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Design of Genetic Circuits that are Robust to Resource Competition

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The ability to engineer genetic circuits in living cells has tremendous potential in a number of applications, from health, to energy, to bio-manufacturing. Although substantial efforts have gone into design approaches that make circuits robust to variable cellular context, context-dependence of genetic circuits still remains a significant hurdle. We review the problem of intra-cellular resource competition, one culprit of context-dependence, and summarize recent efforts toward design approaches to mitigate it. We classify these approaches into two main groups: global control and local control. In global control approaches, the pool of resources is globally regulated to meet the demand by genetic circuits. In contrast, in local control strategies, individual circuit modules are regulated to be robust to variability in the pool of resources. Within each group, both feedback and feedforward regulation methods have been implemented, which we describe by highlighting differences in terms of ease of implementation and performance.

Introduction

The ability to engineer cells for novel functionalities has great promise to revolutionize fields such as medicine, healthcare, and the environment [1, 2, 3, 4, 5, 6, 7]. However, the design of genetic circuits is hampered by context dependence–where circuit components behave differently depending on the cellular and genetic context, which results in lengthy design processes [8]. One major aspect of context dependence in genetic circuits is cellular resource competition: the fact that the operation of a genetic circuit changes the availability of cellular resources, such as ribosomes, RNAP, or dCas9, to other circuit modules. These changes in the availability of cellular resources often results in unwanted coupling between the operation of supposedly independent circuit modules, which can destroy the circuit's intended function [9, 10].

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To address the problem of resource competition, significant effort has recently gone into strategies to mitigate different aspects of resource sharing in genetic circuits. Here, we review recent works that have proposed strategies that either adjust the level of resources to meet demand or that make genetic circuits robust to changes in available resources. We refer to the former as global control and to the latter as local control. Specifically, in the global control strategy, the resource pool is regulated to keep resource availability constant independent of changing demand by genetic circuits [11, 12, 13, 14]. In the local control strategy, genetic modules are regulated to make their operation robust to changes in resource availability [15, 16, 17, 18]. Overall, these approaches set the basis for making the operation of genetic circuits less affected by the intra-cellular context, thus aiding the creation of circuits that behave as intended [7].

The paper is organized as follows. First, we review the molecular basis of resource competition in genetic circuits. Next, we review recently proposed solutions to mitigate the effects of resource competition on circuits' operation.

Molecular Basis of Resource Competition

Resource competition arises from the fact that cells have a limited amount of resources, which are shared among the various components in a genetic circuit [9, 10, 17, 18, 19, 20, 21, 22, 23, 24]. Therefore, when one genetic module in a circuit increases its usage of cellular resources, other modules are left to operate with less available resources. Critical shared resources that any genetic module requires for operation include the RNA polymerase (RNAP) and the ribosome, since they are needed by every gene for transcription and translation, respectively [10, 22]. Additional resources that are also limited and are shared by many genes, especially in mammalian cells, include specific co-activators and general transcription factors [17, 18]. Finally, in genetic circuits where transcriptional regulation occurs through CRISPRi/a, dCas9 is also a shared resource required by multiple modules for transcriptional activation or repression [25, 26, 27].

As an example of ribosome competition, the authors of [10] built a simple genetic circuit in *E. coli* with two unconnected genes, shown in Figure 1a. They showed that as the RFP gene was increasingly expressed, the GFP level decreased by more than 70%, despite the absence of any regulatory interactions between the RFP and GFP genes. This drop in GFP level was due to a reduction in the pool of available translational resources caused by the expression of the RFP gene. Resource competition has also been experimentally demonstrated in genetic circuits in mammalian cells at both the transcriptional and translational levels [18] and in CRISPRi-based genetic circuits [25, 26, 27].

Resource competition leads to subtle effects in the emergent behavior of a genetic circuit beyond simply coupling the expression of unconnected genes through the resource pool. Specifically, Figure 1b depicts two genetic circuit modules (Modules 1 and 2), each with monotonically increasing input-output transfer curves in the absence of other modules. When Module 1 and 2 are connected in a cascade within a single circuit, Module 1 and 2 compete for cellular resources thereby changing each other's input-output transfer curve.

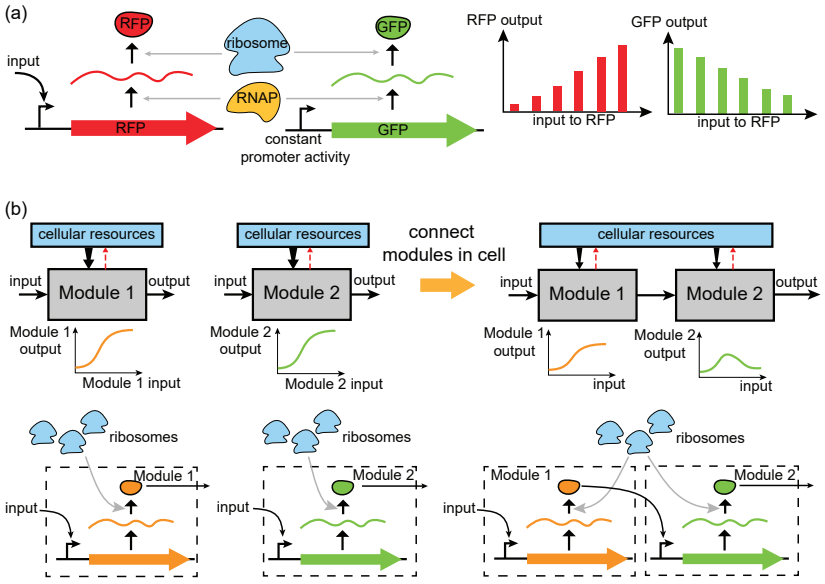

Figure 1: **Examples of experimentally observed consequences of resource competition in genetic circuits.** (a) Two genetic modules sharing ribosomes and RNAP. A red fluorescent protein (RFP) gene is activated by an input to its promoter, while a green fluorescent protein (GFP) gene is constitutively expressed [10]. As the input to the RFP gene is increased, the output of the GFP gene is decreased. (b) Resource competition changes the emergent behavior of connected genetic modules [9]. Module 1, represented by the orange gene, and Module 2, represented by the green gene, are characterized in a cell in isolation and both have increasing input-output transfer curves. When Modules 1 and 2 are connected within the same cell in a cascade structure, they compete for cellular resources, and the input-output transfer curve of the connected module is no longer monotonically increasing.

This results in a cascade's input-output transfer curve that is not monotonically increasing. This can be explained by noticing that when Module 1's gene is expressed upon presentation of the input, even though Module 2's promoter becomes activated, ribosomes are sequestered away from Module 2, which can overall lead to a decrease in Module 2's expression. This was experimentally demonstrated in [9]. Additionally, resource competition may also change other aspects of the qualitative behavior of genetic circuits such as the number of stable equilibrium points as demonstrated in [28, 29, 30, 31].

Cellular resources such as ribosomes are also shared between genetic circuits and the host cell metabolism [32]. Therefore, high resource demand by the genetic circuit can decrease resource availability to the host, resulting in a decreased growth rate, which may also disrupt circuit function [29, 30, 33, 34, 35, 36]. Accordingly, in [22], the authors developed a sensor to estimate the burden on the host cell by the genetic circuit. Whole-cell mechanistic mathematical models have also been developed to capture ribosome redistribution [37].

Other experimentally demonstrated consequences of resource competition that may also significantly affect a genetic circuit's behavior include competi-

tion for proteases [38] and for dCas9 [25, 26, 27]. Specifically, the dCas9 enzyme is a shared resource as it binds with different engineered guide RNAs (gRNAs) in CRISPRi/a-based genetic circuits to produce functional gRNA-dCas9 regulatory complexes. When the production of one gRNA is increased, less dCas9 is available for other gRNAs. This sharing of dCas9 has been shown to result in decreased gRNA-dCas9 activity of other, competing, gRNAs by more than 10-fold [26].

Design approaches for making circuits robust to resource competition

Here, we review experimentally verified approaches to improve circuit robustness in the face of resource competition. We group solutions by whether a controller acts directly on the pool of resources, which we call *global control*, or acts on each module separately, which we call *local control*.

Local control

The objective of the local control approach is to make each genetic module in the circuit insensitive to fluctuations in the amount of available resource by incorporating a controller into the module. In this way, the behavior of the synthetic circuit is made robust to competition for resources among modules within the circuit itself, and to competition with genetic modules outside the circuit. The two types of controllers that have been used for this purpose are feedback controllers and feedforward controllers.

Feedback control is ubiquitous in engineering applications and involves regulating a system by making continuous readings of its output and applying adjustments, which keep the system's output near the desired value independent of perturbations. The local feedback control architecture is shown in Figure 2a. It is useful to split each Module i into two components, the *plant* P_i and the *controller* C_i. The controller C_i uses a measurement of the output of the plant P_i, then computes and applies a corrective action to the plant P_i. A feedback controller should result in a module where the output does not change when the amount of available resource changes. In order to obtain zero steady state change in the module output when the level of available resource changes, an integral controller is required. One example of this approach is the quasi-integral controller experimentally implemented using RNA molecules which bind and degrade each other in [15] and shown in Figure 2b. Other circuits have been constructed using similar principles with other goals, such as the circuit in [39], which used RNA molecules which bind to each other and degrade in the feedback path to reduce noise and improve tunability. Additionally, a feedback controller constructed using protein species instead of RNA species has also been constructed, and has been called antithetic feedback control [40]. Previously, transcriptional feedback control was used to reduce the sensitivity to fluctuations in available resources, as demonstrated in [16].

An alternative approach to the design of controllers for robustness to cellular resource fluctuations is to utilize feedforward controllers within each module.

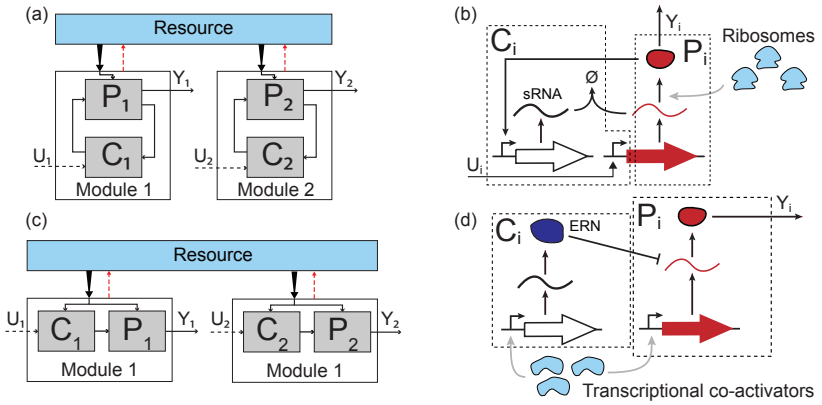

Figure 2: **Local control design approaches for engineering robustness to resource competition.** (a) Block diagram representation of local feedback control. Modules 1 and 2 use the same common pool of resources and have outputs Y_1 and Y_2 as well as regulatory inputs U_1 and U_2, respectively. Each module is composed of the plant P_i and the controller C_i connected in a feedback configuration. C_i senses the output of P_i and applies a corrective control action to P_i, which compensates for changes in the availability of the resource (vertical black arrow from resource to module). The regulatory input U_i, which may not be present in all implementations, acts on C_i to produce a "desired" value of the module output Y_i. (b) Local feedback control implementation example [15]. P_i consists of a gene coding for the output protein (red). C_i contains the gene for an sRNA which is activated by the output of P_i and binds to and mutually degrades the mRNA coding for the output of interest. C_i also containts the promoter for the gene of interest, which must be tuned to compensate for the decrease in Y_i level due to the sRNA. In this case the resource of interest is the ribosome. (c) Block diagram representation of local feedforward control. Each module has an output Y_i and possibly a regulatory input U_i. Each module consists of the plant P_i and controller C_i connected in a feedforward configuration. The available level of the resource acts as a disturbance input on both P_i and C_i. This disturbance positively affects the output of P_i. This disturbance also acts through the intermediary C_i to decrease the output of P_i. The regulatory input from U_i to C_i (dashed arrows) does not exist in current implementations, but would allow Y_i to change in a desired way based on U_i. (d) Local feedforward control. One example implementation of local feedforward control is an incoherent feedforward loop (iFFL) constructed using an endoribonuclease (ERN) [17]. P_i consists of a gene coding for the protein of interest (red). C_i consists of a gene with the same promoter as P_i, which codes for an ERN that degrades the mRNA of P_i. In this case, the resource of interest is a transcriptional co-activator or general transcription factor, which promotes transcription of both the output protein and the ERN. The combination of the activating path from transcriptional co-activator to Y_i, and the repressing path from transcriptional co-activator to Y_i through the ERN forms an iFFL.

In this scheme, as shown in Figure 2c, a feedforward controller C_i senses the available resources and uses this information to apply a control input to P_i to compensate for any change in the output of P_i caused by a change in the available resources. Feedforward controllers have been extensively used before to provide robustness to plasmid copy number variation [41, 42, 43], which appears as a disturbance similar to fluctuations in available transcriptional resources. In the context of robustness to resource competition this feedforward control structure results in an incoherent feedforward loop (iFFL) characterized

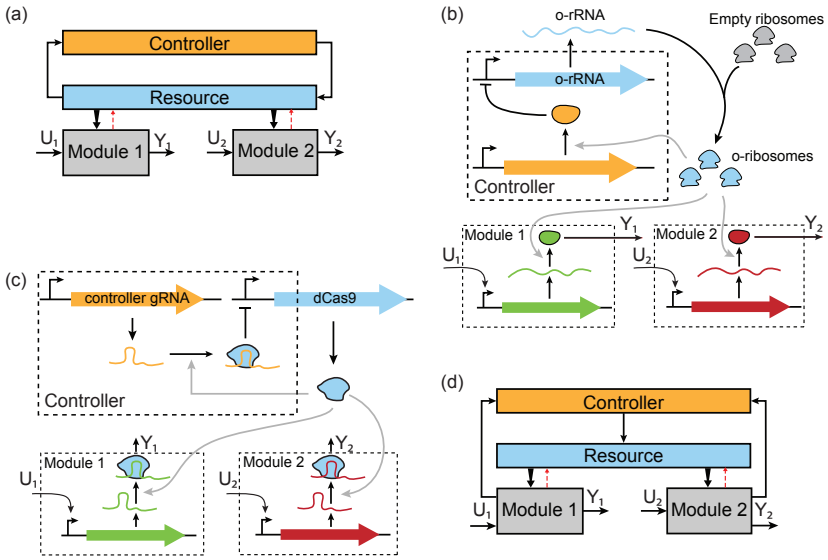

Figure 3: **Global control design approaches for engineering robustness to resource competition.** (a) Block diagram representation of global feedback control. A feedback controller regulates the resource such that the available amount is constant no matter how much is used by the modules. (b) Global feedback control for orthogonal ribosomes (o-ribosomes). o-ribosomes are ribosomes where the 16s ribosomal RNA (rRNA) is an engineered version orthogonal to the wild type rRNA (o-rRNA). The empty ribosomes shown are ribosomes with no 16s ribosomal RNA. The controller is composed of a repressor which is translated by o-ribosomes and represses the production of o-rRNA [11]. (c) Global feedback control for dCas9. dCas9 is used to produce the output of each module, Y_i, a dCas9-guide RNA complex which is a repressor whose target is determined by the guide RNA (gRNA). The controller is composed of a controller gRNA which binds to the dCas9 protein and forms a complex which represses the production of dCas9 [14]. (d) Block diagram representation of global feedforward control. Modules 1 and 2 share a common resource. Each module uses resources (dashed red arrows) and also sends a signal to the controller indicating its level of resource usage (black arrows). The controller then increases the available level of the resource to cancel out the module's usage.

by two paths from the resource to the output: an activating path and a repressing path [17]. If properly designed, the effects of the two paths cancel at steady state, thus making the module's output robust to changes in the availability of the resource. A potential limitation of iFFL-based implementations is that, to ensure robustness to transcriptional resources, the same transcriptional resource must be used to express both the output gene and the gene that represses the output [17]. Therefore, it is still unexplored how the design may be extended to modules with regulatory inputs. On the other hand, iFFL-based designs are often simpler to implement than feedback-based designs. Figure 2d depicts a feedforward controller constructed using an endoribonuclease (ERN) [17]. Feedforward controllers that use a micro RNA in place of an ERN have also been constructed to engineer robustness to transcriptional resources [43], although

these cannot achieve robustness to changes in translational resources.

Global control

Here we review approaches that utilize a controller in order to regulate the cellular resources in a global manner, e.g. by regulating the resource pool across the entire genetic circuit. If the amount of available resource adapts to resource usage by each module, the output of each module will be robust to usage of resources by any other module. Global controllers can be constructed using either feedback, as shown in Figure 3a, or feedforward architectures, as shown in Figure 3d. In both control architectures, the control design aims to keep the available amount of resource constant, despite modules using variable amounts of available resources, which prevents modules from seeing a fluctuation in the available concentration of the resource.

In a global feedback controller, the controller measures the available amount of resource and applies a regulatory action to compensate for measured changes in the available level of the resource due to resource usage by the modules. In [11], the authors implement a global feedback controller regulating ribosome concentration based on closed loop regulation of orthogonal ribosomes (o-ribosomes), as shown in Figure 3b. This solution, further explored via mathematical modeling in [44] and [45], makes modules robust to competition for o-ribosomes, which are ribosomes where the 16s ribosomal RNA is replaced by an orthogonal ribosomal RNA (o-rRNA), which targets ribosome binding sites different from those targeted by the host cell's 16s ribosomal RNA. Similarly, the authors of [14] constructed a global controller for regulating dCas9 concentration as shown in Figure 3c. In such a system, we consider each gene coding for a guide-RNA (gRNA) to be a module, each of which uses dCas9 to produce its output, a gRNA-dCas9 complex. The global controller regulates the amount of dCas9 so that the dCas9 available for each module is constant despite the sequestration of dCas9 by other modules. This makes the output of each module robust to the use of dCas9 by other modules.

Global controllers can also be constructed using feedforward control, as depicted in Figure 3d. Here, the controller measures the resources used by every module and actuates the resource pool to compensate for such use. An example of this implementation is reported in [13], in which a global feedforward controller for ribosomes is proposed that increases the production of ribosomes as a gene is overexpressed. This, in turn, compensates for the changes in resource availability due to overexpression of genes within a given module.

Two global control methods with different goals from making a synthetic circuit robust to resource usage by other modules have also been demonstrated. Feedback systems have been demonstrated that reduce the expression level of synthetic genes when the host cell's stress response is activated by high resource usage [46, 47]. Such systems can maintain constant growth rate at the expense of reducing exogenous gene expression. In [12], the authors demonstrated a system that degrades the host cell's mRNA to free up translational resources for a genetic circuit of interest, resulting in higher and more robust gene expression for the genetic circuit.

Other approaches

While the global and local control approaches are the most common approaches to constructing genetic circuits that are robust to resource competition, there are other conceptually distinct approaches. If each module uses its own of pool of the resource, which is orthogonal to the pools of all other modules, then resource competition among modules is eliminated. However, this approach requires a large library of orthogonal resources. Indeed, Darlington et. al. demonstrated that, in the absence of the global controller, a module using o-ribosomes did not compete for resources with a module using host ribosomes [11]. However, two modules that both use o-ribosomes will still compete with each other. Recent investigations have engineered ribosomes to be orthogonal to wild-type ribosomes [48, 49] and have studied the function of o-rRNA from divergent species in *E. coli* [50]. However, thus far there has been no demonstration of a library of orthogonal resources with sufficiently many orthogonal elements to enable engineering circuits with multiple modules. While not directly solving the problem of modules being coupled through resource competition, methods that free up cellular resources for the synthetic circuits via genetic interventions [51] or tuning growth conditions [52] have been proposed. Alternative approaches include reducing the amount of resources used by a synthetic circuit by genomic integration [53]. Both approaches can mitigate some of the effects of resource competition, but genetic modules will continue to be coupled via resource competition.

Discussion

We have reviewed several recent design approaches for increasing the robustness of synthetic circuits to resource competition. The local control strategies show promise, with multiple solutions demonstrating near perfect adaptation to the available level of the resource. However, since each module contains a controller, each module is substantially more difficult to engineer. In addition, it remains to be investigated whether the controlled modules use more resources than their uncontrolled counter-parts, which could increase the burden on the host cell and potentially lead to design limitations [54]. On the other hand, global control strategies require the engineering of only one controller while each module can be simpler. However, engineering a global controller can be more challenging due to the interaction with the natural resource regulation system. This is consistent with the current absence of an experimentally validated controller capable of making the available level of a transcriptional or translational resource almost perfectly adapt to changes in resource usage. It is plausible that for circuits with many components, such as networks of logic gates [14] the global control approach is simpler, since only one controller is needed. However, in other applications the designer may care more about the robustness of certain modules, and should therefore use a higher performance local controller, at least in the most critical modules in the circuit. Both global and local control strategies may also be used in conjunction with each other to optimize design trade offs.

The implementation of the various control approaches outlined in this paper requires that specific inputs in genetic circuit modules are regulated. There are many possible mechanisms for implementing control strategies depending on the biological context and application [55], which may include sigma/anti-sigma factors [56], sRNA regulators [15, 57], transcriptional regulation through T7 RNA polymerase [58], or transcription-activator-like effectors [41], among others. Additionally, natural biological systems use control strategies to deal with resource competition, and effort has been made to identify these feedforward and feedback loops [59]. Results of these studies may aid in the engineering of controllers for synthetic circuits.

Conflicts of interest

Declarations of interest: none.

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