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In Silico Models for Metabolic Systems Engineering

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17.1 Introduction

Biological systems often display remarkable behaviors that are not easily anticipated or comprehended. One broad example is the ability of cellular systems to maintain phenotypic stability under vast and diverse conditions [1–4]. Also, cellular systems are in constant evolution involving numerous tightly controlled molecular interactions to achieve specific goals; metabolism to balance the cell systems' energy requirement, immune response signalling for tackling invading pathogens etc. Therefore, the properties of cellular systems cannot be understood if we treat biological entities in isolation, rather we have to consider them as an integrated system. The reliance of only using traditional wet-bench biological techniques to study cellular behavior is therefore insufficient and the investigation of molecular interactions in detail is necessary in order to understand especially time-evolving biological properties, such as morphology, growth, metabolism, and disease progression.

It is easy to imagine that cellular systems are complex and that the many cellular processes occur at random. However, we now realise that cellular interactions are structurally organized and can be interpreted in physical terms. Recent studies have revealed that large-scale biological networks are organized in a scale-free manner and their construction consists high degree of modularity [5–7]. It has also been proposed that at the elementary level the network consist of the building blocks of life, the network motifs, and these are connected into modular groups and the modular groups are hierarchically arranged [8–11]. The overall network structure of a complex system is thus built to ensure stability, or robustness to perturbations, and display emergent properties such as phenotypic oscillations that act as biological switches [4,12,13].

We can accept the notion that system dynamics and network construction have a close relationship. When we consider network to network communications, e.g., the interactions between intracellular signalling with transcriptional phenotype or protein expression with metabolic network behavior, we know that an understanding of all these interactions is vital in explaining the holistic behavior of cells. Moreover, cellular interactions are not static and are constantly evolving. In order to interpret network properties such as feedback control/regulation and oscillatory behavior, it is therefore important to temporally quantify the relevant biological entities, such as gene expression or metabolite concentration. Only through the analysis of such time variant interactions phenotype can we understand the dynamic cellular behavior. As dynamic cellular phenotypes cannot be comprehended by visual inspection or simple statistical or linear approaches, the development of appropriate complex network theory is thus essential.

Over the last few years, there has been active development of systemic methodologies to decipher dynamic cellular behavior. This phenomenon led to the creation of a new interdisciplinary field, called systems biology, inviting scientist across various fields to actively participate in joint research. Although interdisciplinary research involving biology has been in existence for a long time, in a rather *ad hoc* manner, only in the last 5–6 years that we have witnessed a global consorted effort [14,15]. The goal of systems biology is to generate, integrate, and analyze biological data, both in time and space, (i) for the understanding of molecular circuit design in detail and (ii) to predict the response of cellular system to various extracellular and intracellular perturbations.

A typical cellular system consists of hundreds of thousands of molecular interactions and to consider them in entirety, though desirable, is an overwhelming and impossible task. Therefore, to reduce such complexity, it would be appropriate to modularize cellular systems into layers of biological interest, for example, modularizing pathways of gene regulation system, for the determination of gene to gene interaction, signal transduction cascades for the understanding of extracellular signal propagation into the nucleus, and metabolic pathways for calculating the redistribution of fluxes to a given concentration perturbation. Although this kind of modularization concept preceded well before systemic approaches were evaluated, the detailed molecular machinery that govern the interactions between biological components along each layer and between layers cannot be understood without the introduction of theoretical concepts. (Figure 17.1).

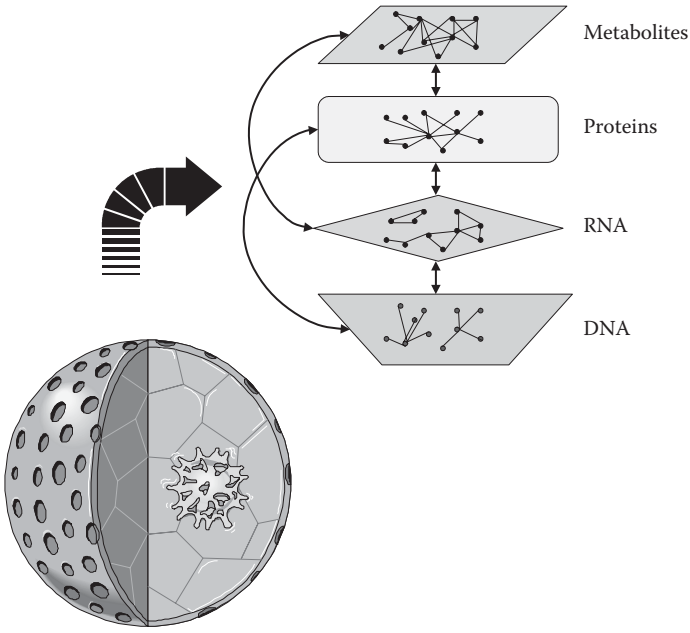


FIGURE 17.1 Schematic depicting the levels or layers of interactions found in biological system.

System biology methods are based upon formalized theories, in most cases utilizing physico-chemical laws. They are intended to provide better insights into the underlying molecular circuitry that controls complex biological systems. Another advantage of systems biology is the possible reduction of time and cost associated with traditional biological research, as the ability to perform optimized experiments *in silico* becomes an increasing reality [16–18].

In the field of metabolic engineering, the desire has been to manipulate the cellular system so as to optimize or improve cellular properties leading to an increased industrial output, for example, optimal production of ethanol for beer brewery. However, using simple intuition or linear approaches to manipulate the metabolic pathways involving the desired substance often leads to failure [19,20]. This is not surprising as we now know that the biological system consists of highly nonlinear regulatory properties and hence targeting just one step in a network may not yield a beneficial outcome [11]. It is thus inevitable to consider the complexity of cellular system in a systemic manner and this is only possible if we consider the use of mathematical and computational approaches to supplement the ongoing wet-bench experimental research.

In this chapter, we briefly introduce the concept of metabolic systems engineering (Section 17.2). We mention some of the popularly used theoretical approaches and introduce our very own computational platform, the E-Cell systems (Section 17.3), which can be used for metabolic engineering studies. In Section 17.4 we perform simple theoretical examples to show the utility of dynamic analysis of metabolic networks. Also in Section 17.5, we provide some practical examples of dynamic models that could benefit the metabolic engineering community. We end the chapter by mentioning some of the future trends and requirements for the field.

17.2 Metabolic Systems Engineering

Metabolic engineering is aimed at improving the biological properties of a cell, by the exploitation of its metabolic network design. In the past, selective breeding of better yielding strains were used for industrial and medical gain, the production of penicillin by *Penicillium chrysogenum* is a good example. The process involved several iterations between selection of new strains and mutating them. As the success of this field started to expand, it attracted scientists from various disciplines; biochemists, chemical engineers, analytic chemists, microbiologists, and physiologists. This resulted in the development of better analytical methodologies such as, recombinant DNA technology, which introduces purposeful intermediary pathways or genetic changes that usually result in better yielding strains [21,22].

The concept of metabolic engineering can be broken down into steps. The initial step is the selection of appropriate metabolic pathways which involve the cellular substance that is desired to be increased, for example, glycolysis and related pathways for the production of alcohol. As mentioned in the introductory section, since it is daunting to evaluate pathways involving thousands of reactions, metabolic engineers usually modularized their interest into specific pathways or network constituting of manageable size, usually in tens of reactions. The next step is the identification of the most effective target within this framework that could be modified for improved specific transport, increased product formation, or optimized conversion of substrate. This then requires the development or utilization of methods and tools to achieve the intended result, for example reducing an inhibitor enzyme concentration by using PCR-based gene deletion strategy [23,24]. However, there often exists unknown or unexpected metabolic regulation that eventually does not lead to the required result or does not yield the intended production volume.

Schaaff et al. investigated the production rate of ethanol by overexpressing eight glycolytic and fermentative pathway enzymes of *Saccharomyces cerevisiae* by placing their genes on multicopy vectors [20]. By doing so, they increased specific enzyme activities between 3.7 and 13.9-fold in logarithmically growing cultures. Surprisingly, at that time, the increases in the activities of the different glycolytic enzymes did not affect the rate of ethanol production significantly as compared with wild type. This experiment was perhaps one of the early experiments that demonstrated that living cells are robust

to diverse perturbation. Metabolic engineering thus cannot just rely on recombinant DNA techniques alone for success, it also requires the effort from systems biology. Therefore, it is important to review this field as metabolic systems engineering.

In metabolic systems engineering, we assess the prospects of utilizing computational models to further refine and optimize the current metabolic engineering design. The aim is to initially develop a dynamic computational or *in silico* model that simulates metabolic fluxes, the amount of product that accumulates in a cell or efflux out of the cell, the strength of enzyme activities that participate in the system and their directions under various perturbation conditions, using existing knowledge of the metabolic system of interest, including linear and nonlinear regulatory features. Basically, the initial model (with system parameters) is built based on what we know currently about the system. The next step is to perform computational analysis to determine the optimal target reaction (Figure 17.2). We can perturb the model (e.g. *in silico* enzymatic inhibition) at multiple steps or specific known key regulatory steps and by the analysis of the simulation result, we determine the combination of perturbation that yields the most beneficial *in silico* result. We then perform genetic changes to verify whether the

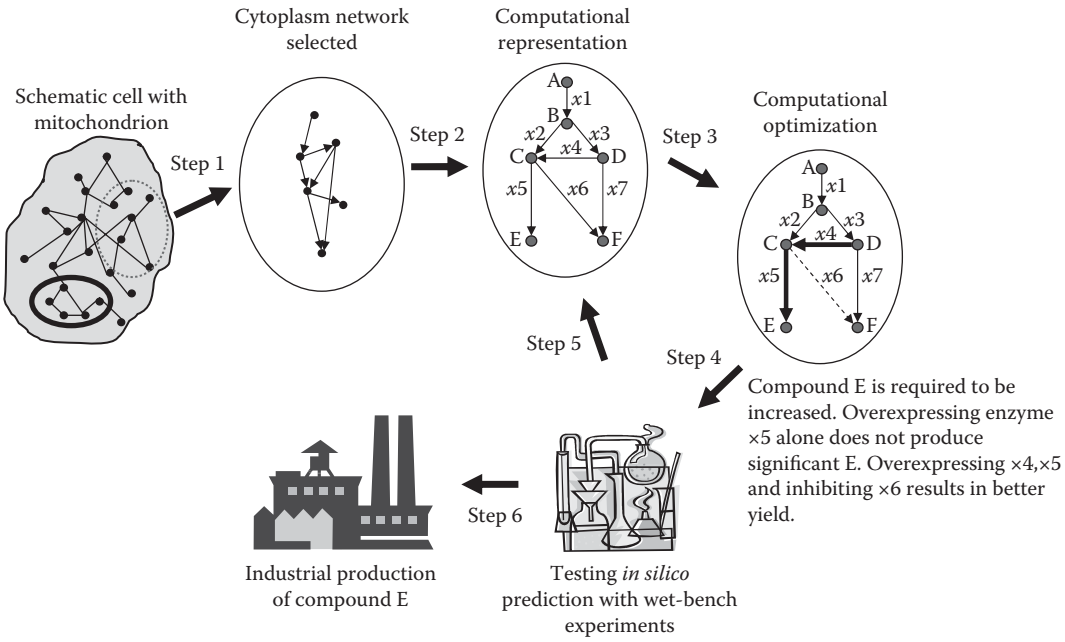


FIGURE 17.2 *In silico* analysis and optimization of metabolic networks for better product yield. Although a metabolic system consists of an entire cell or organism, some degree of isolation is necessary in order to analyze it. Step 1: Model Abstraction. This involves selecting pathways of manageable size in which the component(s) that exert strongest control over metabolic flux is(are) present. Step 2: Model Construction. Computational model (reference model) of the selected pathways is developed based upon existing knowledge, and system parameters are usually chosen to match experimental results of control (e.g., wild type). Step 3: Model Optimization. The reference model is first perturbed (e.g., mutation) at selected locations (single or multiple) using intuition for optimal production of required substance(s) *in silico*. The model simulation is iterated until a satisfactory intended *in silico* outcome is obtained. Step 4: Experimental Testing. The simulation result is used to design new experiments that would produce the intended result. Usually, there will be a need to fine tune the computational model with wet-bench experiments (Step 5). Step 5: Model Iteration. The *in silico* models are tuned until satisfactory experimental system behavior, due to perturbations, is obtained. This is a cyclic process. Step 6: Industrial Production. Once desired optimized result is obtained, the experiments are scaled up for industrial volume output. (From Selvarajoo, K., *FEBS Lett.* 580, 1457, 2006. With permission.)

desired outcome is actually produced. In many cases the model simulations may not look satisfactory to the experimental findings, as we may not yet know other key regulatory features of the system. In such cases, we have to perform iterative work between *in silico* prediction and experimental findings (model refinement), before a desired outcome is achieved [25] (Figure 17.2).

In the following section, we introduce some of the theoretical methods popularly used to model metabolic systems.

17.2.1 *In Silico* Methods

There are a number of computational and mathematical approaches popularly used to model metabolic network behavior. These include kinetic methodologies such as enzyme kinetics [26] and metabolic control analysis (MCA) [27], stoichiometric approaches, such as metabolic flux analysis (MFA) [28] and flux balance analysis (FBA) [29], and power law formalism such as the biochemical systems theory (BST) [30].

In kinetic methodologies, mathematical models of metabolic networks are created with the aid of detailed enzyme kinetic equations. The successful simulation of such models requires the system parameters (usually rate constants) to be known *a priori*. For example, to create a metabolic network model, ordinary differential equations (usually composed of Michaelis–Menten type equations) are set up to describe the fluxes for each metabolite. These are then integrated to obtain the metabolite concentrations over time. As most metabolic reactions are complex involving cofactors or other substrate regulation, the resultant ordinary differential equations are usually complex and not solvable using analytic approaches. Often numerical schemes are introduced to overcome this difficulty [31]. The main problem with kinetic methodologies, however, is the determination of the system parameters which are highly limited. Therefore, simplifying assumptions are generally used to make model simulation [32]. This often results in poor prediction of cellular response and requires improvement by iterative work involving experimental work (Figure 17.2). Kinetic methodologies are often used to determine key regulatory steps of metabolic pathways.

In MCA, the philosophy of modelling metabolic reactions is different. It is not intended to be used to discover a single rate limiting or key regulatory step. Rather, its use focuses on discovering the collective control of a series of interconnected reactions. Hence, it introduces the concept of control, that is, a measure to determine the effect one reaction has on all the interconnected metabolic pathway reactions. It thus defines and incorporates terms like flux and flux control in traditional enzyme kinetics. Instead of assuming the existence of a unique rate-limiting step, it assumes that there is a definite amount of flux control and that this is spread quantitatively among the component enzymes [27]. That is, MCA proposes the idea that the regulation of a cell requires the coordinated activity changes of multiples enzymes by analyzing how the control of fluxes and intermediate concentrations in a metabolic pathway is distributed among the different enzymes that constitute the pathway. The applications of MCA have resulted in notable successes in metabolic engineering usually involving detailed flux calculations with rationalized strain improvements [22].

Stoichiometric methodologies such as MFA and FBA are used when detailed kinetic information of metabolic interactions are not available. These models are therefore, not usually of a kinetic nature, rather they rely upon mass-action constraints to mathematically represent the direction for metabolic modulation. Therefore, they are mostly suited for steady-state analysis of a biological system under a given perturbation. In FBA, metabolic fluxes are represented using stochiometry and assembled into matrices. This usually results in a greater number of metabolic fluxes than the number of mass balances, implying a plurality of feasible flux distributions. Objective functions in metabolic essence, for exxample optimal growth rate, are introduced and chosen to explore the best use of the metabolic network within a given metabolic genotype [33].

In genetic perturbations studies, such as knockouts or overexpressions, flux profiles are determined by the use of an optimizing function, the minimization of metabolic adjustment (MOMA) [34]. Stephanopoulos's group used MOMA as an additional constraint to study heterologous expression of

lycogene in *E. coli* using stoichiometric modelling [22,35]. They performed both single and multiple *in silico* gene knockouts to optimize the production of lycopene. Their simulation trends were subsequently verified through experiments [36].

Another theoretical method that has been used widely in modelling biochemical network but has not gained much popularity within the metabolic engineering community is the BST. BST is the original work of Savageau [37] and is aimed at addressing the characterization of integrated biological systems that cannot be represented to a large extent by linear systems. BST, hence, is a mathematical representation of nonlinear biological systems. The main essence is to consider reaction rates by general power-law expressions:

$$\frac{dX_i}{dt} = \alpha_i \prod_{j=1}^{n+m} X_j^{g_{ij}} - \beta_i \prod_{j=1}^{n+m} X_j^{h_{ij}} \tag{17.1}$$

where $X_1, \ldots, X_n$ are dependent variables (dynamic concentrations of internal metabolites), $X_{n+1}, \ldots, X_{n+m}$ are external variables (fixed concentrations of external metabolites), g_{ij}, h_{ij} are kinetic orders, which may be noninteger and nonpositive, and α_i, β_i are rate constants. In logarithmic coordinates, Equation 17.1 can be interpreted as a linearization of nonlinear kinetics, and as such BST claims to be a better approximation of reaction kinetics than linear expressions. BST suggests that all reactions that generate a metabolite X_j are combined into a single reaction with net v_i, and all reactions that consume the same metabolite similarly are combined into another reaction with net rate v_{-i}. The rate of each of these combined reactions is approximated by power-law expressions and from mass balances around all the metabolites, a system of differential equations can be written that can be studied in detail for its control characteristics [30].

17.2.2 Stochastic Spatiotemporal Dynamics

The methodologies discussed so far are only concerned with static or temporal molecular concentration variations, neglecting the fact that molecular concentration can also vary in space. *In vivo* systems often consist of well defined intracellular compartments such as mitochondrion, nucleus, and golgi apparatus. Intracellular molecules can be localized within these cellular structures through membrane anchoring or sequestration. Glucokinase, for example, is sequestered with glucokinase regulatory protein and predominantly remains in the nucleus of hepatocytes prior to glucose intake [38].

In addition, the molecular accessibility and mobility at different regions of the cellular environment is subjected to cytosol viscosity, dynamic subcellular structure and intracellular molecular crowding [39,40]. Such heterogeneity in temporal molecular distribution can highly influence the reaction kinetics of interacting molecules. In saponin-skinned cardiac fibres studies, for example, the K_m value for ADP to ATP conversion *in situ* mitochondria was found to be an order of magnitude higher than in isolated mitochondria because of *in vivo* diffusion limited reactions [41]. As such, to accurately determine the reaction kinetics of the molecular species that is known to participate with several intracellular compartments, spatial consideration to metabolic reaction modelling is an important future direction.

The observation that certain biological networks are inherently stochastic by nature has lead to the discovery of selective phenotypic switching behavior of cellular systems [42]. Stochastic effects are usually observed when molecules are present in low-copy numbers, for example mRNA levels. This condition is usually not true for many metabolic systems and hence this approach has often been neglected. However, we know that certain metabolites or enzymes within a metabolic framework can be in low concentrations, for example 1,3-BPG in glycolysis [43]. It will be interesting to observe *in silico* how such specific low concentration spots could affect the propagation of downstream metabolism if we consider stochasticity aspects.

There are several simulation approaches that consider both stochastic and spatiotemporal aspects for cellular systems. Takahashi et al. and Lemerle et al. provide comprehensive up-to-date reviews on these approaches [44,45]. More recently, Tolle and Le Novere argued that among the many approaches, particle based simulation with individual molecule resolution can best reproduce *in vivo* phenomena such as substrate channelling and colocalization of molecules [46,47].

In Section 17.3, we introduce the concept of space and noise in metabolic networks through theoretical examples using our newly developed particle based simulation approach with single molecule resolution [48]. We demonstrate that the simultaneous coupling of space and noise can significantly alter the phenotypic outcomes of metabolic pathways.

17.3 Simulation Tools: E-Cell for Metabolic Systems Engineering

As systems biology approaches become increasingly appreciated and adopted, there is a need for the development of systemic tools to perform theoretical analysis of biological processes. As most mathematical models developed to represent biological processes involve large number of reactions or interactions often involving highly nonlinear equations with multiple parameters, it daunting to solve them without proper computational tools. Furthermore, these computational tools must be available in a form appreciated by biologists, whom are usually not well versed in detailed programming, with ease of use and analysis. For example, model construction, parameter selection or estimation, simulation results comparison with experimental findings, model modifications etc. should be done with ease without the requirement to possess programming skills or to know the detailed background architecture of the computational tools. In this light, there have been numerous efforts across the globe to develop user friendly computational simulation platforms. As of today, there are more than 90 such tools freely available (http://sbml.org/index.psp) and among the many, one of the earliest and pioneering computational tool developed is the E-Cell simulation platform [49].

The E-Cell simulation system was induced in 1995 at our institute with the aim to perform simulation and analysis of an organism's entire metabolic reaction kinetics. This ongoing effort also incorporates several other methodologies, apart from reaction kinetics, such as MCA, FBA and S-systems, into one simulation platform (Table 17.1). As a consequence, the E-Cell system can be used to model several biological processes albeit metabolic pathways modelling, such as membrane transport, transcription, translation, DNA replication, signal transduction etc. [50].

We know that certain cellular process like metabolic reactions can be treated as deterministic processes while others like gene regulation networks are usually considered stochastic events [51,52]. Using modern simulation tools, like E-Cell, we can selectively use deterministic approaches to model protein interactions at the receptor and cytoplasm and stochastic processes in the nucleus for gene expression output for a more accurate representation of the entire signalling process.

The E-Cell platform also provides the user with freedom to combine and test various methodologies into one model simulation. For example, Nakayama et al. developed the hybrid dynamic and static simulation (HDSS) method to combine the simulation technique of kinetic with stoichiometric methods (Figure 17.3). They claim that such hybrid methods could optimize the benefits of both methods to yield faster and improved simulation results with lesser reliance on detailed kinetic parameters which are often difficult to obtain [53].

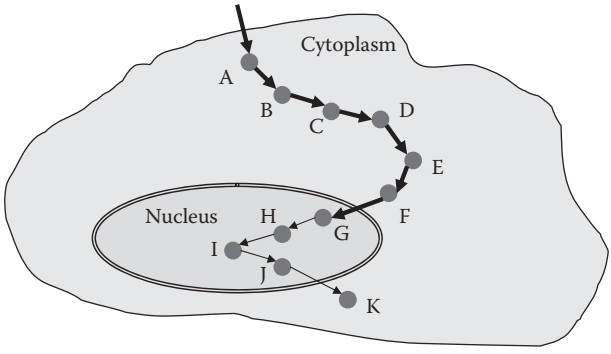
Apart from model creation and simulation, the E-Cell system also features intelligent built-in optimization processes such as genetic algorithm and genetic programming for the determination of system parameter values (e.g., rate constants) and the selection of system mechanism type (e.g., type of enzyme regulation), respectively when dynamic experimental information (e.g., temporal metabolite concentration profiles) are available [54,55].

In the next section, using E-Cell, we show the development of simple models, to demonstrate the utility of dynamic computational models that can be used to interpret biological network properties.

TABLE 17.1 Core Features of E-Cell System

Type of Feature	Description
Modelling capabilities	Stochastic and deterministic events
Model types	Enzyme kinetics Metabolic control analysis Flux balance analysis Biochemical systems theory (S-systems) Spatial simulation* Hybrid dynamic/static simulation*
Algorithms	Gillespie-Gibson (stochastic) Explicit/implicit Tau-leap (stochastic) Langevin method (stochastic) Radau5/Dormand-Prince adaptive Dormand-Prince 4(5)7M explicit Fehlberg 2(3) explicit Euler explicit Radau5 implicit
Model optimization schemes	Genetic algorithm Genetic programming*
Computing optimization schemes (parallel computing)	Distributed computing
User interface	Real time user intervention and visualization Python scripting for automation of simulation Graphical Model Editing
Platform	Linux and Microsoft Windows XP
Source code	Object Oriented C++/Python with GPL license
File types	SBML and EML

*Under implementation.



Thick lines – kinetic analysis (cytoplasm)
Thin lines – flux balance analysis (nucleus)

FIGURE 17.3 Combining multiple simulation algorithms is the idea behind hybrid dynamic/static simulation (HDSS) process. Metabolic fluxes between A to K can be simulated using two methods, kinetic reaction analysis for metabolites A to G and stoichiometric flux determination for metabolites G to K. This method calculates the metabolic fluxes for all reactions without the necessity to obtain all kinetic parameters. (From Yugi, K., Nakayama, Y., Kinoshita, A., and Tomita, M., *Theor. Biol. Med. Model.,* 2, 42, 2005. With permission.)

17.4 Dynamic *in Silico* Simulation

17.4.1 Theoretical Illustration

In this section, we introduce basic ideas to illustrate the usefulness of studying dynamic models to understand regulatory behavior of biological networks. The intention is to show how analyzing system dynamics may change the way we view or understand biological phenotype. For example, how do we understand feedback regulation controlling the flux distribution of a metabolic system? To illustrate, let us consider a simple theoretical metabolic pathway system, consisting of five metabolites, with a negative feedback mechanism. Figure 17.4a shows that increasing concentration of metabolite D negatively controls the flux through metabolite C.

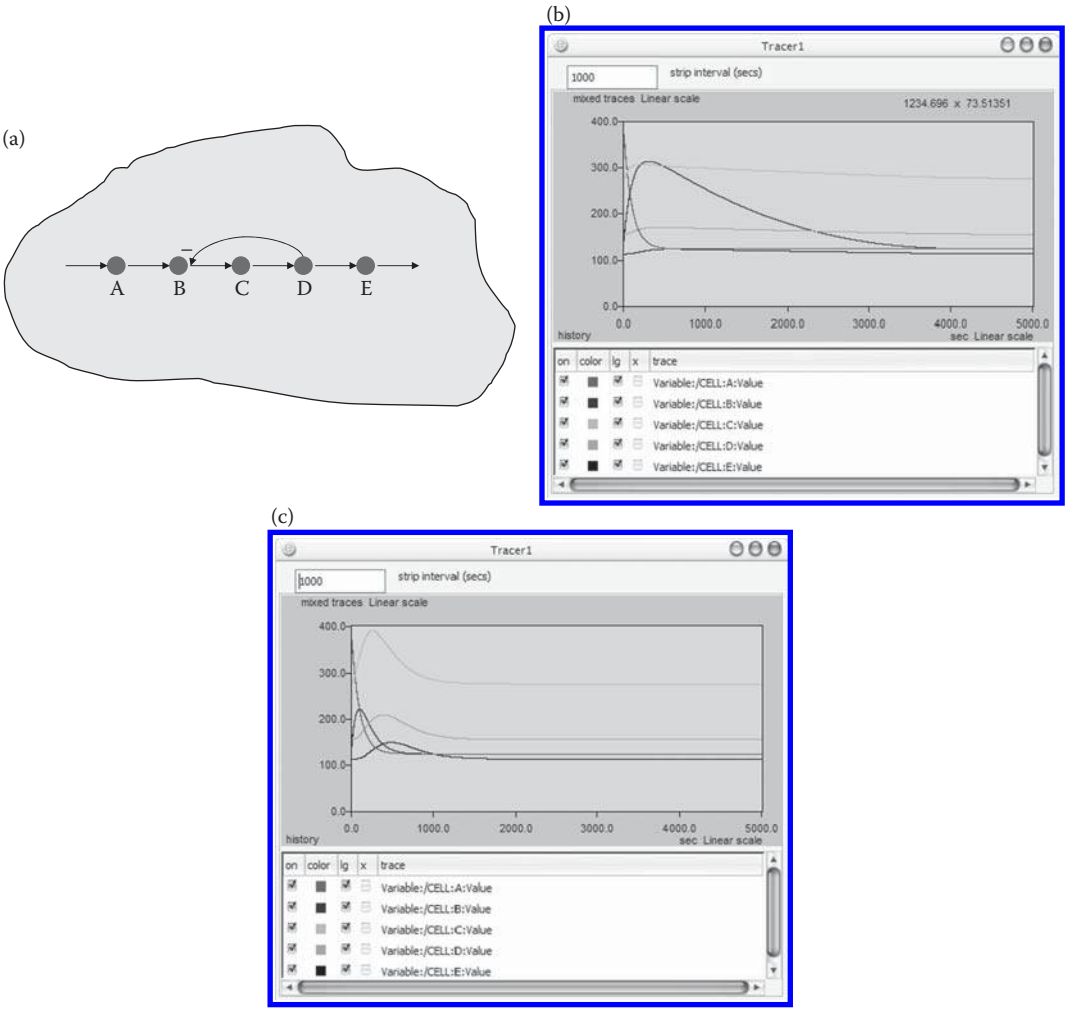


FIGURE 17.4 **(See color insert following page 13-20.)** (a) A simple schematic of negative feedback system in metabolic pathways. (b) The temporal simulation profile of metabolite concentrations of a hypothetical system as depicted in Figure 17.4 and Table 17.2a. (c) The temporal simulation profile of metabolite concentrations of a hypothetical system as depicted in Figure 17.4 and Table 17.2b (without negative feedback mechanism). In (b) and (c) all metabolites are initially at steady-state levels and at $t = 0$ s, the concentration of A is increased instantaneously (perturbed) by 266 molecules or 0.44 mM. The x-axis represents time in seconds and the y-axis represents the number of metabolites. (Using an assumed volume of 1e–18 l, we could covert the y-axis to metabolite concentration, if necessary.) All simulations were carried out using the E-Cell system version 3.

TABLE 17.2 Initial Concentration, Transient Concentration, Kinetic Reaction Formulae and the Parameter Values: (a) for Linear Pathway with Feedback Mechanism; (b) without Feedback Mechanism, as Depicted in Figure 17.4

Metabolite, S	S_0 Steady-State Conc. (mM)	S_1 Kinetic Formulae	Parameter Values (1/s)	S Trans. Conc. $t=253$ s (mM)
(a)				
A	0.21	$\frac{dE_1}{dt} = -k_5E_1 + k_4D_1$	$k_1=0.01$	0.24
		$\frac{dA_1}{dt} = -k_1A_1$		
B	0.21	$\frac{dB_1}{dt} = \frac{-k_2B_1}{q(D-D_o+1)} + k_1A_1$	$k_2=0.01$, $q=1$ (1/mM)	0.52
C	0.46	$\frac{dC_1}{dt} = -k_3C_1 + \frac{k_2B_1}{q(D-D_o+1)}$	$k_3=0.0045$	0.51
D	0.26	$\frac{dD_1}{dt} = -k_4D_1 + k_3C_1$	$k_4=0.008$	0.28
E	0.19	$\frac{dE_1}{dt} = -k_5E_1 + k_4D_1$	$k_5=0.011$	0.20
(b)				
A	0.21	$\frac{dA_1}{dt} = -k_1A_1$	$k_1=0.01$	0.24
B	0.21	$\frac{dB_1}{dt} = -k_2B_1 + k_1A_1$	$k_2=0.01$	0.30
C	0.46	$\frac{dC_1}{dt} = -k_3C_1 + k_2B_1$	$k_3=0.0045$	0.65
D	0.26	$\frac{dD_1}{dt} = -k_4D_1 + k_3C_1$	$k_4=0.008$	0.33
E	0.19		$k_5=0.011$	0.22

The model parameters, k values, were selected so that the metabolites reach designated (hypothetical) steady-state levels with a constant source of 0.0023 mM/s given to metabolite A. Once the steady-state levels were reached, we reset the simulation time to zero and pulse the metabolite A by 266 molecules. So, a typical metabolite, S, is represented by: $S = S_0 + \int_0^\infty (dS_1/dt)\,dt$.

We performed two types of simulations with this system, one in which the feedback regulation is "switched on" and the other with the feedback regulation "switched off". Table 17.2 shows the simulation details such as kinetic formula, parameter values and end simulation result for the various metabolites shown in Figure 17.4a. (The actual theoretical models can be downloaded from http://e-cell.org/community/models.). When we compare the simulations between the two cases, we notice that transiently the flux through metabolite C to E is reduced due to the negative feedback mechanism. However, eventually at larger simulation time, the differences between the two cases for all metabolites ceases (Figure 17.4b and c). That is to say, the steady-state levels are similar in the presence or absence of negative feedback regulation in such a metabolic pathway. This simple analysis of metabolic phenotype suggests that steady-state condition alone is insufficient for the discovery of novel regulatory network features of metabolism.

We next extend this illustration to include a slightly modified scenario that results in profound difference between the steady-state levels in the presence or absence of negative feedback mechanism. Figure 17.5a includes an additional reaction for metabolite B, which converts it to metabolite F. In this renewed scenario, the flux through metabolite C to E is noticeably reduced under negative feedback control (Figure 17.5b and c and Table 17.3). *In silico* dynamic models can, therefore, allow us to predict

metabolic network behavior under various types of conditions. Such models should be increasingly used as part of metabolic engineering design.

17.4.2 Stochastic Spatiotemporal Simulations

The issue of biochemical movement within cells, especially intercompartmental exchanges, could be an important aspect of biological or metabolic regulation that is often left out due to lack of availability of experimental information or theoretical expertise. One such example is the translocation of pyruvate, in mammalian cells, from cytoplasm to mitochondrion.

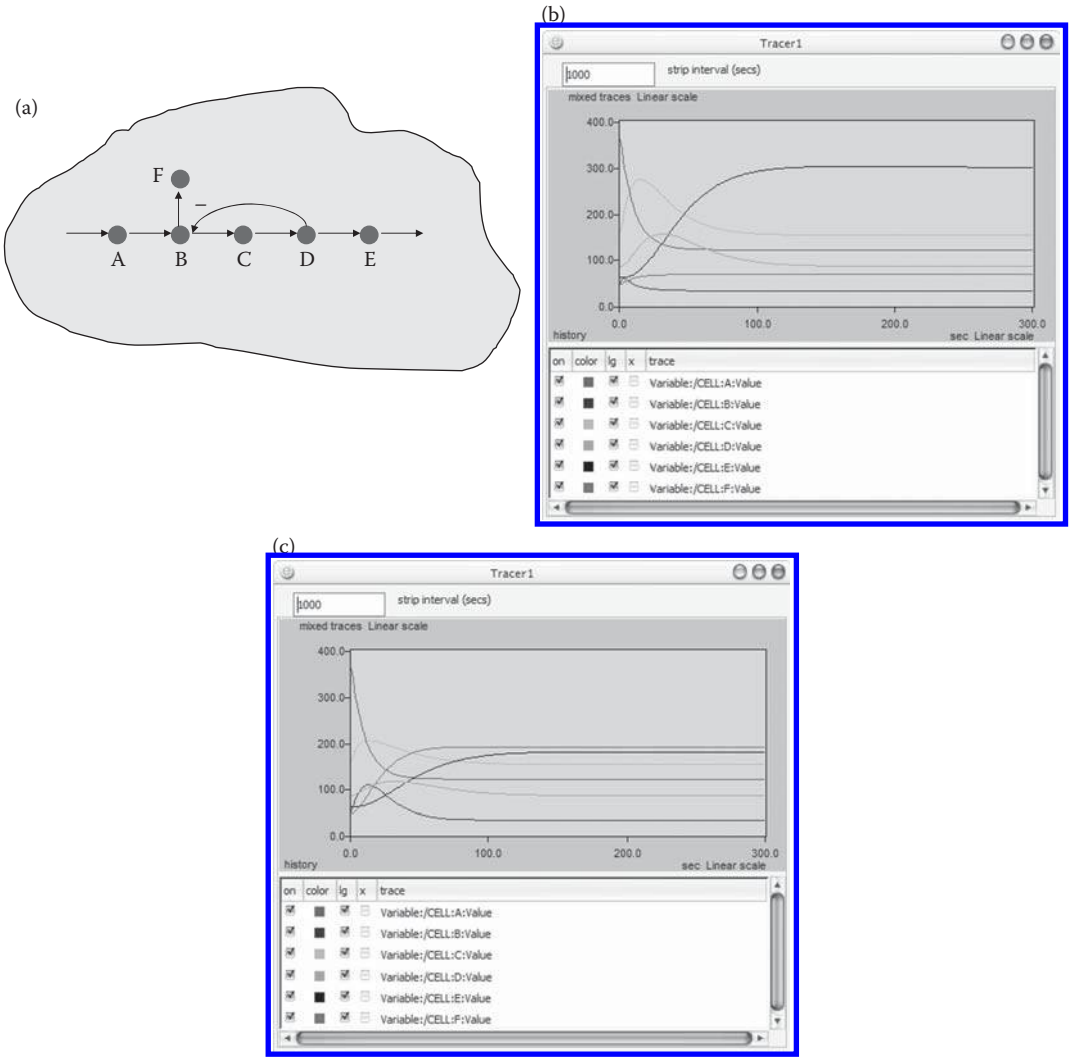


FIGURE 17.5 **(See color insert following page 13-20.)** (a) Metabolite B having an additional reaction that converts it to metabolite F. The temporal simulation profile of metabolite concentrations, (b) without and (c) with negative feedback mechanism, a hypothetical system depicted in Figure 17.6 and Table 17.3. Initially all metabolites remain at steady-state condition and at $t=0$ s, the concentration of A is increased instantaneously (perturbed) by 266 molecules or 0.44 mM (volume of cell is assumed to be 1e−18 l). The x-axis represents time in seconds and the y-axis represents the number of metabolites. All simulations were carried out using the E-Cell system version 3.

TABLE 17.3 Initial Concentration, Final Concentration, Kinetic Reaction Formulae and the Parameter Values: (a) for Branching Pathway with Feedback Mechanism and (b) without the Feedback Mechanism, as Depicted in Figure 17.6

Metabo-lite, S	S_0 Steady-State Conc. (mM)	S_1 Kinetic Formulae	Parameter Values (1/s)	S Quasi-Steady-State Conc. $t=300$ s (mM)
(a)				
A	0.65	$\frac{dA_1}{dt} = -k_1 A_1$	$k_1 = 0.1$	0.21
B	0.06	$\frac{dB_1}{dt} = \frac{-k_2 B_1}{q(D-D_o+1)} - k_6 B_1 + k_1 A_1$	$k_2 = 0.7$, $k_6 = 0.06$, $q = 1$ (1/mM)	0.06
C	0.26	$\frac{dC_1}{dt} = -k_3 C_1 + \frac{k_2 B_1}{q(D-D_o+1)}$	$k_3 = 0.05$	0.26
D	0.14	$\frac{dD_1}{dt} = -k_4 D_1 + k_3 C_1$	$k_4 = 0.06$	0.14
E	0.11	$\frac{dE_1}{dt} = -k_5 E_1 + k_4 D_1$	$k_5 = 0.0001$	0.30
F	0.08	$\frac{dF_1}{dt} = k_6 B_1$		0.32
(b)				
A	0.65	$\frac{dA_1}{dt} = -k_1 A_1$	$k_1 = 0.1$	0.21
B	0.06	$\frac{dB_1}{dt} = -k_2 B_1 - k_6 B_1 + k_1 A_1$	$k_2 = 0.7$, $k_6 = 0.06$	0.06
C	0.26	$\frac{dC_1}{dt} = -k_3 C_1 + k_2 B_1$	$k_3 = 0.05$	0.26
D	0.14	$\frac{dD_1}{dt} = -k_4 D_1 + k_3 C_1$	$k_4 = 0.06$	0.14
E	0.11	$\frac{dE_1}{dt} = -k_5 E_1 + k_4 D_1$	$k_5 = 0.0001$	0.50
F	0.08	$\frac{dF_1}{dt} = k_6 B_1$		0.12

A typical metabolite, S, is represented by: $S = S_0 + \int_0^\infty (dS_1/dt)\, dt$.

To demonstrate computationally how intercompartmental diffusion and localization of biochemical molecules can affect the overall reaction kinetics of a metabolic network, we developed two three-dimensional *in silico* models of a single cell consisting of a few reactions. In the first model, the cell constitute of only a single compartment, the cytoplasm and all the metabolites are free to diffuse and react anywhere within this compartment (Figure 17.6a). When the metabolite A is pulse perturbed, the concentration of metabolites C and D reached steady state levels of 0.74 mM and 0.75 mM respectively, at around $t=750$ s (Figure 17.6c). The details of the model are shown in Table 17.4. In the second model, we introduced another compartment, like the mitochondrion, and localized one of the reaction's enzyme, E2, within this compartment (Figure 17.6b). This means that the enzyme E2 exclusively reside only in mitochondrion and cannot travel outside the compartment. Under this renewed situation, with metabolite A perturbed in the same way, the steady-state levels of C and D reached, 0.64 mM and 0.83 mM, respectively and the time to reach steady-state is $t=1500$ s (Figure 17.6d and e). These simulations reveal, even for a simple situation, considering spatial effects produce significant changes in the time to reach steady-state conditions. In addition, the steady-state levels for C and D also differ perceptibly. Theoretically, the delay and changes in the steady-state levels are caused by (i) the intercompartmental diffusion of metabolites

and due to the absence of enzyme E1 and E3 in the mitochondrion compartment and (ii) enzyme E2 being located in the mitochondrion only; metabolite B, which diffuses through Brownian motion, is unable to be catabolized by E2 as frequently as it could under the noncompartmental situation.

Our example, even though minimal, demonstrates the utility of spatiotemporal effects when incorporated with *in silico* models representing multiple reactions across multiple intracellular compartments. (Similar results can also be shown for noncompartmental localization of molecules at different regions of the cell.) Though the present usage spatial simulation is limited due to the general lack of quantitative experimental data, the advent of fluorescence correlation spectroscopy, immunoelectron microscopy and other related technologies may change the situation in the future [56,57].

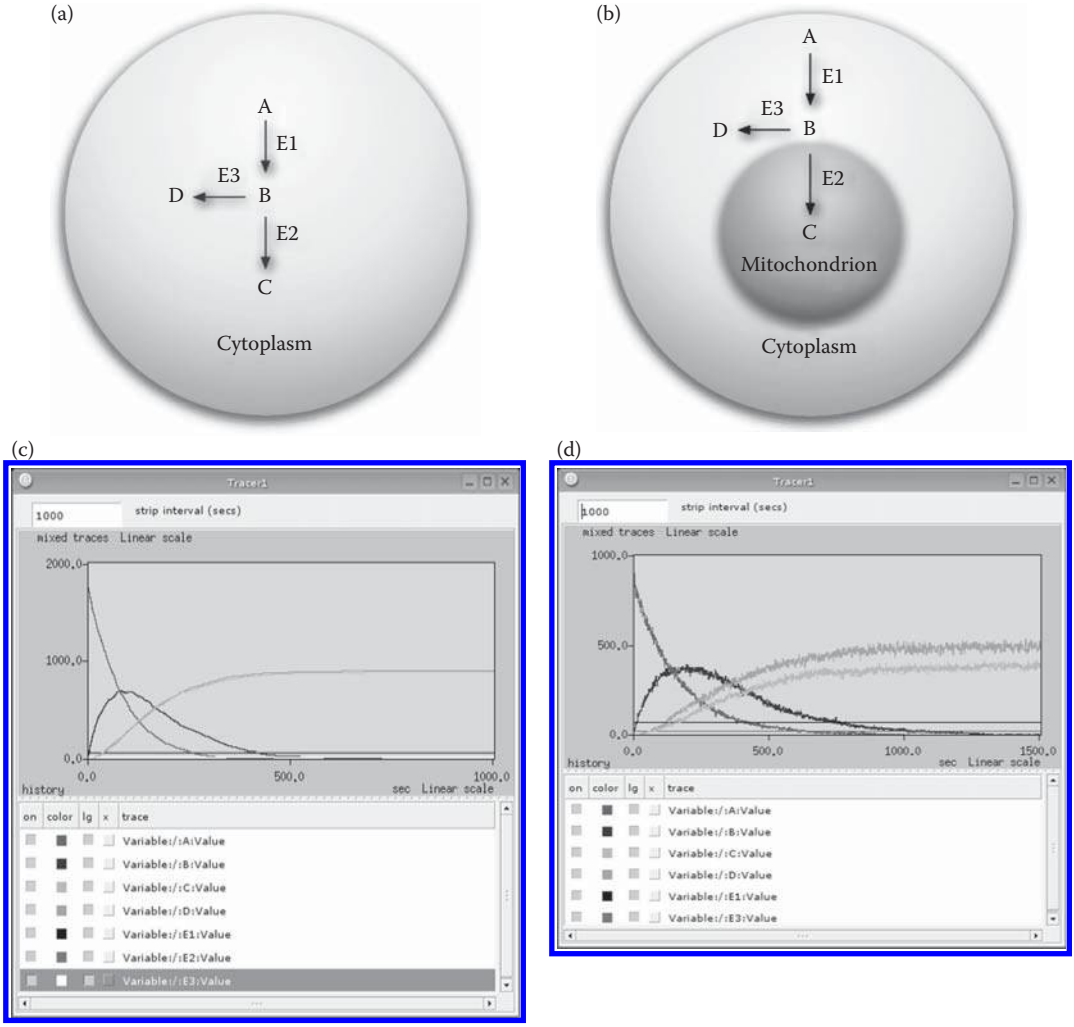


FIGURE 17.6 A schematic of four reactant metabolic pathway developed using spatial simulation algorithm (Box 17.1). (a) A hypothetical cell with only one compartment, the cytoplasm. (b) A hypothetical cell with two compartments, cytoplasm, and mitochondrion. Reactions A to B to D takes place within the cytoplasm and reactions B to C occurs in the mitochondrion. (c) The dynamic *in silico* simulations of the various reactants concentration in the cytoplasm for model represented in (a). (d) The dynamic *in silico* simulations of the various reactants concentration in the cytoplasm, (e) in the mitochondrion and (f) both combined (overall), obtained using model represented in (b). The x-axis represents time in seconds and the y-axis represents the number of metabolites. The volume of cell used is 2e−18 liter. All simulations were carried out in E-Cell system version 3.

(e) (f)

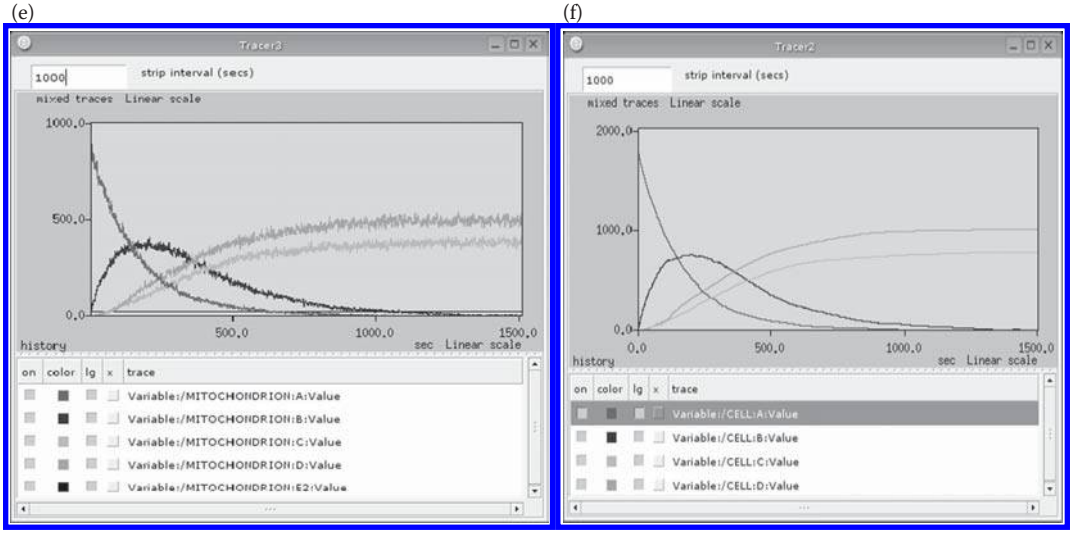


FIGURE 17.6 (continued)

Box 17.1 Spatiotemporal Stochastic Simulation Algorithm

We developed a novel spatial simulation algorithm, which describes the three-dimensional cell space and components such as intracellular compartments, and performed our simulations using Monte–Carlo technique [48]. The three-dimensional space, corresponding to the simulated cell volume, is discretized into a lattice of spheres arranged in hexagonal close-packing (Figure 17.B1). A molecule can occupy a single sphere in the lattice and diffuse based on its diffusion probability to one of its 12 adjacent spheres in a time step (Figure 17.B2). The selection of a destination sphere out of the 12 adjacent spheres is performed randomly. After a large number of time steps, the diffusion of each molecule converges into a Brownian motion. The time step interval is determined from the diameter of the sphere, i.e., the molecule's displacement in a time step. The diameter of sphere on the other hand, is determined from the diffusion coefficient of the fastest moving molecular species, such that the diffusion probability is unity in a time step. The diffusion probability of other slower species is computed from its diffusioncoefficient and the time step interval.

During diffusion, if the destination sphere is occupied by another molecule and the molecule is its reaction partner, both molecules can form a complex probabilistically based on their reaction rate (Figure 17.B3). If the molecule in the destination sphere is not a reaction partner, the molecule stays at its currently occupied sphere. On the other hand, if the molecule is a complex, it can also dissociate based on its dissociation probability into two separate molecules, with one occupying the currently occupied sphere and the other, occupying one of the free neighboring spheres that is randomly selected (Figure 17.B4).

Box 17.1 (continued)

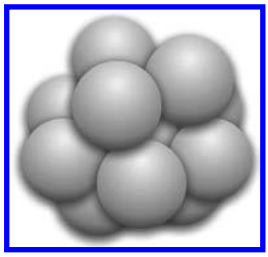


FIGURE 17.B1

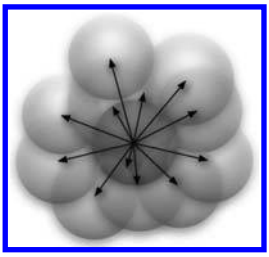


FIGURE 17.B2

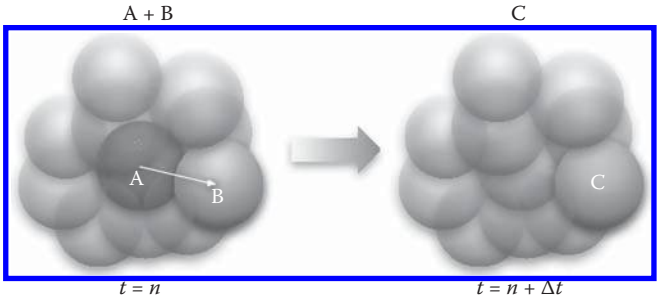


FIGURE 17.B3

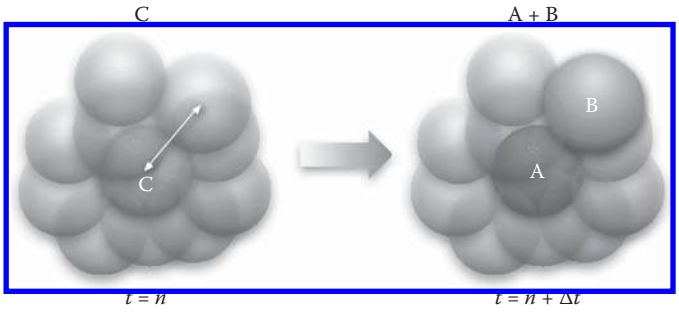


FIGURE 17.B4

TABLE 17.4 Model Details for *in Silico* Cell with (a) One Compartment, Cytoplasm (b) with Two Compartments, Cytoplasm and Mitochondrion; (c) Represents the System Parameters and Reactions

(a) Cytoplasm Compartment (Total Vol.: 2.0e–18 l)

Metabolite/ Enzyme	Initial Value (t=0 s) mM	Steady-State value (t=750 s) mM
A	1.49	0
B	0	0
C	0	0.74
D	0	0.75
E1	0.06	0.06
E2	0.03	0.03
E3	0.03	0.03

(*continued*)

TABLE 17.4 Model Details for *in Silico* Cell with (a) One Compartment, Cytoplasm (b) with Two Compartments, Cytoplasm and Mitochondrion; (c) Represents the System Parameters and Reactions (Continued)

(b) Cytoplasm Compartment (Total Vol.: 1.85e–18 l)

Metabolite/Enzyme	Initial Value (t=0 s) mM	Steady-State Value (t=1500 s) mM
A	1.34	0
B	0	0
C	0	0.33
D	0	0.42
E1	0.06	0.06
E2	0.03	0.03
E3	0	0
Mitochondrion compartment (Total Vol.: 1.5e–19 l)		
A	0.15	0
B	0	0
C	0	0.31
D	0	0.41
E1	0	0
E2	0	0
E3	0.03	0.03

(c)

System Parameters (Reaction Probability)	System Reactions
$p1$=0.004	A+E1→B+E1
$p2$=0.006	B+E2→C+E2
$p3$=0.006	B+E3→D+E3

17.5 Practical Applications

17.5.1 Budding Yeast Metabolism

Metabolomics is an emerging science that aims at temporal quantification of metabolites in cellular system [58]. Although the field still faces many challenges to produce accurate high-throughput metabolic profiling, there have been recent successes when considering smaller network quantification. For example, Theobald et al. and Visser et al. have temporally quantified the primary energy metabolites and adenine nucleotides of *Saccharomyces cerevisiae* in pulse perturbed experiments [59,60]. The generation of such *in vivo* "snap shot" of metabolism is indispensable as it allows one to check *in silico* predictions over a period of time rather than just comparing at steady-state conditions.

In this section, we discuss an example of how dynamic models can be used to decipher key regulatory steps of metabolic pathways. Figure 17.7a through c, adapted from Theobald et al. shows a section of glycolytic phenotype of *Saccharomyces cerevisiae* [59]. By simple visual inspection or static analysis, we are unable to understand the mechanism underlying the dynamic changes in the glycolytic phenotype. For example, in *Saccharomyces cerevisiae* glycolysis we expect glucose pulse to be predominantly metabolized into pyruvate, lactate, ethanol, and glycerol (end products). However, this prediction using stoichiometry does not allow us to comprehend the results shown in Figure 17.7b and c. We would not expect, for instance, phosphoenol pyruvate (PEP) levels to reduce after glucose pulse experiments. Also, by using stoichiometry, we would expect glycerol levels to rise significantly for the amount of glucose pulse given (Figure 17.7a).

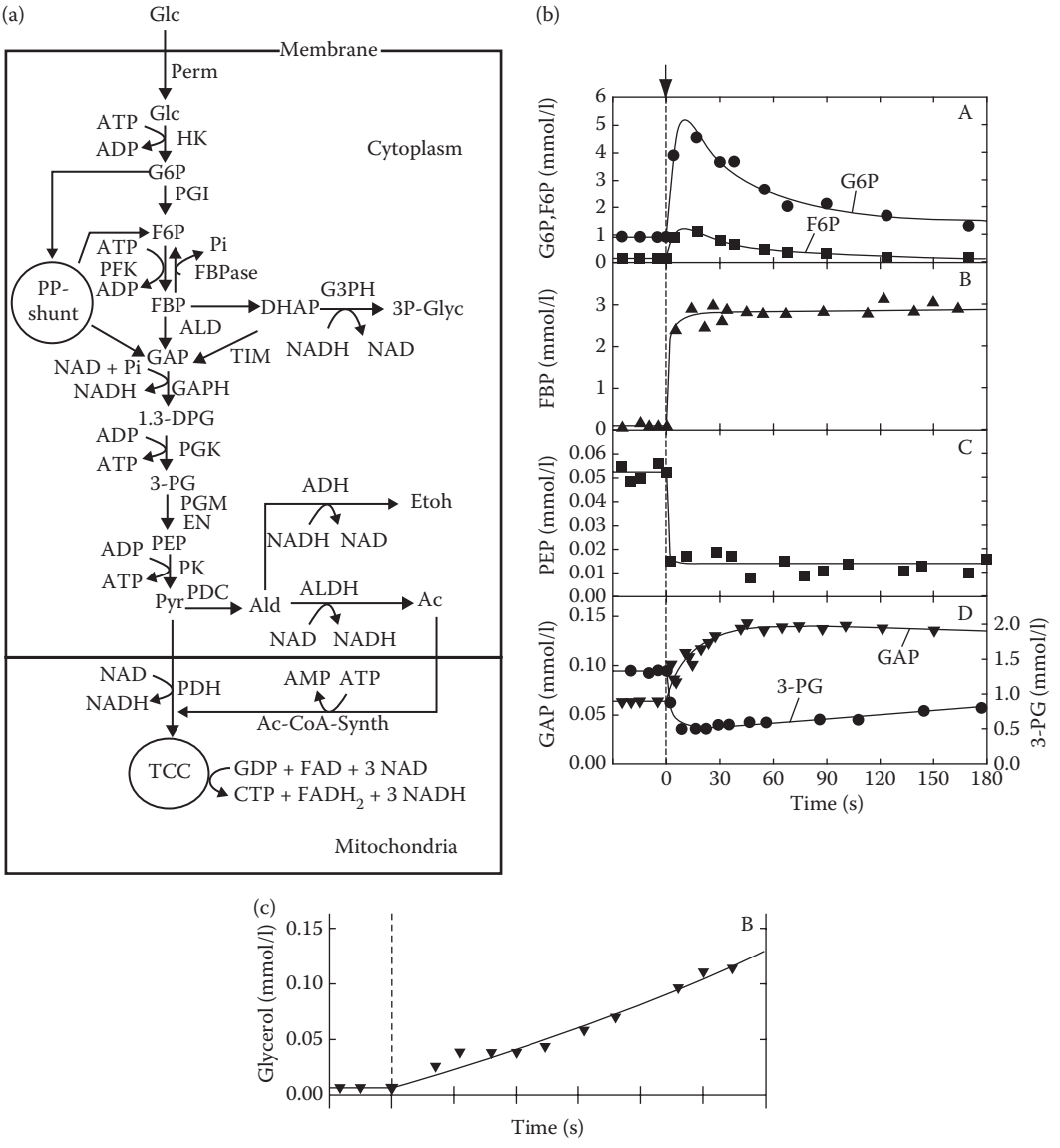


FIGURE 17.7 (a) The glycolytic pathway of *Saccharomyces cerevisiae*. (Adapted from Theobald, U. et al *Biotechnol Bioeng.,* 55, 305, 1997. Reprinted with permission of John Wiley & Sons, Inc.) (b) The temporal changes in the levels of glucose-6-phosphate (G6P), fructose-6-phosphate (F6P) (A), fructose 1,6-bisphosphate (FBP) (B), PEP (C), Glyceraldehyde phosphate (GAP)/3-phosphoglycerate (3PG) (D) from steady-state conditions after a glucose pulse perturbation. (c) The changes in the levels of glycerol from steady-state conditions after a glucose pulse perturbation. We represent GAP as G3P throughout the text. (a) and (b) have the same time scale.

The certain answer to these misnomers is that we do not yet understand the glycolytic pathway regulations well enough to use it for industrial optimization. There still exist novel regulatory features that require elucidation. The best way that we could approach the problem is to analyze the dynamic glycolytic phenotype of the *Saccharomyces cerevisiae* using systemic *in silico* methods.

Recently, Selvarajoo and Tsuchiya analyzed the result shown in Figure 17.7 using a novel dynamic network analysis method [61]. They suggest an unassuming location of glycolysis, the reaction catalyzed

by aldolase, may have become saturated and thus causes the lower than expected production of glycerol. To verify this result, they performed an additional test using traditional mass-action kinetic analysis with pulse perturbation and obtained similar result (Figure 17.8). Although their prediction that aldolase might be a novel key regulator of glycolysis has not been validated with subsequent wet experiments, there is the hope that *in silico* models could be utilized to decipher previously undiscovered key regulatory steps which may turn up to benefit metabolic engineering field, such as targeting the suggested novel steps for increased/decreased production of substances of interest/concern.

17.5.2 Innate Immune Signaling

In innate immunity, the Toll-like receptors (TLRs) play a central role in combating invading pathogens by the induction of proinflammatory chemokines and cytokines [62]. The activation of TLR receptors are self-limiting but in certain cases, aberration of the signalling mechanism leads to

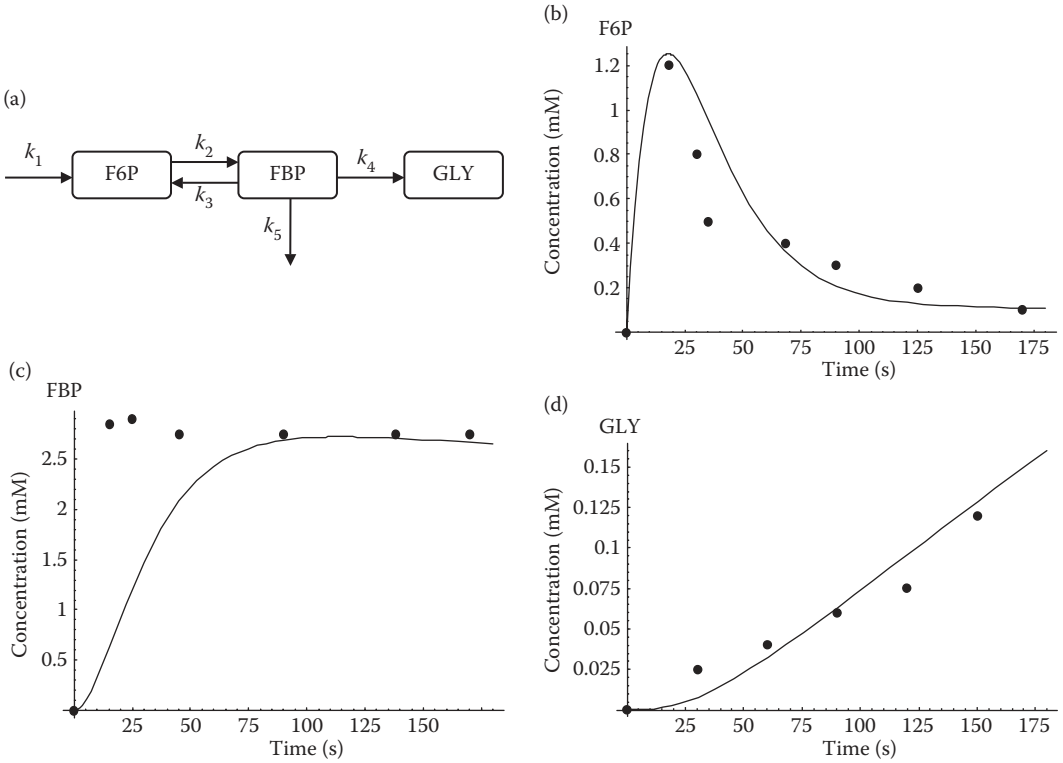


FIGURE 17.8 Mass-action kinetics analysis for local network involving fructose-6-phosphate (F6P), fructose 1,6-bisphosphate (FBP) and glycerol (GLY). A unit pulse perturbation is given to F6P. The aim was to determine the various rate constants of the model, by making a close fit to the experimental data, and then comparing their values to infer the presence or absence of any rate-limiting phenomenon. In order to fit the model to experimental data, the value of k_2 has to be much larger than that of k_4 and k_5 (k_2=0.05, k_4=0.0004, k_5=0.0002). This mathematically indicates that the reaction aft of FBP causes bottleneck, which suggests that the enzyme responsible, aldolase, is a key regulatory enzyme for glucose pulse experiments. (Solid lines indicate simulation result and dotted points indicate experimental result obtained.) (From Selvarajoo, K. and Tsuchiya, M. Systematic determination of biological network topology: Non-integral connectivity method (NICM). Humana Press, Totowa, New Jersey, 449–471, 2007; Tolle, D. and Le Novère, N. *Curr. Bioinform.,* 1, 315, 2006; Theobald, U., Mailinger, W., Baltes, M., Rizzi, M., and Reuss, M. *Biotechnol. Bioeng.,* 55, 305, 1997. With permission.)

inflammation which eventually results in chronic diseases such as asthma, rheumatoid arthritis, multiple sclerosis etc. Although understanding proinflammatory signalling is key in resolving inflammation, there is little known about the regulatory role of the various intracellular signalling molecules. One example is IκB kinase (IKK) α. Recently, Lawrence et al. implicated that IKKα limits the activation of NF-κB in macrophages and therefore could be one of the candidate target for downregulating inflammation [63].

Lawrence et al. performed various time-course experiments. Although they report very interesting and fascinating results, they did not perform systemic analysis that may have potentially influenced their final conclusion. For example they relatively quantified mRNA levels of several chemokines and cytokines at various time points for both wild type and IKKα mutant macrophages under lipopolysaccharide (LPS) stimlus (Figure 4a of Ref. [63]). Many of the mRNA levels show distinct features. For example, Bfl-1, A20, GADD45β have similar response profiles while KC and MIP-2 have similar response profiles. By grouping similar mRNA response profiles together and using systemic approaches, we could possibly determine the connectivity of genes, or gene networks which may eventually help in the better understanding of signalling network [64].

To understand TLR4 signaling mechanism in a more systemic manner we developed a computational model of TLR4 signaling pathway using a deterministic approach [65,66]. The *in silico* model was designed to simulate, for both wild type and myeloid differentiation primary-response protein 88 (MyD88) knockout macrophages, the expression of all known protein interactions in the cytoplasm and the mRNA levels of two genes encoding the proteins, IP-10 and TNF-α [65]. We proposed, through our systemic model, that the kink observed for the temporal phenotype of TNF-α mRNA in the IKKα wildtype may not be an experimental artefact, but rather it displays the behavior of the TLR4 signaling network; the kink is a consequence of superposition of two signals, one coming from the MyD88-dependent pathway and the other delayed signalling from the MyD88-independent pathway (Figure 17.9).

We subsequently also performed an *in silico* IKKα mutant simulation which resulted in increased mRNA expression of TNF-α in accordance with experimental findings (data not shown). However, in contrast to the concept that IKKα is a negative regulator of NF-κB, we observed IKKα mutant causes a bottleneck at the signaling upstream of IKK complex thus resulting in more flux through the alternative pathway of JNK and p38 which then results in increased expression of TNF-α. Although this prediction is preliminary and requires further investigations, this alternative explanation to the role of IKKα brings us to consider systemic approaches that could elucidate nonintuitive behavior of biological networks. The resultant in silico hypothesis should then be complemented with wet-bench reality.

17.6 Future Prospects

Although metabolic engineering field is constantly advancing with the introduction of modern combinatorial tools that explores cellular behavior, the quantitative optimization of biochemical products has yet to take huge strides forward. This is partly due to the fact that our knowledge of biological network behavior is still very limited. One way to overcome this difficulty is to use the knowledge gained from studying network architectures found in nonbiological fields and apply such insights to biological interactions. Barabasi et al. studied the "wiring" of world-wide-web and social networks and found that complex networks are often designed in a scale-free manner [5,6]. The protein interaction network of *Saccharomyces cerevisiae* was later shown to possess similar characteristics [67]. Such observations, which suggests that certain (dominant) elements in a network are much more highly connected than others, could pave way for the discovery of key "hubs" of biological (or metabolic) network. The "hubs" could then be targeted, say, by drugs for eliminating key disease progression. Therefore, uncovering the design principle of biological network construction is very important.

Another challenge lies with wet-bench research. Even though today, we are presented with a deluge of biological information gathered from high throughput experimental sources such as microarray and

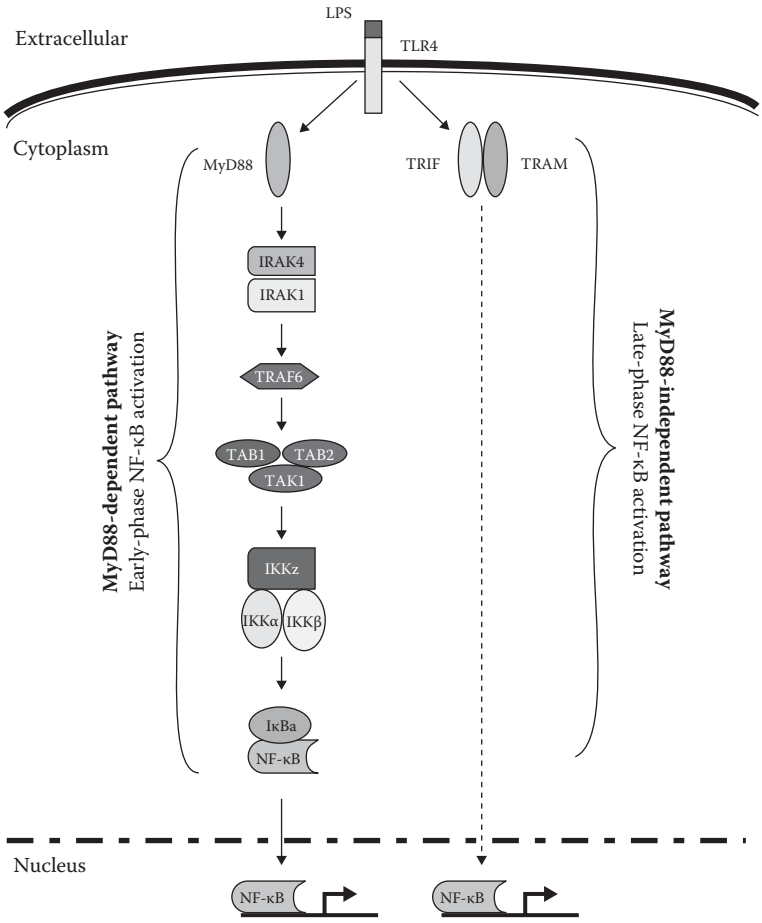


FIGURE 17.9 Schematic of TLR4 signaling pathways. Upon LPS binding to TLR4, NF-κB is activated through two pathways, the MyD88-dependent and MyD88-independent pathways. The MyD88-dependent pathway consist of MyD88, IRAK1 and 4, TRAF6, TAB/TAK and IKK complexes. The MyD88-independent pathway is less understood, hence, at this stage that TRIF activates NF-κB is the only universally accepted mechanism. (From Miggin, S.M. and O'Neill, L.A., *J. Leukoc. Biol.,* 80, 226, 2006. With permission.)

mass spectrometry, the raw data generated are usually not in a form that could easily be used for *in silico* model analysis. Often there are issues to remove experimental artefact such as noise (especially, for low concentration species) and accurate deconvolution of spectra peaks (mass spectrometry). In addition, the reproducibility of high throughput data is also a major challenge. Nevertheless, the quantitation of metabolic phenotype is gradually improving. The slow but steady progress of systems biology will eventually result not only in the advancement of metabolic engineering but also revolutionize the industrial bioprocess output.

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