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Dynamics and Control of Synthetic Bio-molecular Networks

D. Del Vecchio and E. D. Sontag

Abstract— **This tutorial paper presents an introduction to systems and synthetic molecular biology. It provides an introduction to basic biological concepts, and describes some of the techniques as well as challenges in the analysis and design of biomolecular networks.**

I. Introduction

Biologists have long employed phenomenological and qualitative models in order to help discover the components of living systems and to describe their behaviors. On the other hand, the analysis in living organisms of the dynamical properties of complex molecular reaction networks composed of interacting genes, mRNA, proteins, and metabolites requires a more quantitative and systems-level knowledge. Thus, in recent years the field of *systems biology* has emerged, whose focus is the quantitative analysis of cell behavior, with the goal of unraveling the basic dynamic processes, feedback control loops, and signal processing mechanisms underlying life,

Complementary to systems biology is the engineering discipline of *synthetic biology*. The goal of synthetic biology [3] is to extend or modify the behavior of organisms, and control them to perform new tasks. Through the *de novo* construction of simple elements and circuits, the field aims to foster an engineering discipline for obtaining new cell behaviors in a predictable and reliable fashion. The ultimate goal is to develop synthetic circuitry to be employed in targeted drug design, in the construction of molecular computers, and in other applications. In the process, synthetic biology plays a role in improving the quantitative and qualitative understanding of basic natural phenomena, since one approach to the testing of mathematical models of biological systems is to design and construct instances of the system in accordance to hypothesized models. Discrepancies between expected behavior and observed behavior highlight either research issues that need more studying, or knowledge gaps and inaccurate assumptions in models.

While tools from controls and dynamical systems theory, such as systems identification and robustness analysis, have been put to great use in systems biology for the analysis of naturally occurring biological systems, the use of this theory for the design of synthetic biological circuits is still emerging. The pioneering work of several biologists and physicists [4], [5], [8], [10] shows the potential and the need for such tools when tackling the challenges of biological design. The experimental results of [5] and of [20] on a negatively auto-regulated gene agree with the mathematical predictions obtained by using straightforward feedback control analysis. However, more complicated systems such as the oscillators built in [8] and in [4] do not provide experimental results that match well the theoretical predictions. In particular, intrinsic and extrinsic noise sources [21] seem to disrupt the oscillating behavior of the repressilator [8]. In [4], the oscillations are only damped, which suggests that the parameters of the constructed system may not be inside the theoretically computed range of parameters that guarantee oscillations. From these results, the need emerges for *robust, model based design*.

Control and systems theory have much to offer to synthetic biology. But, conversely, one may look forward to technologies inspired by biological research: evolution has resulted in systems that are highly fault-tolerant, nonlinear, feedback-rich, and truly hybrid in the sense that the digital information encoded in DNA controls chemical concentrations in cells. Advances in genomic research are continually adding to detailed knowledge of such systems architecture and operation, and one may reasonably argue that they will constitute a rich source of inspiration for innovative solutions to problems of control and communication engineering, as well as sensor and actuator design and integration.

This paper is organized as follows. In Section II, the basics on living organisms and on gene expression are introduced. In Section III, the basic control mechanisms of gene expression are introduced as they are among the key elements on which synthetic biological design relies. In Section IV, we introduce the modeling formalism used to describe the behavior of average molecule quantities. Synthetic biological design heavily relies on such models. In Section V, some analysis tools are reviewed. In Section VI, we briefly review the basic technology that has enabled the development of synthetic biology in the past few years. In Section VII, we provide an overview of the main network motifs that have been designed and built in living cells. In Section VIII, we discuss the fundamental issue of *modular design*, and propose a technique that, in principle, may be used in order to achieve modularity.

II. Fundamentals on molecular biology

A. Prokaryotes, Eukaryotes, Archaea, and Viruses

At the highest level, biologists classify life forms into prokaryotes, eukaryotes, and archaea. *Prokaryotes* are organisms whose cells do not have a nucleus nor other well-

D. Del Vecchio is with the Systems Laboratory, University of Michigan, Ann Arbor. E. D. Sontag is with the Department of Mathematics and BioMaPS Institute for Quantitative Biology, Rutgers University, New Brunswick. EDS supported in part by NSF Grants NSF DMS-0504557 and DMS-0614371. DDV supported in part by the Rackham Faculty Research Grant, University of Michigan. E-mail: ddv@umich.edu, sontag@math.rutgers.edu

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defined compartments; their genetic information is stored in chromosomes (typically circular) as well as in smaller circular DNA molecules called *plasmids*. *Eukaryotes* have cells with organized compartments; their genetic material is stored in chromosomes (typically linear) that lie in the nucleus. Most prokaryotes, with few exceptions, are unicellular, and most are bacteria. *Escherichia coli* is an example bacterium. Eukaryotes might be unicellular (e.g., yeast) or multicellular (e.g., plants and animals). *Archaea* were proposed as a third life form in the mid-1970s, and they share many characteristics with both prokaryotes and eukaryotes.

Eukaryotic cells are enclosed in a plasma membrane, which is made up of lipids and also contains proteins and carbohydrates, and acts as a protective barrier and gatekeeper, permitting only selected chemicals to enter and leave the cell. (In addition to membranes, plant cells also have a rigid cell wall.) Their interior is called the cytoplasm, and many types of organelles (specialized compartments) the cell (mitochondria, responsible for energy production through metabolism, and containing a very small amount of DNA; chloroplasts for photosynthesis; ribosomes, responsible for protein synthesis, and made up themselves of proteins and RNAs; endoplasmic reticulum; and so forth). The cytoskeleton, made up of microtubules and filaments, gives shape to the cell and plays a role in intracell substance transport. Prokaryotic cells, on the other hand, are surrounded by a membrane and cell wall, but do not contain the usual organelles.

Viruses consist of protein-coated DNA or RNA, and are not usually classified as living organisms, because they cannot reproduce by themselves, but rather require the machinery of a host cell in order to replicate. In particular, bacteriophages (λ-phage and 434-phage are two examples) are viruses that infect bacteria.

B. Gene expression

Research in molecular biology, genomics, and proteomics has produced, and will continue to produce, a wealth of data describing the elementary components of intracellular networks as well as detailed mappings of their pathways and environmental conditions required for activation. The genome, that is to say, the genetic information of an individual, is encoded in double-stranded deoxyribonucleic acid (DNA) molecules, which are arranged into chromosomes. It may be viewed as a "parts list" which describes all the proteins that are potentially present in every cell of a given organism. Genomics research has as its objective the complete decoding of this information, both the parts common for a species as a whole and the cataloging of differences among individual members. The key paradigm of molecular biology: "DNA makes RNA, RNA makes protein, and proteins make the cell" is called the central dogma of molecular biology (Crick, 1958). See Figure 1. A separate process, replication, occurs more rarely, and only when a cell is ready to divide (S phase of mitosis, in eukaryotes), and results in the duplication of the DNA, one copy to be part of each of the two daughter cells. The term gene expression refers to the process by which genetic information is ultimately transformed into

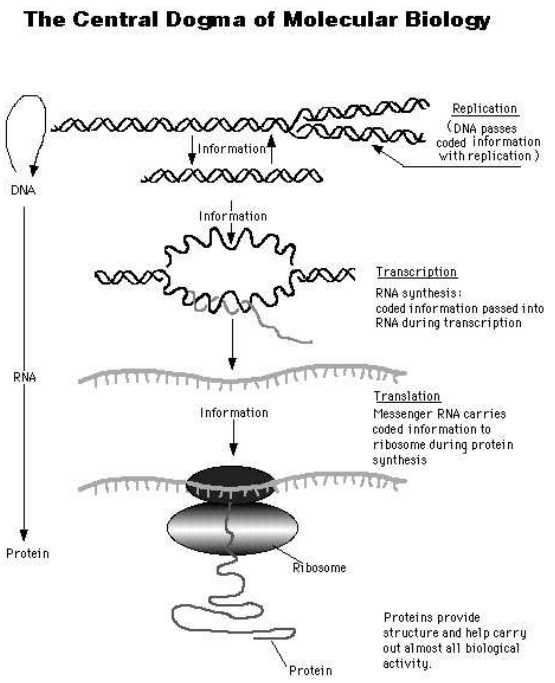

Fig. 1. Central dogma of molecular biology: Gene expression.

working proteins. The main steps are transcription from DNA to RNA, translation from RNA to linear amino acid sequences, and folding of these into functional proteins, but several intermediate editing steps usually take place as well. (Sometimes the term "gene expression" is used only for the transcription part of this process.) At any given time, and in any given cell of an organism, thousands of genes and their products (RNA, proteins) actively participate in an orchestrated manner.

The DNA molecule is a double-stranded helix made of a sugar-phosphate backbone and nucleotide bases (Figure 2). Each strand carries the same information, which is encoded in the 4-letter alphabet {A,T,C,G} (the nucleotides Adenine, Thymine, Cytosine, and Guanine), in a "complementary" form (A in one strand corresponds to T in the other, and C to G). The two strands are held together by hydrogen bonds between the bases, which gives stability but can be broken-up for replication or transcription. One describes the letters in DNA by a linear sequence such as:

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gcacgagtaaacatgcacttcccaggccacagcagcaag...
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and genes (instructions that code for proteins) are substrings of the complete DNA sequence. (Besides genes, there are regulatory and start/stop regions that help delimit genes as well as determine if and when they should be "active". In addition, there are also regions that have other roles, such as coding for RNA that may not lead to proteins.) Because of its double-stranded nature, DNA is chemically stable, and serves as a good depository of information. One might think of DNA storage as a "hard disk" in a vague computing

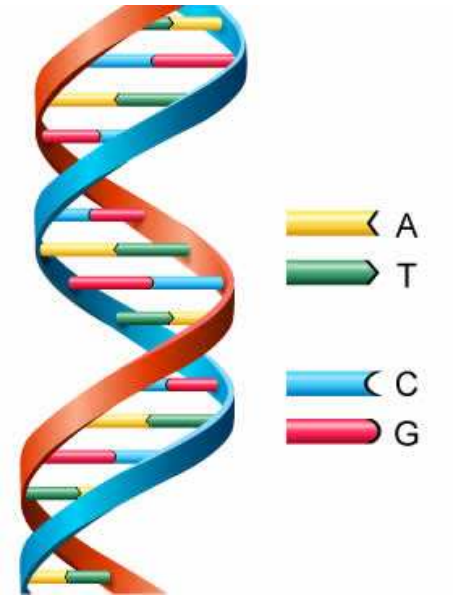

Fig. 2. DNA helix.

analogy. The "read-out" of genetic information (bringing-in the instructions into working memory for execution, in our computer analogy) begins when DNA information is transcribed letter by letter into "RNA language." *Ribonucleic acid* (RNA) is a nucleic acid very similar to DNA, but less stable than DNA, and almost exclusively found in single-stranded form (with exceptions such as the RNA in some viruses). RNA language is basically the same as DNA's, with the minor (for us) detail that in RNA, the amino acid thymine is replaced with uracil, symbolized by the letter U. This process is known as *transcription*. The "copying-machine" is called *RNA polymerase*. A polymerase is, generally speaking, an *enzyme* - a type of protein that acts as a catalyst - that helps in the synthesis of nucleic acids. RNA polymerase is, thus, a polymerase that helps make RNA, more precisely messenger RNA (mRNA). A *promoter region* is a part of the DNA sequence of a chromosome that is recognized by RNA polymerase. In prokaryotes, the promoter region consists of two short sequences placed respectively 35 and 10 nucleotides before the start of the gene. Eukaryotes require a far more sophisticated transcriptional control mechanism, because different genes may be only active in particular cells or tissues at particular times in an organism's life; promoters act in concert with enhancers, silencers, and other regulatory elements.

C. Proteins: the control elements

Proteins are the primary components of living systems. Among other roles, they form receptors that endow the cell with sensing capabilities, actuators that make muscles move (myosin, actin), detectors for the immune response, enzymes that catalyze chemical reactions, and switches that turn genes on or off. They also provide structural support, and help in the transport of smaller molecules, as well as in directing the breakdown and reassembly of other cellular elements such as lipids and sugars. Ultimately, one might say that cell life is about proteins and how and when they are produced. After transcription, translation is the next step in the process of protein synthesis and it is performed by the *ribosomes*. The information in the mRNA is read, and proteins are assembled out of amino acids (with the help of *transfer RNA* (tRNA), which help bring in the specific amino acids required for each position). RNA language is translated into protein language by a mapping from strings written in the RNA alphabet Σ_n = {U,A,G,C} into strings written in the amino acid alphabet:

$$\Sigma_a = \{A,R,D,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V\}.$$

Every sequence of three letters in the RNA alphabet Σ_n is replaced by a single letter in the alphabet Σ_a. The genetic code explains how triplets of bases map into individual amino acids [1]. The shape of a protein is what largely determines its function, because proteins interact with each other, and with DNA and metabolites, through lego-like fitting of parts in lock and key fashion, transfer of small molecules, or enzymatic activation. Therefore, the elucidation of the three-dimensional structure of proteins is a central goal in biochemical research; this subject is studied in the fields of proteomics and structural biology. The *Protein Data Bank* (http://www.rcsb.org/index.html) based at Rutgers University, USA, serves as an online catalog of protein structures. Sometimes, protein structure can be gleaned through physical methods, such as X-ray crystallography or NMR spectroscopy. Very often, however, the structure of a protein P can only be estimated, based upon a comparison with an homologous protein Q whose structure has been already determined (as chemists say, "solved"). One says that P and Q are homologous if they are, in an appropriate sense, close in amino acid sequence, or equivalently, in the DNA sequences for the genes coding for P and Q. One measure of closeness is Hamming distance (by how many "letters" do P and Q differ?), but more sophisticated measures used in practice include allowance for deletions and insertions of letters in P and Q. The rationale behind homology-based protein shape determination is that homologous proteins probably share a common evolutionary or developmental ancestry, and hence perform similar functions. Mathematical methods of computational biology (bioinformatics) play a central role in homology approaches; the critical assessment of structure prediction methods (CASP) competition compares methods from different researchers. Yet another set of techniques for elucidating the shape of proteins from their description as a linear sequence of amino acids is that of energy minimization methods. One views the protein-folding process as a gradient dynamical system, of which steady states are the stable configurations. This method is very difficult to apply, because of the complexity of the energy function, but has been useful for comparatively small proteins.

After translation, proteins are typically subjected to *post-translational* modifications, such as the addition of phosphate or methyl groups, or, in eukaryotic cells, ubiquitination, the process by which a protein is inactivated by attaching ubiquitin to it. Ubiquitin is a protein whose function is to

mark other proteins for proteolysis (degradation), a process which occurs at the proteasome. One of the key properties of proteins is that their shape (conformation) can be modified in a predictable fashion, as the consequence of interactions with other molecules. One often says that the protein has been "activated" as a result of such an interaction.

III. Basic control mechanisms of transcription networks

The cell is an integrated device made of thousands of interacting proteins. For example, the bacterium *Escherichia coli* is a cell that contains about $4,000$ different types of proteins. Each protein is a nano-meter size machine that carries out specific control tasks in a precise fashion. For example, when the cell is damaged, repair proteins are produced. When sugar is sensed, the cell produces proteins that can transport sugar, etc. The cell continuously monitors its internal state and decides which proteins are needed and when. This complex monitoring and control mechanism occurs mainly through **transcription networks**. In these networks, special proteins called transcription factors are usually designed to transit rapidly between active and inactive states. Each active transcription factor can bind to the DNA to regulate the rate at which some target genes are *transcribed* into messenger RNA (mRNA), which is then *translated* into protein. This protein can in turn act in the environment by regulating the transcription of other genes. The fundamental building-block of a transcription network is then the process of transcriptional regulation.

A. Transcriptional regulation

The interaction between transcription factors and genes is described by the phenomenon of transcriptional regulation. The rate at which the gene is transcribed is controlled by the *promoter*, a regulatory region of DNA that precedes the gene (Figure 3). RNA polymerase binds a defined site (a specific DNA sequence) at the promoter. The quality of this site specifies the transcription rate of the gene (the sequence of the site determines the chemical affinity of RNA polymerase to the site). RNA polymerase acts on all of the genes. However, each transcription factor modulates the transcription rate of a set of target genes. Transcription factors affect the transcription rate by binding specific sites on the promoter region of the regulated genes (Figure 3). When bound, they change the probability per unit time that RNA polymerase binds the promoter region. Transcription factors thus affect the rate at which RNA polymerase initiates transcription. A transcription factor can act as a *repressor* when it prevents RNA polymerase from binding to the promoter site. A transcription factor acts as an *activator* if it facilitates the binding of RNA polymerase to the promoter. Usually, if the transcription factor X is an activator for the expression of the protein Y, the interaction is denoted by "$X \to Y$". If, instead X is a repressor for the expression of Y, one denotes this interaction by "$X \dashv Y$". Transcription factors are themselves proteins encoded by genes that may be regulated by other transcription factors. These forms of interaction form a transcription network.

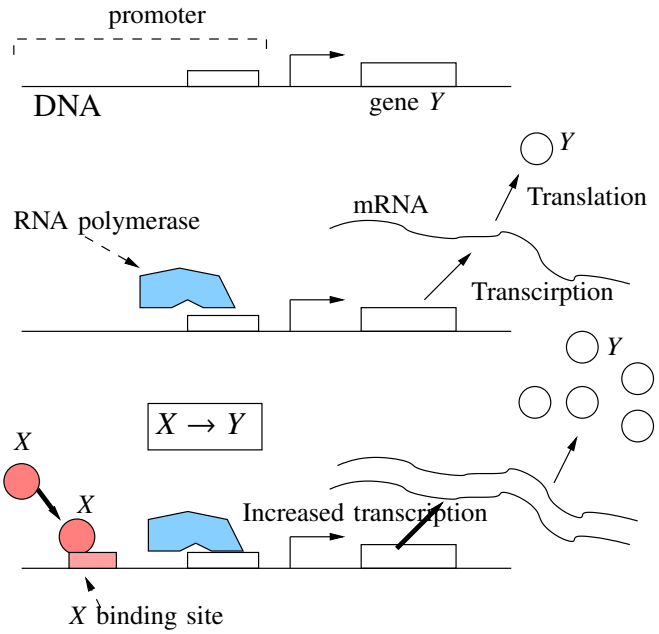

Fig. 3. Transcriptional regulation. Each gene is preceded by a regulatory region on the DNA called *promoter*. The promoter contains a specific site (DNA sequence) that can bind RNA polymerase. An activator X is a transcription factor that binds to a specific site on the promoter and increases the rate at which RNA polymerase binds its own site to initiate transcription.

Transcription networks are characterized by a strong *separation of timescales*: the binding of the transcription factor to the DNA promoter site reaches the equilibrium in seconds, while transcription and translation of the target gene takes minutes to hours. Thus, the dynamics describing the binding and unbinding of the transcription factor to its site can be considered at steady state when compared to the dynamics that describes the transcription and translation processes.

IV. Modeling transcriptional networks

A. The Hill function

For the sake of designing simple networks of activation and repression interactions, ordinary differential equations describing the rate at which protein and mRNA concentrations change in time are often employed. Let us consider first the rate of production of protein Y controlled by a single transcription factor X. When X regulates Y, the number of molecules of protein Y produced per unit time is a function of the concentration of X in its active form. With abuse of notation, we denote this concentration by X. Thus,

$$\text{rate of production of } Y = f(X).$$

The regulation function $f(X)$ is usually a monotonic function. It is an increasing function when X is an activator, and a decreasing function when X is a repressor (Figure 4). A function that well describes the regulation function is the *Hill function*. The Hill function can be derived by considering the equilibrium binding of the transcription factor to its site on the promoter. This is possible by virtue of a singular perturbation argument due to the separation of timescales, as

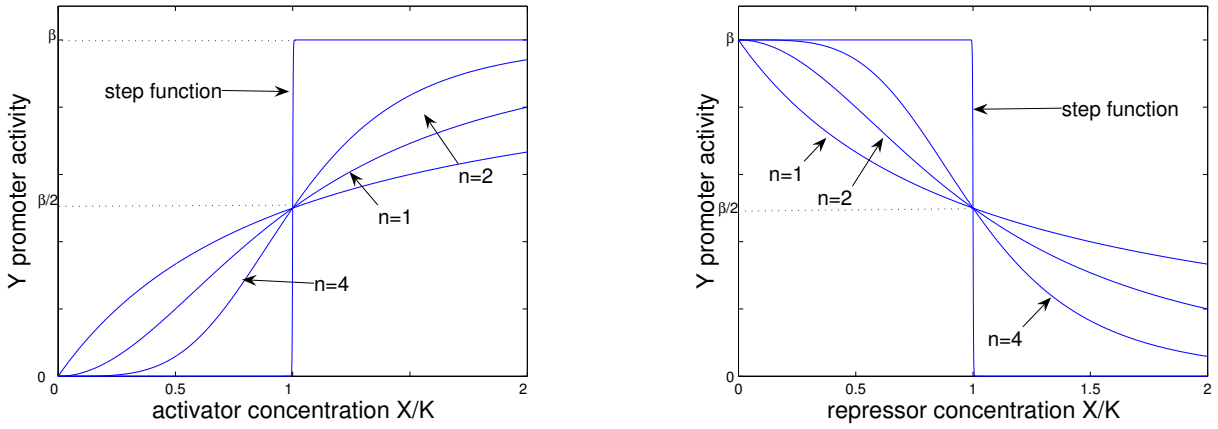

Fig. 4. Hill function for an activator (left side plot) and for a repressor (right side plot).

explained in Section III. The Hill function for an activator ($X \longrightarrow Y$) is thus given by

$$f(X) = \frac{\beta X^n}{K^n + X^n} \text{ (Hill function for activator),}$$

and it is depicted in the left side plot of Figure 4. The Hill function has three parameters: K, β, and n. The parameter K is the *activation coefficient* and defines the concentration of X needed to significantly activate expression. From Figure 4, it is clear that half-maximal expression is reached when $X = K$. The value of K is related to the *chemical affinity* between X and its site on the promoter. The parameter β is the maximal expression level of the promoter. Maximal expression is reached at high activator concentrations because at high concentrations X binds to the promoter with higher probability. The *Hill coefficient* n establishes how close the Hill function is to a step function. Typically, n is between 1 and 4. The Hill coefficient is often called the *degree of cooperativity* of the reaction, as it often arises from molecular reactions that involve multiple ("cooperating") copies of the protein X. When $n = 1$, a Hill function is also called a *Michaelis-Menten* function. For $n = 1$, the graph is hyperbolic, but for $n > 1$ it takes a sigmoidal of S-shaped form. For a repressor ($X \dashv Y$), the Hill function becomes

$$f(X) = \frac{\beta}{1 + (\frac{X}{K})^n} \text{ (Hill function for repressor),}$$

and it is shown in the right side plot of Figure 4.

Many genes also have a non-zero minimal expression level, the *basal expression level* called also leakiness. This can be taken into account by adding a term β_0 to the Hill function.

We just described how the Hill function can model the regulation of a gene by a single transcription factor. However, several genes can be regulated by multiple transcription factors, some of which may be activators and some may be repressors [2]. There are a number of different forms for the regulation function depending on the number of its inputs and on whether they activate or repress. For example, for the case in which Y is activated by A, i.e., $A \rightarrow Y$ and it is repressed by R, i.e., $R \dashv Y$, then we may have

$$f(A,R) = \frac{\beta(\frac{A}{K_A})^n + \beta_0}{1 + (\frac{A}{K_A})^n + (\frac{R}{K_R})^m}, \quad (1)$$

in which the parameters K_A and K_R are the activation coefficient and the repressor coefficient for A and R, respectively. The term β_0 is the basal expression rate that occurs when no activator and no repressor are present.

A transcriptional network is composed of multiple edges with activation or repression functions between several species.

B. Dynamics of simple gene regulation

In the simple transcriptional activation $X \rightarrow Y$ or repression $X \dashv Y$ described by the transcriptional regulation function $f(X)$, there are two main dynamic steps involved: transcription and translation. Protein production is balanced by two processes, *degradation* and *dilution*. Degradation occurs when the protein is destroyed by specialized proteins in the cell that, for example, recognize a specific part of the protein and destroy it. Dilution is due to the reduction in concentration of the protein due to the increase of cell volume during growth. In a similar way, mRNA production is also balanced by dilution and degradation processes. Thus, the dynamics of gene regulation is often well captured by the following ordinary differential equations:

$$\begin{aligned} \frac{dm_Y}{dt} &= f(X) - \alpha_1 m_Y \\ \frac{dY}{dt} &= \gamma\, m_Y - \alpha_2 Y, \end{aligned} \quad (2)$$

in which m_Y denotes the concentration of *mRNA* translated by gene Y, the constants α_i's incorporate the dilution and degradation processes, and γ is a constant that establishes the rate at which the mRNA is translated. Usually, the value of α_1 is about 10 times larger than α_2, that is, the mRNA is degrading much faster than the protein itself. Therefore,

often it is possible to consider the first of the equations (2) at the equilibrium by singular perturbation arguments.

V. Analysis of gene transcriptional networks

As the number of species involved in the network of activations and repressions increases, the dynamics of the overall system becomes potentially more complex and harder to analyze. Thus, research has largely focused on designing subsystems that serve as building blocks for the entire network, and on establishing rules for interconnections. The ultimate goal is that of predicting the behavior of the entire network from the behavior of the component blocks. This approach is a standard one in the design and fabrication of electronic circuitry. In particular, researchers in the field of synthetic biology have mainly focused on designing and fabricating *self-repression modules* [5], *mutual repression modules* [10], and *oscillator modules* [4], [8]. Before going into the details of each of these already fabricated modules, we first concentrate on the main features of their behaviors. The first type of module is characterized by a unique stable state, the second one by multiple attracting states, and the third one by stable limit cycles. These three types of behavior are intimately linked, and often give rise to each other as we now discuss.

Uniqueness of steady states, and global asymptotic stability, are quite common among simple biochemical reactions, although it is not always easy to prove theorems insuring this behavior. Systems with multiple attractors arise in many forms, a typical one of which is the interaction between two processes, such as formation and degradation, each of which by itself would lead to global stability. Relaxation, or hysteresis-driven, oscillators are those in which to a system with multiple attractors one adds a slow parameter adaptation law. Other oscillators arise through a Hopf bifurcation phenomenon, which consist basically of an unstable linear oscillator combined with nonlinear terms that prevent escape to infinity, and thus confine trajectories in systems whose linearization has been made unstable by means of negative feedback loops. The transitions (bifurcations) between qualitative behaviors such as mono and multiple-stability, or the onset of oscillations, are phenomena which frequently arise when parameters in systems are modified.

It is especially relevant to understand how the parameters can be tuned so as to obtain a desired behavior when one wants to build a synthetic circuit with prescribed behavior. A parameter can be, for example, the promoter affinity, the decay constant of a protein or of an mRNA molecule, and the Hill coefficient. We briefly discuss in Section VI how one can control the value of these parameters in the biological substrate.

For example, consider the simple activation network $X \to Y$. If we model the dynamics of the protein, only, considering the *mRNA* dynamics to be at the equilibrium, we obtain that

$$\dot{Y} = \frac{\beta X}{K + X} - \alpha Y,$$

for the case in which the cooperativity is equal to one, that is, $n = 1$. The first term is the Michaelis-Menten formation term. Let X be at a constant value X_0, then the concentration $Y(t)$ will, from any initial condition $Y(0)$, converge to the steady state $Y_0 = \frac{(\beta/\alpha)X}{K+X}$. In a typical set of experiments, a biologist will set the concentration of the inducer X to a given value X_0, let the system relax to the corresponding steady state Y_0, and repeat for various values of X_0, thus obtaining a plot of Y_0 against different such X_0. The corresponding plot is as the left side plot of Figure 4 for $n = 1$. We will call this graph, using control-theory terminology, the steady state response to step inputs, where we think of X_0 as the magnitude of a constant input applied to the system. The response in this example is graded in the sense that it is proportional to the parameter X_0 , at least over a large range of values of X_0, even though it eventually saturates. It is said to be a hyperbolic response, in contrast to a sigmoidal response as in the left side plot of Figure 4 for $n > 1$.

Sigmoidal responses are characteristic of many signaling cascades, which display what biologists call an ultrasensitive response to inputs. If the purpose of a signaling pathway is to decide whether a gene should be transcribed or not, depending on some external signal sensed by a cell, for instance the concentration of a ligand as compared to some default value, such a binary response is required. Cascades of enzymatic reactions can be made to display ultrasensitive response, as long as at each step there is a Hill coefficient $n > 1$ [13], because the derivative of a composition of functions $f_1 f_2 ... f_k$ is, by the chain rule, a product of derivatives of the functions making up the composition (thus, the slopes get multiplied, and a steeper nonlinearity is produced). In this manner, a high effective cooperativity index may in reality represent the result of composing several reactions, perhaps taking place at a faster time scale, each of which has only a mildly nonlinear behavior. Synthetic cascades of transcriptional activation have been designed and fabricated to obtain this type of behavior [3].

We mentioned that systems with multiple attractors sometimes arise through the interaction of formation and degradation/dilution processes. For example, consider now the case in which $Y = X$, that is, Y is activating its own transcription:

$$\dot{Y} = \frac{\beta Y^n}{K^n + Y^n} - \alpha Y.$$

We plot in Figure 5, both the first term in blue (formation rate) for $n = 1$ and for $n > 1$, and the second one in red (decay rate). Let us analyze the solutions of the differential equation. In the first case, $n = 1$, for small Y the formation rate is larger than the decay rate, while for large Y the decay rate exceeds the formation rate; thus, the concentration $Y(t)$ converges to a unique intermediate value. In the second case, however, the situation is more interesting: for small Y the degradation rate is larger than the formation rate, so the concentration $Y(t)$ converges to a low value, but, in contrast, for large Y the formation rate is larger than the degradation rate, and so the concentration $Y(t)$ converges to a high value instead. In summary, two stable states are created, one low and one high, by this interaction of

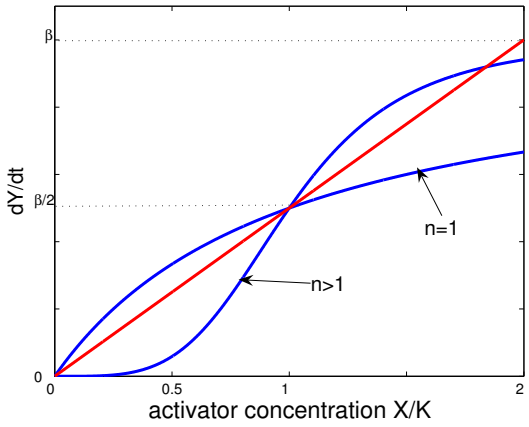

Fig. 5. Bistability arises from high Hill coefficients.

formation and decay, if one of the two terms is sigmoidal. (There is also an intermediate, unstable state.) These facts are elementary, but they serve to motivate a theory based upon monotone systems [24], which provides a far-reaching generalization. Whether, under feedback, a mono-stable or a multi-stable system results, depends thus on the shape of the transcription regulation curves, which in turn is determined by the numerical values of the parameters. For example, the hyperbolic case corresponds to $n = 1$, while $n > 1$ tends to generate multi-stability as shown in Figure 5.

VI. Enabling technology for synthetic networks

The discovery of mathematical logic in gene regulation [17] and the early achievements in genetic engineering in the 1970s, such as recombinant DNA technology, set the stage for today's synthetic biology. Recent advances in molecular biology provide the ability to translocate and fuse promoters, operators, binding sites, and genes in almost any fashion on a size-wise-compatible plasmid. Most importantly, a key enabler to synthesize DNA in amounts large enough to be used for transfection (or transformation) and for various measurement procedures has been the *Polymerase Chain Reaction* (PCR). This molecular biology technique allows a small amount of DNA to be amplified exponentially. As PCR is an *in vitro technique*, it can be performed without restrictions on the form of DNA, and it can be extensively modified to perform a wide array of genetic manipulations.

Another key enabling technology has been the development of *in vivo* measurement techniques that allow to measure the amount of protein produced by a target gene X. For instance, green fluorescent protein (GFP) is a protein with the property that it fluoresces in green when exposed to UV light. It is produced by the jellyfish *Aequoria victoria*, and its gene has been isolated so that it can be used as a reporter gene. The GFP gene is inserted (cloned) into the chromosome, adjacent to or very close to the location of gene X, so both are controlled by the same promoter region. Thus, gene X and GFP are transcribed simultaneously and then translated, so by measuring the intensity of the GFP light emitted one can estimate how much of X is being expressed. Other fluorescent proteins, such as yellow fluorescent protein (YFP) and red fluorescent protein (RFP) are genetic variations of the GFP.

Just as fluorescent proteins can be used as a read out of a circuit, inducers function as external inputs that can be used to probe the system. Inducers function by disabling repressor proteins. Repressor proteins bind to the DNA strand and prevent RNA polymerase from being able to attach to the DNA and synthesize mRNA. Inducers bind to repressor proteins, causing them to change shape and making them unable to bind to DNA. Therefore, they allow transcription to take place. Two commonly used inducers are IPTG and aTc. Isopropyl-β-D-1-thiogalactopyranoside (IPTG) induces activity of beta-galactosidase, which is an enzyme that promotes lactose utilization, through binding and inhibiting the lac repressor. The anhydrotetracycline (atc) binds the wild-type repressor (TetR) and prevents it from binding the Tet operator.

For engineering a system with prescribed behavior, one has to be able to change the physical component features so as to change the values of the parameters of the model. This is now possible. For example, the binding affinity (K in the Hill function model) of a transcription factor to its site on the promoter can be affected by single or multiple base pairs substitutions. The protein decay rate (constant α_2 in equation (2)) can be increased by adding degradation tags at the end of the gene expressing protein Y (*http://parts.mit.edu/registry/index.php/Help:Tag*). (Degradation) Tags are genetic additions to the end of a sequence which modify expressed proteins in different ways such as marking the protein for faster degradation. Promoters that can accept multiple input transcription factors (called combinatorial promoters) to implement regulation functions such as the one in equation (1) can be easily obtained by combining the operator sites of several simple promoters [4]. For example, the operators $O_{R1} - O_{R2}$ from the λ promoter of the λ bacteriophage can be used as binding sites for the λ transcription factor [19]. Then, the pair $O_{R2} - O_{R1}$ from the 434 promoter from the 434 bacteriophage [6] can be placed at the end of the $O_{R1} - O_{R2}$ sequence from the λ promoter. Depending on the relative positions of these sites and on their distance from the RNA polymerase binding site, the 434 transcription factor may act as a repressor as when this protein is bound to its $O_{R2} - O_{R1}$ sites it prevents the polymerase to bind, while the λ transcription factor may act as an activator.

The "cut and paste" procedure described in the previous paragraph is called *cloning* [1]. Cloning of any DNA fragment involves four steps: *fragmentation, ligation, transfection, and screening/selection*. The DNA of interest is first isolated. Then, a ligation procedure is employed in which the amplified fragment is inserted into a vector. The vector (which is frequently circular) is linearized by means of restriction enzymes that cleave it at target sites called restriction sites. It is then incubated with the fragment of interest with an enzyme called *DNA ligase*. After ligation,

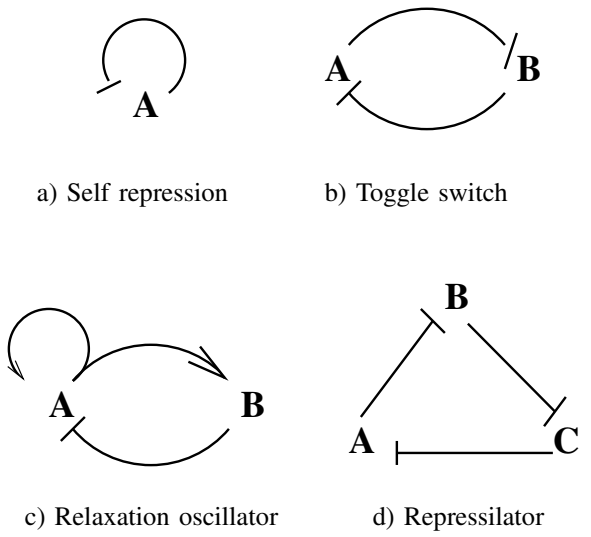

Fig. 6. Transcriptional network modules that have been fabricated in bacteria *Escherichia coli*. The self-repression circuit a) has been designed and analyzed by [5], the toggle switch b) has been designed and analyzed by [10], the relaxation oscillator has been designed and analyzed by [4], and the repressilator d) has been designed and analyzed by [8].

the vector with the insert of interest is transformed into cells by means of electroporation, for example. Finally, the transformed cells are cultured. These procedures are of very low efficiency and hence there is a need to identify the cells that have been successfully transformed with the vector construct containing the desired insertion sequence. Modern cloning vectors include thus antibiotic resistance markers, which allow only cells in which the vector has been transformed, to survive and grow.

VII. Synthetic networks: Autoregulated circuits, toggle switches, and oscillators

Enabled by the recent technological developments briefly summarized in Section VI, a number of simple synthetic circuits with prescribed behaviors have been built in *E. coli*. The basic motifs are shown in Figure 6. Naturally occurring transcriptional networks are very complex, however biologists have been discovering recurrent patterns of interconnection that appear frequently. These patterns are called *motifs* [16]. Mutations in the DNA sequence that may create or delete some of the network connections happen at very high rates. This suggests that if a network motif appears in a natural network more often than in a randomly generated network, evolution must have selected it based on some advantage it gives to the organism. If the motif did not offer selective advantage, it would have been washed out. Thus, synthetic biologists have been focusing mainly on synthetically reproducing these network motifs to study their behavior in isolation with the hope to (1) understand their role and features and to (2) create a number of understood building blocks that can be interconnected to create more complex networks with predictable behavior. At the heart of this approach is the concept of modularity, which we describe in Section VIII.

A. Self repressed gene

Negative autoregulation occurs when a transcription factor A represses its own transcription (diagram a) of Figure 6). The dynamics of A is then described by (neglecting the mRNA dynamics)

$$\frac{dA}{dt} = \frac{\beta}{1 + (A/K)^n} - \alpha A.$$

This system has been fabricated and two major findings resulted from the measurements: negative autoregulation speeds the response time [20], and negative autoregulation promotes robustness to fluctuations in production rates [5]. By using standard linear control theory, one can immediately predict that an increased negative feedback increments the robustness of the equilibrium point with respect to fluctuations. The interesting part is that this result has been confirmed by experiments performed on a simple negative feedback loop cloned on a plasmid and then transformed inside a bacterium. This fact is encouraging, as it means that the adopted modeling framework may be good enough to suggest design guidelines for circuitry to be implemented in the biological substrate.

B. Toggle switch

A genetic toggle switch (diagram b) of Figure 6) is a bistable system in which reliable switches between the two steady-states are induced through an input signal. Any such genetic toggle switch typically needs particular behavioral characteristics in order to be considered a true "memory component". First, the toggle switch must exhibit bistability over a wide range of parameter values (transcriptional rates, translational rates, decay constants, etc.) that tend to fluctuate in a living cell. Second, the two steady-states must be highly tolerant of random fluctuations in molecular-species concentrations, so that noise-induced transitions between the two states are virtually non-existent [10].

One approach to constructing bistable systems was discussed theoretically in Section V: bistability can be accomplished with a single auto-activating gene with a positive feedback loop.

A different approach to bistability employs two repressible promoters that mutually repress each other. Neglecting the mRNA dynamics, this type of toggle switch can be modeled by the following equations

$$\begin{aligned} \frac{dA}{dt} &= \frac{\beta_1}{1 + (B/K_1)^n} - \alpha_1 A \\ \frac{dB}{dt} &= \frac{\beta_2}{1 + (A/K_2)^n} - \alpha_2 B. \end{aligned}$$

The nullcline for the first equation is shown in red in Figure 7, while the nullcline for the second equation is shown in blue in Figure 7.

Within appropriate parameter ranges, one unstable and two stable steady-states exist for the system (see Figure 7). At the stable steady-states, one of the repressors is dominant over the other, while the other repressor is shut down.

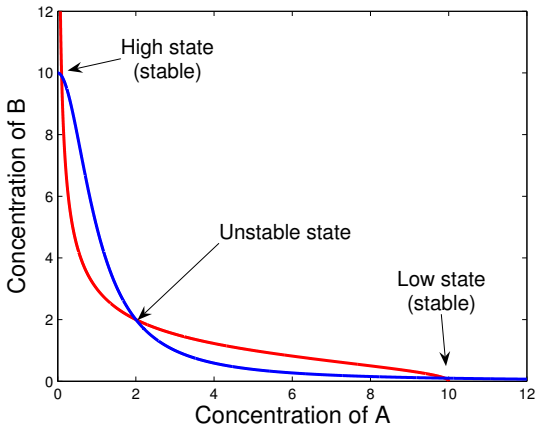

Fig. 7. Set of equilibria of the toggle switch of diagram b) of Figure 6.

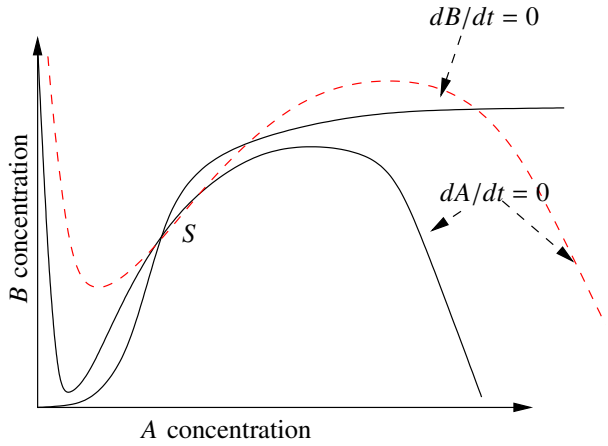

Fig. 8. Nullclines for the equations (3). The equilibrium S is unique when $dA/dt = 0$ is given by the black plot. There are multiple equilibria when $dA/dt = 0$ is given by the red plot.

A switch of the dominance-toggle is induced by externally repressing the dominant repressor, let us say B, so it cannot bind any longer to the target promoter for the other gene, let us say A. The effect is to boost the expression of the formerly low-expressed repressor A, which thus returns to its higher constitutive expression rate (set $B = 0$ in the equation for dA/dt). This method will allow A to now grow in concentration uninhibited, and eventually repress its target promoter (B), which will cause the two repressors to switch relative concentrations. At that time a state transition to the other steady-state has taken place, and this must remain permanent until the next intentional signal that indicates a transition back again. The external repressing of B is experimentally accomplished by means of an *inducer*. An inducer (section VI) is a molecule (such as IPTG) which binds to the repressor protein B and disables its repressing function, rendering it incapable of binding to DNA. Two inducers are used, one for each of A and B, so as to be able to switch back and forth.

There is experimental evidence that bistable systems made up of two mutually repressing genes are more robust than those made up of a single auto-activating gene, and are therefore better candidates for toggle switches. In addition, there is a good supply of well-characterized repressible genes that may be used as components, compared to activable genes. For induction purposes (switching to a different steady state), it appears to be easier to switch the former type of system, simply by repressing each of the repressors separately by means of an inducer.

C. Relaxation oscillator

An implementation of the simple motif shown in diagram c) of Figure 6 was proposed in [4]. The system generates relaxation oscillations by virtue of the competition arising between the strong self-activation of gene A and the transcriptional repression of A due to the transcription factor B. The system can be described by the following model (again neglecting the mRNA dynamics):

$$\begin{aligned} \frac{dA}{dt} &= \frac{\beta_1 A^n + \beta_0}{1 + (A/K_1)^n + (B/K_2)^m} - \alpha_1 A \\ \frac{dB}{dt} &= \frac{\beta_2 A^n + \beta_0'}{1 + (A/K_1)^n} - \alpha_2 B, \end{aligned} \tag{3}$$

in which the Hill coefficients are greater than 2. Since the model is two-dimensional, one can apply the Poincaré-Bendixson Theorem. It is thus possible to determine the parameter range that guarantees the existence of a periodic solution just by computing the parameter range that guarantees a unique and unstable (not a saddle) equilibrium point. From Figure 8, it appears that there is one equilibrium when the nullcline $dA/dt = 0$ is given by the black plot. Such an equilibrium S can be seen to be unstable for α_1 sufficiently larger that α_2. If instead β_1 is too large compared to β_2, the configuration of the nullcline $dA/dt = 0$ is given by the red plot of Figure 8. Thus, one cannot infer that the ω-limit set will be given by periodic solutions as there are multiple equilibria some of which are stable and some are unstable.

The data obtained by the experiments in [4] show damped almost sinusoidal oscillations. The fact that the oscillations are damped means that the equilibrium point is stable (other behaviors are ruled out by the Poicaré-Bendixson Theorem). To make the equilibrium point unstable, one can increase α_1 with respect to α_2 by adding a degradation tag to the gene of A. Since the oscillations are almost sinusoidal, the system could be operating in the proximity of a Hopf bifurcation. In such a case, increasing α_1 with respect to α_2 would increase the real part of the pair of eigenvalues that cross the imaginary axis, making the equilibrium point unstable [7].

D. Repressilator

Elowitz and Leibler [8] constructed the first operational oscillatory genetic circuit consisting of three repressors arranged in ring fashion, and coined it the "repressilator" (See diagram d) of Figure 6). The repressilator exhibits sinusoidal,

limit cycle oscillations in periods of hours, which are slower than the cell-division life cycle. Therefore, the state of the oscillator is transmitted between generations from mother to daughter cells. In the repressilator, the protein lifetimes are shortened to approximately two minutes (close to mRNA lifetimes). Thus, the dynamical behavior of the repressilator is driven by mRNA and protein dynamics and it can be described after re-arranging the parameters by:

$$\begin{aligned}
\frac{dm_A}{dt} &= \frac{\beta_1}{1+(C/K_1)^n} + \beta_0 - \alpha_1 m_A \\
\frac{dA}{dt} &= \gamma_1(m_A - A) \\
\frac{dm_B}{dt} &= \frac{\beta_1}{1+(A/K_1)^n} + \beta_0 - \alpha_1 m_B \\
\frac{dB}{dt} &= \gamma_1(m_B - B) \\
\frac{dm_C}{dt} &= \frac{\beta_1}{1+(B/K_1)^n} + \beta_0 - \alpha_1 m_C \\
\frac{dC}{dt} &= \gamma_1(m_C - C),
\end{aligned}$$

in which it is assumed that the three components are equal. Since the regulation functions have all negative slope, and there is an odd number of them in the loop, there is only one equilibrium. One can then invoke Mallet-Paret's Theorem [15] or Hastings' Theorem [12] to conclude that if the equilibrium point is unstable, the ω-limit set of any bounded trajectory is a periodic orbit [9]. Thus, one can search for parameter values to guarantee the instability of the equilibrium point. This procedure was followed by [8] in the design of the repressilator.

VIII. Modular analysis and design

The fundamental systems engineering paradigm is that of larger systems made up of simpler components, interconnected according to well-defined rules. This idea permits the recursive verification of important properties through the use of standard analysis tools, for example the study of stability by means of passivity, small-gain, or input to state stability. It also allows the synthesis of larger systems from well-characterized and validated components.

Similarly, one of the important themes in current molecular biology and especially synthetic biology [3], [11], [14], is that of understanding and engineering cell behavior in terms of cascades and feedback interconnections of elementary *modules*. Cells themselves can be seen as composed of a large number of subsystems, involved in various processes such as cell growth and maintenance, division, and death. The hope is that one should be able to decompose into such, hopefully simpler, subsystems, and then study the emergent properties of interconnections. Similarly, for purposes of design, it is desirable to be able to build complicated circuitry on the basis of a number of characterized components or motifs.

Underlying the use of modularity is the principle that the input/output (dynamic) behavior of components should not change when components are connected together. It has been

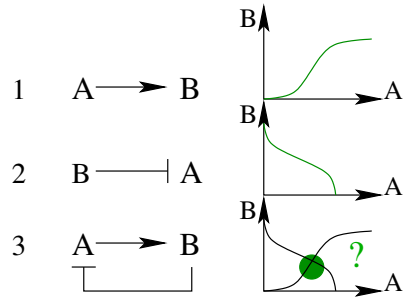

Fig. 9. Module 3 is the feedback interconnection of modules 1 and 2. The green plots denote characterization data obtained by experiments. The black plots on the right side of module 3 are the same as the ones on the right side of modules 1 and 2, but plotted on the same axis. If the input/output steady state behavior of modules 1 and 2 is not affected by their interconnection, the equilibrium value of A and B in module 3 (green spot in the plot on the right of module 3) should be close to the intersection point of the black curves.

frequently observed, on the other hand, that many natural and engineered systems cannot be neatly decomposed into subsystems with "inputs" and "outputs", see for instance the discussion in [25]. In practice, in order to enforce modularity, one must insert active mechanisms such as operational amplifiers, in order to buffer and otherwise prevent "back effects" from downstream elements.

The issue of how modules should be delimited in a biomolecular network remains an unanswered question (see [3], [22], [23] for discussion). As in engineering applications, there are two main issues that arise when dealing with this problem: in analysis, checking modularity, and in design, enforcing modularity.

A. Checking modularity

It is easier to discuss the questions that arise here by an informal simple example. Suppose that two subsystems A and B have been characterized, perhaps describing concentrations of two proteins, and an output signal from A is also viewed as a signal (activating) for B, and, conversely. an output signal from B is a repressing input signal for A. We may ask how the dynamical behavior of the feedback loop in Figure 9(3) relates to that of the individual components. The simplest such question concerns the location of steady states of the feedback system. Mathematically, one proceeds as follows. We consider the subsystem B as having an external input signal that can be manipulated (and which we denote as A in Figure 9(1)), and we consider the subsystem A as driven by an external input (B in Figure 9(2)). We would expect that graphing the equilibrium input/output responses of each of the individual systems (recall the discussion in Section V) the intersection of the two plots will predict the location of the equilibrium of the feedback composition. To experimentally check this prediction, one must carry out several experiments, for the individual subsystems as well as for the closed-loop; for example, the latter is obtained by directly measuring the equilibrium concentration of both

A and B in the interconnection. A key point is what the input and output signals of the modules are. In this case, we have considered protein concentrations as inputs and outputs. "Opening the loop" in such systems is not a trivial matter, and might involve genetic engineering intervention, such as replacing a wild-type gene by a mutant that is not subject to repression (for A) or activation (in the case of B). An example of this approach can be found in [18], where a cell-cycle relaxation oscillator was broken down so as to expose an underlying bistable subsystem.

B. Enforcing modularity

A basic feature of modules is that their input-output behavior should not change when several modules are interconnected.

To illustrate the difficulties that may arise in attempting modular design in synthetic biology, let us consider the following typical situation. Suppose that a synthetic oscillator has been designed, which involves a negative feedback loop involving the genes coding for three proteins X, Y, and W. We would like to use the periodic behavior generated by this oscillator as a "clock signal" that times the behavior some other downstream module. Let us say that this downstream module is driven by the level of another protein, Z. For concreteness, let us say that the feedback loop for the oscillator is given by $Y \to W \dashv X \to Y$, and that we wish to use the protein X as a transcription factor regulating the expression of the gene that codes for Z. Thus, one module consists of X, Y, and W with X considered as an "output" signal, and a second module consists of X, Z (and possibly other proteins), with the protein X considered as an "input" signal.

Intuitively, the interconnection of the two modules should be achieved by simply putting both genes on the same plasmid or chromosome. However, there is a major problem with this idea: since we have in the same environment (the given cell) both $X \to Y$ and $X \to Z$, the promoters of Y and of Z will be in competition for X. In other words, the protein X is a physical object, which can be bound either to the promoter of Z or to the promoter of Y, but not to both simultaneously. Unless X is kept at a saturation level (which it cannot be, since the whole purpose is for X to oscillate), this competition will mean that the effective rate of binding to the promoter of Y will change, and hence the behavior of the oscillator will be disrupted. In electrical engineering terms, the "load" on the system has not been considered in the design of the oscillator.

One solution to this problem would be to design a feedback control system, similar to an integral controller in an engineering system, that automatically adjusts the oscillator amplitude and frequency so as to compensate for the different possible "loads". Another solution, proposed here, is to create a mechanism that in effect decouples the connections $X \to Y$ and $X \to Z$. We proceed as follows.

We introduce a new protein, X_2, which does not bind to the promoter region for Y, and modify the promoter for Z in such a manner that X_2 binds to it but X does not. We now splice a gene for X_2 next to X, which we denote as X_1 in Figure 10, in such a manner that both X_1 and X_2 are under the control of the same promoter (repressed by W).

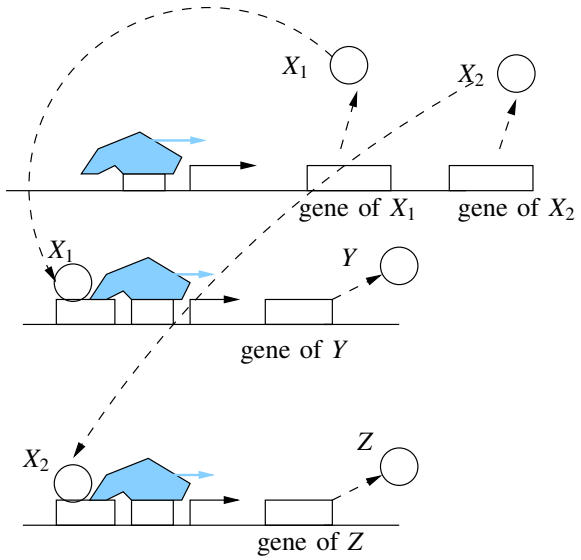

Fig. 10. Enforcing modularity by preventing competitivity.

The net effect of this construction is that, since X_1 binds only to the promoter of the gene of Y, and X_2 binds only to the promoter of the gene of Z, we eliminated the competition problem that we had initially. The oscillator's characteristics will not change in any way depending on the presence or absence of the second module (driven by X_2).

IX. Remarks and Conclusions

We presented an introduction to general concepts in molecular biology and synthetic biology, and discussed a number of appealing dynamics and system-theoretic questions. The rapidly developing field is tremendously exciting, and full of opportunities and challenges. The reader will, hopefully, take on some of the latter. We refer the reader to [24] for a discussion of theoretical challenges in control and systems theory raised by research in systems biology.

References

[1] B. Alberts, D. Bray, J. Lewis, M. Raff, and K. Roberts. *Molecular Biology of the Cell*. Garland Publishing, 1989.

[2] U. Alon. *An Introduction to Systems Biology: Design Principles of Biological Circuits*. Chapman & Hall, 2006.

[3] E. Andrianantoandro, S. Basu, D. K. Karig, and R. Weiss. Synthetic biology: New engineering rules for an emerging discipline. *Molecular Systems Biology*, pages 1–14, 2006.

[4] M. R. Atkinson, M. A. Savageau, J. T. Meyers, and A. J. Ninfa. Development of genetic circuitry exhibiting toggle switch or oscillatory behavior in *Escherichia coli* . *Cell*, pages 597–607, 2003.

[5] A. Becskei and L. Serrano. Engineering stability in gene networks by autoregulation. *Nature*, pages 590–593, 2000.

[6] F. D. Bushman and M. Ptashne. Activation of transcription by the bacteriophage 434 repressor. *PNAS*, pages 9353–9357, 1986.

[7] D. DelVecchio. Design and analysis of an activator-repressor clock in E. coli. In *American Control Conference (To appear)*, 2007.

[8] M. B. Elowitz and S. Liebler. A synthetic oscillatory network of transcriptional regulators. *Nature*, pages 339–342, 2000.

[9] H. ElSamad, D. DelVecchio, and M. Khammash. *Repressilators and promotilators: Loop dynamics in synthetic gene networks. In *American Control Conference*, pages 4405–4410, 2005.

[10] T.S. Gardner, C.R. Cantor, and J.J. Collins. Construction of the genetic toggle switch in *Escherichia coli* . *Nature*, page 339342, 2000.

[11] L. H. Hartwell, J.J. Hopfield, S. Leibler, and A. W. Murray. From molecular to modular cell biology. *Nature*, 402:C47C52, 1999.

[12] S. Hastings, J. Tyson, and D. Webster. Existence of periodic solutions for negative feedback cellular systems. *J. of Differential Equations*, pages 39–64, 1977.

[13] J.E. Ferrell Jr. Tripping the switch fantastic: How a protein kinase cascade can convert graded inputs into switch-like outputs. *Trends Biochem. Sci.*, page 460466, 1996.

[14] D. A. Lauffenburger. Cell signaling pathways as control modules: Complexity for simplicity. *PNAS*, 97:5031–5033, 2000.

[15] J. Mallet-Paret and H.L. Smith. The Poincaré-Bendixson theorem for monotone cyclic feedback systems. *J. of Dynamics and Differential Equations.*, 2:367–421, 1990.

[16] R. Milo, S. Shen-Orr, S. Itzkovitz, N. Kashtan, D. Chklovskii, and U. Alon. Network motifs: Simple building blocks of complex networks. *Science*, pages 824–827, 2002.

[17] J. Monod and F. Jacob. Teleonomic mechanisms in cellular metabolism, growth, and differentiation. In *Cold Spring Harb. Symp. Quant. Biol. 26*, page 389401, 1961.

[18] J. R. Pomerening, E.D. Sontag, and J. E. Ferrell. Building a cell cycle oscillator: hysteresis and bistability in the activation of cdc2. *Nat Cell Biol*, 5(4):346–351, April 2003. Supplementary materials 2-4 are here: http://www.math.rutgers.edu/(tilde)sontag/FTPDIR/pomerening-sontag-ferrell-additional.pdf.

[19] M. Ptashne. *A genetic switch*. Blackwell Science, Inc., 1992.

[20] N. Rosenfeld, M. B. Elowitz, and U. Alon. Negative autoregulation speeds the response times of transcription networks. *J. Mol. Biol.*, pages 785–793, 2002.

[21] N. Rosenfeld, J. W. Young, U. Alon, P. S. Swain, and M. B. Elowitz. Gene regulation at the single-cell level. *Science*, pages 1962–1965, 2005.

[22] J. Saez-Rodriguez, A. Kremling, H. Conzelmann, K. Bettenbrock, and E. D. Gilles. Modular analysis of signal transduction networks. *IEEE Control Systems Magazine*, pages 35–52, 2004.

[23] E.D. Sontag. Some new directions in control theory inspired by systems biology. *IEE Proc. Systems Biology*, 1:9–18, 2004.

[24] E.D. Sontag. Molecular systems biology and control. *Eur. J. Control*, 11(4-5):396–435, 2005.

[25] J.C. Willems. Behaviors, latent variables, and interconnections. *Systems, Control and Information*, pages 453–464, 1999.