFN can be changed to a cooperative dynamical system (30). Let $\hat{y}$ be the constant function equal to y for all values of its argument, i.e. $\hat{y}(\theta) \equiv y$ where $-r \leq \theta \leq 0$. Since $G(y_M) < 0 \leq G(y_N)$ and (30) is a cooperative and irreducible dynamical system, $y_t(\hat{y}_M)$ converges monotonically to a large equilibrium E_M, and $y_t(\hat{y}_N)$ converges monotonically to a small equilibrium E_N, with $E_N \leq E_M$, according to [23] (Corollary 5.2.2). Therefore, by monotonicity, for any y satisfying $y_N < y < y_M$, we have:

$$y_N < y_t(\hat{y}_N) < y_t(\hat{y}) < y_t(\hat{y}_M) < y_M, \quad t > 0 \tag{31}$$

Since both (y_N, y_M) and (E_N, E_M) are bounded, for each $\hat{y}_N < \phi < \hat{y}_M, y_t(\phi)$ is defined and bounded for $t \geq 0$ for (30). Moreover, since $\mathbb{P}$ is a reversible and one-to-one linear map, the PFN of (1) is qualitatively equivalent to (30). Therefore, the solution of (1) is also bounded.

As a simple example of a PFN, we can verify that assumptions 1 and 2 and irreducibility are satisfied with two negative interaction edges for the following system

$$\dot{z}_1(t) = \frac{1}{1+z_2^2(t-\tau_{12})} - z_1(t) \tag{32}$$

$$\dot{z}_2(t) = \frac{1}{1+z_1^2(t-\tau_{21})} - \frac{4}{5}z_2(t) \tag{33}$$

where $z_1, z_2, \tau_{12}, \tau_{21} \geq 0$. According to (29), we have $\mathbb{P} = \text{diag}(1,-1)$. Hence, the transformed system is

$$\dot{y}_1(t) = \frac{1}{1+y_2^2(t-\tau_{12})} - y_1(t) \tag{34}$$

$$\dot{y}_2(t) = -\frac{1}{1+y_1^2(t-\tau_{21})} - \frac{4}{5}y_2(t) \tag{35}$$

where $y_1 \geq 0$ and $y_2 \leq 0$. It is easy to verify that all interaction edges of (34)–(35) are positive and (32)–(33) is qualitatively equivalent to (34)–(35). In fact, an easy calculation shows that $G(y_M) < 0 \leq G(y_N)$, where $y_M = (100, 0)$ and $y_N = (0, -100)$ with $y_M > y_N$. With (31) and the definition of $\mathbb{P}$, clearly solutions of (34)–(35) or (32)–(33) are bounded. By using $\mathbb{P}, z_M = (100, 0)$ and $z_N = (0, 100)$. Note that $x < y$ means $x \leq y$ and $x \neq y$ for $x, y \in \mathbb{R}^{+n}$.

8.3 Proof of theorem 4

Here, we give the detailed proof of theorem 4. Consider a singular perturbed system, i.e. (10) and (11):

$$\dot{x}(t) = f(x_t, y_t) \tag{36}$$

$$\dot{y}(t) = \frac{1}{\epsilon}g(x_t, y_t) \tag{37}$$

Define

$$Q_H := \{\psi \in \mathbb{C}[-r, 0] : \|\psi\| < H\} \tag{38}$$

where $H > 0$ and $\|\psi\| = \sup_{-r\leq\theta\leq 0}|\psi(\theta)|$. Let $V : Q_H \to \mathbb{R}$ be a continuous function. By definition, the derivative of V along the solution of (36)–(37) is:

$$\dot{V}(\psi) = \lim_{h\to 0^+}\sup\frac{1}{h}[V(z_h(\psi)) - V(\psi)] \tag{39}$$

With assumption 4, the fast sub-network, whose chemical components are represented by y, is a PFN. Kobayashi *et al.* proved that a general biological network with only positive feedback loops has no dynamical attractors except stable equilibria [7, 8]. Therefore, for the fixed x, the solution of fast chemical reactions (37), i.e. $y(t) = \Psi(t, x)$ converges to its equilibria $y_0(x)$ rapidly.

Let $y(t) = \Psi(x_t) + \Gamma(x_t, t, \epsilon)$ be the solution of (37) for changing x_t with t, where $\Gamma(x_t, t, \epsilon)$ is an unknown function. To obtain the structure information about $\Gamma(x_t, t, \epsilon)$ we consider the derivative of $y(t)$, namely,

$$\begin{aligned}\epsilon\frac{d\Psi}{dx_t}\frac{dx_t}{dt} + \epsilon\left(\frac{\partial\Gamma}{\partial x_t}\frac{dx_t}{dt} + \frac{\partial\Gamma}{\partial t}\right) &= g(x_t, \Psi(x_t) + \Gamma(x_t, t, \epsilon)) \\ &= \frac{\partial g}{\partial y}(x_t, \Psi(x_t) + \theta\Gamma(x_t, t, \epsilon))\Gamma(x_t, t, \epsilon) + g(x_t, \Psi(x_t))\end{aligned} \tag{40}$$

where $0 < \theta < 1$. Note that in g and $\partial g/\partial y$, we substitute $y(t)$ for y_t, although there are time delays in y_t, because we only want to know the order of Γ. By using theorem 1 for the PFNs, for a sufficiently large t, $g(x_t, \Psi(x_t)) \to 0$ and, therefore, it can be rewritten as $g(x_t, \Psi(x_t)) = \epsilon\bar{g}(x_t, \Psi(x_t))$. Then:

$$\epsilon\left(\frac{\partial\Gamma}{\partial x_t}\frac{dx_t}{dt} + \frac{\partial\Gamma}{\partial t}\right)\frac{\partial g}{\partial y}\Gamma(x_t, t, \epsilon) + \epsilon\bar{g}(x_t, \Psi(x_t)) - \epsilon\frac{d\Psi}{dx_t}\frac{dx_t}{dt} \tag{41}$$

By equating the order of ϵ for a sufficiently small ϵ, we obtain

$$\Gamma(x_t, t, \epsilon) = \epsilon R(x_t, t) + O(\epsilon^2) \tag{42}$$

where $R(x_t, t)$ is a bounded function. Therefore, we have the expression, by omitting terms of the higher order ϵ

$$y(t) = \Psi(x_t) + \epsilon R(x_t, t) \tag{43}$$

Next, we consider a slow subsystem:

$$\dot{x}(t) = f(x_t, \Psi(x_t) + \epsilon R(x_t, t)) \tag{44}$$

Expand $f(x_t, \Psi(x_t) + \epsilon R(x_t, t))$ as a Taylor series:

$$\begin{aligned}&f(x_t, \Psi(x_t) + \epsilon R(x_t, t)) \\ &\quad = f(x_t, \Psi(x_t)) + \epsilon\frac{\partial f(x_t, \Psi(x_t))}{\partial y}R(x_t, t) + O(\epsilon^2)\end{aligned} \tag{45}$$

To study (45), we consider the system at $\epsilon = 0$, i.e.

$$\dot{x}(t) = f(x_t, \Psi(x_t)) \tag{46}$$

which is called the *reduced system* of the singular perturbed system (36) and (37). With assumption 5, (46) constitutes a CFN, whose only possible omega limit sets are equilibria or periodic orbits. Now we assume the reduced system (46) has a periodic solution

$$x = \Phi(t) \tag{47}$$

with a minimal period $T > 0$.

A sufficiently small neighbourhood $S(M, \delta)$ of the orbit M for the periodic solution (47) is defined by:

$$S(M, \delta) \triangleq \{u \in \mathbb{C}[-h, 0] : \ \|u - \Phi\| \ < \delta\} \tag{48}$$

In parallel with (46), we consider the perturbed system (45), which can be rewritten as

$$\dot{x}(t) = f(x_t, \Psi(x_t)) + r(t, x_t) \tag{49}$$

where $r(t, x_t)$ is the vector function with $x_t \in S(M, \delta), t \in [0, +\infty)$, that is real and continuous throughout its domain.

Our main result here is that the asymptotically stable periodic solution (47) for (46) is still stable for the singular perturbed system (36) and (37) or (49).

Assumptions 1 to 3 and 4 to 6 hold and ϵ is sufficiently small. Now we are in a position to prove theorem 4, i.e. $x = \Phi(t)$ is a stable periodic solution of (36) and (37).

Proof of Theorem 4: If a periodic solution to (46) is hyperbolically and asymptotically stable, then there are two functions V and W which possess the following properties: function $V(\phi)$ given for $\phi \in S(M, \delta)$ is real, continuous, and satisfies the inequality $V(\phi) > 0$ for $\phi \in S(M, \delta)\backslash M$ and the identity $V(\phi) = 0$ for $\phi \in M$; the function W given in $S(M, \delta)$ is real, continuous, and satisfies the inequality $W(\phi) < 0$ for $\phi \in S(M, \delta)\backslash M$ and the identity $W(\phi) = 0$ for $\phi \in M$. In this case, functions V and W are related to one another by

$$\dot{V} = W(\phi) \tag{50}$$

where $\dot{V}$ is the total derivative of the function V along the integral curve of (46).

Choose a number $\delta > \bar{\epsilon} > 0$ and find a number:

$$\lambda = \inf_{\rho(\phi,M)=\bar{\epsilon}} V(\phi) \tag{51}$$

Because of the continuity of function V, the number λ is positive for any fixed $\bar{\epsilon}$. For the number λ we find a $\epsilon_1 < \bar{\epsilon}$ such that $\epsilon_1 > 0$ and $V(\phi) < \lambda$ for $\rho(\phi, M) < \epsilon_1$. Furthermore, let:

$$\mu = \sup_{\epsilon_1 \le \rho(\phi,M) \le \bar{\epsilon}} W(\phi) \tag{52}$$

Because of the continuity of the function W, the number $\mu < 0$. Choose the number $\epsilon_2 > 0$ so small that the inequality

$$\mu + \epsilon_2 v < 0 \tag{53}$$

holds, where

$$v = \sup_{\epsilon_1 \le \rho(\phi,M) \le \bar{\epsilon}} |[\mathrm{grad}_{\phi(0)} V(\phi)]'| \tag{54}$$

We now show that all the integral curves of (46) starting in the domain $S(M, \epsilon_1)$ for $t = t_0 \ge 0$, stay in the domain $S(M, \bar{\epsilon})$ for all $t \ge 0$ under any choice of $r(t, \phi)$ which is given for $t \ge 0$. $\phi \in S(M, \delta)$ is real, continuous, and satisfies the inequality $\|r\| \le \epsilon_2$ for all $\epsilon_1 \le \rho(\phi, M) \le \bar{\epsilon}, t \ge 0$. Compute the total derivative of function V along the integral curve of (49). If for V, the following linearisation holds:

$$V(\phi + \delta\phi) - V(\phi) = \int_{-h}^{0} K'(\theta, \phi(\theta)) \delta\phi(\theta) d\theta + O(\|\delta\phi\|) \tag{55}$$

as $\|\delta\phi\| \to 0$, where K' is a continuous vector function on $[-h, 0] \times \mathbb{R}^p$, then from (50) we get:

$$\frac{dV}{dt} = W + [\mathrm{grad}_{\phi(0)} V(\phi)]' r(t, \phi) \tag{56}$$

By using (53), (54) and (56), we get:

$$\frac{dV}{dt} \le \mu + v\epsilon_2 < 0 \tag{57}$$

Suppose the above statement is not true, i.e. there exists an integral curve $x = x(t)$ of (46) such that $x_{t'}(\phi) \in S(M, \epsilon_1)$, and there is a number $\bar{t} > t'$ satisfying the equality $\rho(x_{\bar{t}}(\phi), M) = \bar{\epsilon}$. Furthermore, $\bar{t}$ is said to be the least number of t. Denote by $\bar{\bar{t}}$ the time which satisfies the condition $\bar{\bar{t}} > t', \rho(x_{\bar{\bar{t}}}(\phi), M) = \epsilon_1$, such that $\bar{\bar{t}}$ is the greatest number of t. Then the function $V(t) = V(x(t))$ on the interval $(\bar{\bar{t}}, \bar{t})$ is found to have the property $V(\bar{t}) \ge \lambda, V(\bar{\bar{t}}) < \lambda$ and $\frac{dV}{dt} < 0$ for $t \in (\bar{\bar{t}}, \bar{t})$. The latter inequality holds because the arc of the integral curve $x(t)$ joining the points $V(\bar{t})$ and $V(\bar{\bar{t}})$ lies entirely in the closed domain $\epsilon_1 \le \rho(\phi, M) \le \bar{\epsilon}$. Hence we have $V(\bar{t}) > V(\bar{\bar{t}})$ and $\frac{dV}{dt} < 0$ for $t \in (\bar{\bar{t}}, \bar{t})$, which contradicts the assumption. Therefore, all the integral curves of (49) starting in the domain $S(M, \epsilon_1)$ stay in the domain $S(M, \bar{\epsilon})$ for $t \ge t'$. This completes the proof.