Stochastic Effects in Signaling Pathways in Cells: Interaction between Visualization and Modeling

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Abstract. We review a research program under way at Rice University in Houston, TX and University of Texas Medical Branch in Galveston, TX, in the United States and at the Systems Engineering Group at the Silesian University of Technology in Gliwice in Poland. Individual biological cells display stochastic variability in their responses to activating stimuli. This variability can be measured using recent molecular biology techniques, which prove that in many respects no cell in the population behaves like an average cell. In cells taking part in the innate immune response this variability seems very important. In prokaryotes, which are small, importance of stochastic effects at all levels of the transcription/translation process was recognized early on. Eukaryotic cells are much larger and also have more complex mechanisms of transcription initiation. Since stochastic effects arise mainly through interactions of a limited number of discrete entities (such as transcription factors, promoter binding sites, receptors and so forth), it is to be expected that in eukaryotic cells these effects will be mainly due to transcription initiation and to signaling mediated by small numbers of active molecules (such as recognition of foreign antigens by T lymphocytes. We present the biological system which is the subject of our research, as well as an outline of mathematical and computational methods which we use to analyze it. Visualization and modeling are two major elements of this approach.

Keywords: stochastic process, robustness, gene transcription and control, modeling, eukaryotic cells.

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1 Introduction

This paper reviews a research program under way at Rice University in Houston, TX and University of Texas Medical Branch in Galveston, TX, in the United States and at the Systems Engineering Group at the Silesian University of Technology in Gliwice in Poland. The description concerns past and current as well as future research.

Individual biological cells display stochastic variability in their responses to activating stimuli. This variability can be measured using recent molecular biology techniques, which prove that in many respects no cell in the population behaves like an average cell. In cells taking part in the innate immune response this variability seems very important. *It confers a kind of Stochastic Robustness : Subpopulations of cells may react differently to the same stimulus, but all of them react in a well-defined way.* There is a growing interest in stochastic effects in the dynamics of gene transcription and signal transduction in eukaryotic cells [4]. In prokaryotes, which are small, importance of stochastic effects at all levels of the transcription/translation process was recognized early on [1]. Eukaryotic cells are much larger and also have more complex mechanisms of transcription initiation [36]. Since stochastic effects arise mainly through interactions of a limited number of discrete entities (such as transcription factors, promoter binding sites, receptors and so forth), it is to be expected that in eukaryotic cells these effects will be mainly due to transcription initiation and to signaling mediated by small numbers of active molecules (such as recognition of foreign antigens by T lymphocytes, [10]). As a result, variability will arise in cells in the population, which has a dynamic nature, as demonstrated by the experiments of Nelson et al. [25], Sigal et al. [31] and Geva-Zatorsky et al. [13], and which results in a possibly stable and mixing stochastic process [31]. A number of researchers posed questions concerning the importance of stochastic effects for functioning of cell populations and organisms. As an example, Blake et al. [2] considered transcriptional noise in yeast concluding, based on a mathematical model, that there exists a noise level, which optimizes transcriptional efficiency. Raser and O'Shea [29] conclude that noise (variability) among cells has multiple sources, including the stochastic or inherently random nature of the biochemical reactions of gene expression; they also comment on recent investigations into the sources, consequences, and control of noise in gene expression.

Mathematical modeling of stochastic effects in gene transcription and control has a tradition reaching at least as long as Peccoud and Ycart [27], Arkin et al. [1], and Kierzek et al. [19], all using mainly Gillespie's [15] discrete stochastic simulation algorithm. In eukaryotes, the large numbers of mRNA and protein molecules (in excess of 10^2 and 10^4, respectively), require that the system be partly continuous. The breakthrough innovation seems to belong to Kepler and Elston [18]. In the context of NFκB module, Lipniacki et al. [22, 21] introduced their 'continuous' model, essentially a version of a stochastic differential equation (SDE) system. These models explain results of single-cell experiments of Nelson et al. [25] and previous experimental work [21].

As detailed further on, the stochastic nature of transcription initiation is likely due to fluctuation at the level of assembly of the transcription complexes attracting

RNA Polymerase II [28]. There is an open question as to what is the mean period of stochastic oscillations of this process, which was assayed in variety of cell systems. Estimates based on photobleaching experiments have the order of 5-10 seconds [30]. Indirect estimates based on mathematical modeling in [28] imply the order of hours. Constants used in [21] imply 10–20 minutes. Apparent divergence of these estimates may result from estimation inaccuracy or from incomplete understanding of the mechanistic principles underlying transcrition activation.

The biological system we consider is constituted by 3 pathways involving NFκB family of transcription factors playing a decisive role in innate immunity in mammals, with evolutionary roots reaching into the past as deep as *Drosophila* [37].

The innate immune response plays the role of a first line of defense from potentially harmful organisms. In this response, infecting organisms induce cellular signaling pathways to protective cytokines such as interferon. These three well-defined NF-κB signaling pathways, known as the canonical, the RIG-I-MAVS-, and the non-canonical pathways, are activated by distinct stimuli, producing dynamic cytoplasmic-nuclear oscillatory behavior and serve as informative models for computational analysis of sources of stochasticity in a biologically important eukaryotic signaling pathway. We plan to extend the current understanding of these pathways by applying recent advances in dynamic single cell imaging using fluorescent fusion proteins, analysis of transcription at a single mRNA molecule resolution, and chromatin exchange using photobleaching and fluorescence lifetime measurements.

Mathematical tools for model building, analysis, computation and estimation of parameteres in cell systems involving stochastic effects do not seem to be sufficiently developed. Papers such as [18] and [22] are based on special cases or heuristic derivations. The transition between discrete and continuous part in the mixed models is not well-justified. Models of processes at nuclear level lead to difficult partial differential equations of diffusion (FRAP models) and transport type (Gillespie-type models). The only paper known to us concerning systemic approach to estimation is Fujarewicz et al. [12]).

The aim of this research is to develop tools for better understanding and more accurate modeling of stochastic phenomena related to dynamics of gene transcription and signal transduction in eukaryotic cells. This involves analyzing mathematical models of specific signaling pathways, developing computational tools of more general applicability and constructing mathematical framework for statistical inference. The models are based on jointly devised experiments carried out by our biological co-workers and consultants.

2 Biological System Considered and Aims

Mucosal surfaces in the airway, gastrointestinal tract and skin form play an important role in maintaining physiological homeostasis by forming barriers that prevent foreign organisms from entering and causing disease. Here, the innate immune response plays a first line of defense from these potentially harmful substances or organisms. In the innate immune response, products of viruses, fungi, or bacteria

are recognized by mucosal epithelial cells and induce cellular signaling pathways that produce inflammatory and protective cytokines such as interferons [3]. Two important consequences of this inflammatory response are to: 1. induce expression of protective proteins in adjacent epithelial cells; and, 2. recruit effector immune cells to further combat the infection. In this way, the innate immune response serves as a critical signaling pathway in maintaining health.

Studies from our group and others have shown that nuclear factor-κB (NF-κB) is a highly inducible transcription factor that plays a central role in the innate immune response [6], [23]. NF-κB is a family of 11 highly homologous proteins whose activity is controlled by distinct activation pathways and in stimulus-specific manner [5], [14]. Although the signaling pathways controlling innate immune response by NF-κB to infectious stimuli are being intensively studied, the theoretical understanding of stochasticity in these signaling pathways is poorly developed. We propose a series of mathematical and biological experiments on three interrelated signaling pathways regulating the nuclear factor-κB (NF-κB) and interferon response factor (IRF) pathways that can be used to develop more general mathematical tools for understanding the role of stochasticity in biological systems. These pathways include the canonical-, the RIG-I-MAVS-, and the noncanonical NF-κB activation pathways.

1. Measure *time-dependent parameters* of key NF-κB signaling proteins *in cell populations*. Western immunoblots of cytoplasmic and nuclear proteins will be used to measure the major regulatory components of NF-κB at various times after stimulation for the canonical-, the RIG-I-MAVS-, and the non-canonical pathways. In parallel, kinetics of target gene expression will be measured by quantitative RT-PCR (Q-RT-PCR). These data will be used as parameters in deterministic models of each pathway.
2. Measure *dynamics of nuclear oscillations* of signaling proteins and *stochastics of transcriptional response*. In this aim, cytoplasmic and nuclear distributions of fluorescent protein tagged RelA and IRF3 in response to activators of the canonical and non-canonical pathways will be measured. Dose response curves for TNF activation will be measured in *single cells* using a high throughput confocal imager. Measurement of the transcriptional response will be performed in cells at the single molecule level using stably integrated genes detected by fluorescence in situ hybridization (FISH).
3. *Perturb the two major negative feedback loops* and determine their effects on the NF-κB regulatory module. NF-κB activation induces the expression of two autoregulatory loops, known as the IκBα and A20 feedback loops responsible for oscillatory DNA binding and inhibition of the major regulatory IκB kinase. In this aim, *we will construct synthetic feedback loops* incorporating either low or highly inducible IκBα or A20 expression, and determine their effects on fluorescent RelA nuclear translocation and IKK activity.
4. Measure dynamics of NF-κB *binding to rapidly and slowly responding genes* using *FRAP* and fluorescence loss in photobleaching (*FLIP*). Arrays of rapidly (IκBα) and slowly (Naf1) NF-κB inducible genes linked to monomeric red fluorescent protein reporter genes will be introduced into cells containing a regulated green fluorescent protein tagged RelA. The dynamic exchange rates of

RelA binding to each will be measured in stimulated cells by measuring the time required for exchange of the bleached for fluorescent RelA molecules.

3 Mathematical, Statistical, and Computational Aims

1. Identify sources of stochastic effects in gene transcription and regulation on single-cell, nuclear and molecular level and develop *mathematical models* of these effects, taking into account processes and quantities observable and measurable using recent technological advances.
2. Investigate the mathematical *properties of these models* by: (a) Finding deterministic and corresponding stochastic solutions in the form of probability generating functions (discrete models) and distribution densities (continuous models). (b) Developing limit theory, which justifies a wide range of approximate solutions. (c) Investigating qualitative properties of the models.
3. Develop efficient and accurate *computational algorithms* for calculation of model predictions under various experimental scenarios and including variations of parameters within the models. Implement computer programs for these algorithms.
4. Apply formal *statistical methodologies* for estimating parameters in the models from experimental data and making inferences about these parameters, and assess the goodness of fit of the models. We propose to apply existing Bayesian and non-Bayesian methods and to develop new methods for inference with complex computer models.

4 Stochastic Effects in Dynamics of Interaction of Transcription Factors with Binding Sites in Cell Nuclei

Kinetics of binding of transcription factors and co-factors to promoter regions of genes is not very accurately known. It has been recently determined by a series of works utilizing diverse visualization techniques including fluorescence recovery after photobleaching (FRAP) that there may exist a pronounced stochastic component of this kinetics, depending on the number of binding sites and transcription factor molecules interacting. In this section, we will review the relevant evidence for different sources of stochasticity. However, we will start from description of a technique, which has been mostly applied in the cases when the number of molecules and sites is large enough for the system to be considered deterministic.

4.1 Deterministic Approximation, Kinetics of Photobleaching, Diffusion-Type PDEs

Fluorescence recovery after photobleaching (FRAP) is a popular technique that has been used to measure mobilities of fluorescently tagged proteins inside living cells

[20]. In this technique, a small spot in the cytoplasm, nucleus or plasma membrane of a living cell that expresses or is microinjected with a fluorescently tagged protein, is exposed to an intense laser beam at the excitation wavelength of the fluorophore. The intense irradiation causes photobleaching of the fluorescent protein in the spot making it optically invisible, although its binding functions are not altered. Because non-bleached fluorescent molecules present in surrounding areas diffuse into the irradiated region, fluorescence recovery occurs in the spot and this can be used to estimate the diffusion coefficient of the protein. If the photobleached spot contains a significant number of fluorescent molecules that are bound to insoluble scaffolds inside cells, then the recovery curve can be utilized to estimate binding (k_{on}) and unbinding (k_{off}) constants of the proteins, in addition to the diffusion coefficients, provided sufficient measurement accuracy is reached. This requires the formulation of mathematical models that can be used to estimate kinetic rate constants for binding of proteins to scaffolds.

We presume a large (infinite) region that is at rest prior to photobleaching a volume Ω. As such, the initial conditions for the concentrations of the fluorescent protein, f, and its bound state, c, are

$$f(x,0) = f_{\text{eq}}(1-\chi_\Omega(x)) \quad \text{and} \quad c(x,0) = c_{\text{eq}}(1-\chi_\Omega(x)), \tag{1}$$

where χ_Ω is the characteristic function of Ω. These initial concentrations then evolve according to the standard coupled diffusion model

$$\frac{\partial f}{\partial t} = D\Delta f + k_{\text{off}}c - k_{\text{on}}f, \tag{2}$$

$$\frac{\partial c}{\partial t} = k_{\text{on}}f - k_{\text{off}}c. \tag{3}$$

Now (2)–(3) subject to (1) is a well posed boundary value problem. As a result the boundary values of f,

$$F(x,t) \equiv f(x,t), \quad x \in \partial\Omega \tag{4}$$

are uniquely determined by the diffusivity and two rate constants. This model is overdetermined by the fluorescence recording

$$\phi(t) = \int_\Omega \{f(x,t) + c(x,t)\}\,\mathrm{d}x$$

for, on integrating (2)–(3) over Ω it is not hard to see that

$$\phi(t) = D\int_{\partial\Omega} \nabla f(x,t)n(x)\,\mathrm{d}x, \tag{5}$$

where n is the unit inner normal to $\partial\Omega$. In the case that Ω is a ball then (4) and (5) constitute Dirichlet and Neumann data for (2)–(3) and we may draw upon the sizable literature devoted to lateral overdetermination of parabolic systems. In particular, the theory is spelled out in [17], while detailed application is carried out in [7, 11, 8, 9]. Although this literature addresses the questions of identifiability, sensensitivity

to noise, and delivers practical algorithms for efficient recovery of values such as D, k_{on} and k_{off}, the current, most quantitative, studies of FRAP, e.g., [34, 32, 33], have focused on exact solution methods in very special geometries.

4.2 Random RNA Bursts, Due to Small Number of Binding Sites

Under experimental conditions such as in [30], the overall number of molecules of the protein binding to the promoters of tandemly arrayed gene copies was so large that deterministic description using differential equations was appropriate. However, under different circumstances, stochastic effects become important. Raj et al. [28] observed stochastic bursts of transcription from a reporter gene inserted into the genome of Chinese Hamster Ovary (CHO) cells. In this case, there were either 1 or 7 DNA binding sites for the transcription factor of a single gene copy. These observations are indirect, based on observed cell-cell variability in level of the total mRNA in single cells. To obtain estimates of the dynamics of gene activation and transcription, Raj et al. [28] build a mathematical model in which they assume, among other, that the transitions from the inactive (I) to the active (A) state are random and occur with intensities λ and γ, respectively (see Figure). By fitting the complete model ([28], Supplement) to distributions of total mRNA, they estimated the expected times of the reporter gene being transcriptionally active and inactive to be equal to $E(T_A) = \gamma^{-1} = 0.8\,\text{hr.}$, and $E(T_I) = \lambda^{-1} = 2.2\,\text{hr.}$, respectively.

4.3 Stochastic Effects Due to Limiting Co-factors

Stochastic effects may be present even in when there is a large number of arrayed promoter sites with large number of bound molecules. As an example, in the paper by Voss et al. [35], the glucocorticoid receptor (GR) was investigated, which dynamically interacts with response elements in the mouse mammary tumor virus (MMTV) promoter to regulate steroid-dependent transcription. In a clonal mammary carcinoma cell line containing a tandem array of MMTV promoter-reporter gene cassettes integrated at a single genomic locus (total of 800–1200 binding sites in 200 tandemly arrayed gene copies [24], direct binding of a green fluorescent protein (GFP-GR) fusion protein to the MMTV regulatory elements can be observed in living cells. A pronounced cell-to-cell variability was observed in RNA FISH signal and GR-MMTV association within treatment groups. The authors of [35] hypothesize that the GR receptors exist in the nucleoplasmic space in a large variety of multiprotein complexes (Fig. in [35]). These complexes are recruited randomly and stochastically to hormone response elements but remain template associated for brief periods of time. The authors conclude that the transcriptional process induced by nuclear receptor activation involves a series of highly stochastic events, with considerable variation in efficiency possible at each stage.

5 Stochastic Models of Transcription Activation

5.1 Stochastic Model with Multiple Gene Copies and Multiple Binding Sites [26]

The simple model employed in [28] can be used to draft a slightly more general model applicable to systems with many gene copies and many transcription factor binding sites, which will be applicable for biological models in several papers cited above. Let us consider a system of N serially arrayed genes, each with K functional binding sites in the promoter region. Let us notice the following partial list of possibilities: (i) Deterministic approximation, K and/or N large, and/or λ and γ and μ large. (ii) Stochastic effects due to small numbers of binding sites, K and N small. (iii) Stochastic effects due to limiting co-factors and/or low abundance of transcription factors, λ small. Various intermediate and more complicated variants are possible. For example, the model in Raj et al. [28] has $N = 1$, $K = 1$ or 7, and μ large in one of the versions. The models in Hat et al. [16] involve $N = 1, 2$, or 4 and $K = 1$ with large μ. This variety of options is increased when translation of mRNA into proteins is included in the model.

The 'chemical master equation' of Gillespie [15] provides a well-known algorithm for simulation of chemical systems (SSA, 'Stochastic Simulation Algorithm', also see Aim 2) with random interactions of a finite numbers of particles of many types. This method can be used to model systems in which gene activation triggers transcription and translation [19]. In some cases, it may be used to derive analytically tractable differential equations. As an example, Paszek [26] considered several versions of systems of partial differential equations (PDE) of transport type, which describe stochastic dynamics of the transcription and translation process in a simple model involving one gene. In this model, the probabilities of gene activation $(A(t):\ 0 \to 1)$ and deactivation $(A(t):\ 1 \to 0)$ in $(t, t+\Delta t)$ are correspondingly equal to $c\Delta t + o(\Delta t)$ and $d\Delta t + o(\Delta t)$, while those of production $(X(t) \to X(t)+1)$ and degradation $(X(t) \to X(t) - \min[X(t), 1])$ of a mRNA molecule, are correspondingly equal to $A(t)H\Delta t + o(\Delta t)$ and $\Delta t + o(\Delta t)$, whereas those of a protein molecule production and $(Y(t) \to Y(t)+1)$ and degradation $(Y(t) \to Y(t) - \min[Y(t), 1])$ are equal to $X(t)K\Delta t + o(\Delta t)$ and $r\Delta t + o(\Delta t)$. The distributions of the process are described by the PDE system,

$$\frac{\partial F}{\partial t} + [(z-1) - Kz(s-1)]\frac{\partial F}{\partial z} + r(s-1)\frac{\partial F}{\partial s} = -cF + bG, \tag{6}$$

$$\frac{\partial G}{\partial t} + [(z-1) - Kz(s-1)]\frac{\partial G}{\partial z} + r(s-1)\frac{\partial G}{\partial s} = cF + [H(z-1) - b]G, \tag{7}$$

where $F = F(z,s;t), G = G(z,s;t)$, represent the joint probability generating function (pgf) of $(X(t), Y(t))$ when the gene is inactive $(A(t) = 0)$ or active $(A(t) = 1)$. More equations of this type, corresponding to various models are presented in [26]. Solution and qualitative properties of these models constitute considerable mathematical challenge. However, they can be used with ease to generate solvable moment equations.

5.2 Mixed-Type Equations

In eukaryotic cells, the stochastic effects primarily originate in regulation of gene activity [18]. In this approach, the ordinary differential equations for mRNA and protein levels in a single cells are driven by a stochastic term related to gene activation

$$\begin{aligned}\dot{x}(t) &= -x(t) + HA(t),\\ \dot{y}(t) &= -ry(t) + Kx(t),\end{aligned}$$

where intuitively, $x(t) \sim X(t)$, $y(t) \sim Y(t)$ whereas the transitions of $A(t)$ are governed by

$$\begin{aligned}A(t-0) = 0 &\xrightarrow{c} A(t) = 1,\\ A(t-0) = 1 &\xrightarrow{d} A(t) = 0,\end{aligned}$$

where rates c and d may depend on continuous state variables. This system is a counterpart of (6-7) when rates H, K, r are large compared to c, d. These equations yield a system of first-order partial differential equations (PDEs) for two-dimensional joint probability density functions $f(x,y,t)$ and $g(x,y,t)$, of $x(t)$ and $y(t)$, with $A(t) = 0$ and $A(t) = 1$, respectively

$$\begin{aligned}\partial f/\partial t - \partial(xf)/\partial x + \partial[(Kx - ry)f)]/\partial y &= byg - cf,\\ \partial f/\partial t + \partial[(H - x)g]/\partial x + r\partial[(Kx - ry)f)]/\partial y &= -byg + cf.\end{aligned}$$

The model can be considered a set of quasi-deterministic equations with jump-process $A(t)$ forcing, with corresponding Fokker-Planck or Chapmann-Kolmogorov equations for distributions. Verifying validity of this mixed-type approximation is one of the Aims of our proposal. Numerical examples indicate the approximation varies from excellent to poor. The paper of Nelson et al. [25] presents an experimental study of responses of individual cells under a variety of activation levels and patterns. We will continue studying dynamics of such responses, as we did in [22] and [21], based on our own experiments.

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