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Robustness Analysis of Cellular Systems for *In Silico* Drug Discovery

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Abstract: Biological robustness has been recognized as a fundamental organizational principle in cellular behavior. The understanding of robustness trade-off in biology has significant implications in the drug discovery research. Some diseases such as cancer can hijack cellular robustness complicating their treatment. Most of the published robustness analyses in systems biology relate this property to the output parametric sensitivity. A new analysis is proposed in which the sensitivities are evaluated for perturbations on the system states rather than on the model parameters. The result of this analysis can be directly validated in experiments, and further used in the drug discovery research to understand drug effects, to optimize drug dosing and timing, and to identify potential molecules as drug targets. The application to a model of cell death regulation shows the biological insights offered by this analysis.

1. INTRODUCTION

Robustness is a ubiquitous property of biological systems, which has been recognized as a fundamental organizational principle in the evolution of cellular functions (Barkai and Leibler, 1997; Kitano, 2004; Stelling *et al*., 2004a). The term *biological robustness* has multiple definitions in the literature, but the most widely accepted one describes the maintenance of specific functionalities (phenotype) against perturbations in the cellular internal and external conditions (Stelling *et al.*, 2004a). The term does not necessarily imply that the system is static. On the contrary, a wide array of cellular processes, from signalling to gene expression, is orchestrated in response to a perturbation. Robust behaviour has been observed in many biological systems, such as in bacterial λ-phage switching (Little *et al*., 1999), bacterial chemotaxis (Alon *et al*., 1999), and Drosophila circadian rhythms (Stelling *et al*., 2004b). Despite the obvious benefits, robustness property can turn into an Achilles heel if the cellular mechanisms that confer this property are hijacked, such as in cancer and diabetes (Kitano, 2003; Kitano *et al*., 2004). The acquired robustness property gives these diseases the ability to adapt to drug actions and develop drug resistance. Thus, the understanding of robustness and its tradeoffs in cellular systems can greatly benefit the drug discovery efforts for human diseases (Kitano, 2007).

Robustness is an inherently systems-level property which can not be judged by looking at individual component alone. In fact, the concept of biological robustness was adapted from the field of control systems theory, which is the study of the functioning behaviour of a group of objects or units. The use of systems theory in biology is driven by the recent explosion in biological data and knowledge, which has transformed biology from a single molecule reductionist study to a multicomponent systems-level investigation. The marriage between the two fields has given birth to the field of *systems biology*, which focuses on the emergence of cellular functional behaviour (e.g., robustness) from the interactions of many biological components (Liu, 2005). In addition to robustness, other concepts in systems theory have also found great relevance in biology, for example stability, feedback/feedforward, bifurcation, sensitivity, and limit cycle (Sontag, 2004).

The analysis of biological robustness has become an active area of research within systems biology (see for example Aldridge *et al.*, 2006; Chaves *et al*., 2006; Gunawan *et al*., 2005; Gunawan and Doyle, 2006, 2007; Kim *et al.*, 2006). Most of the published analyses relate the property of robustness to the sensitivity of the system output to a specific perturbation. An output is called robust to a perturbation when it is relatively insensitive to such change. On the other hand, there also exist perturbations that can cause a large output modification, pointing to system *fragility*. Some diseases such as cancer are thought to hijack cellular robustness through the fragility points in the cells (Kitano, 2003). Many systems in engineering and nature that are optimized through design or evolution to be robust against common perturbations are known to possess such fragility or high sensitivity to unexpected perturbations (Carlson and Doyle, 2002). This robust-yet-fragile characteristic manifests in the large discrepancy between the overall insensitivity of cellular function to most perturbations and the extreme sensitivity to a few parameters (Stelling *et al.*, 2004b).

The most common method to analyze biological robustness is the parametric sensitivity analysis, which represents one type of local sensitivity analyses (Varma *et al.*, 1999). This method quantifies sensitivity as the change in the output with respect to perturbations in the system (model) parameters through the sensitivity coefficient $S_{i,j}$ given by:

$$S_{i,j} = \frac{\partial x_i}{\partial p_j} \tag{1}$$

where x_i denotes the i-th state and p_j is the j-th parameter. This analysis is *local* since the parameter perturbation is infinitesimally small. Such sensitivity measurements have been extended to investigate relevant dynamics in biology, such as oscillatory behaviour in circadian rhythms (Gunawan and Doyle, 2006; Ingalls, 2004) and stochastic noise in gene expression (Gunawan *et al*., 2005; Plyasunov et al., 2007). The calculated coefficients highlight the key parameters in the system having strong effects on the output (i.e., fragile points), as well as those whose values do not matter considerably with respect to the output. In other words, the sensitivities allow the demarcation of system parameters to which the outputs are robust and fragile. Parametric sensitivity analysis has been applied to many biological problems, from metabolic engineering of microbes (Fell, 1992) to cell death in human (Bentele *et al*., 2004) to Drosophila circadian rhythm entrainment (Gunawan and Doyle, 2007). Aside from its application in robustness analysis, parametric sensitivity coefficients are also used in the model identification of cellular networks (Gadkar *et al*, 2005) and in the design of new biological systems in synthetic biology (Andrianantoandro *et al*., 2006).

Other methods for robustness analysis arise from using various definitions of system output (state variable, stability, bifurcation) and different types of perturbation (global vs. local). Global parametric sensitivity analysis typically consists of repeated local analyses around parameter values sampled from a given region in the parameter space, and the consolidation of the local analysis results into global sensitivity indexes (Stelling *et al.*, 2004b). Bifurcation analysis has also been used to assess biological robustness, in which the robustness is measured as the distance to the nearest bifurcation point (Lu *et al*., 2006; Ueda, 2001) or as the stability margin of linear or linearized models (Kim *et al.*, 2006, Schmidt and Jacobsen, 2004).

Despite the differences, most of the existing robustness analyses share a common feature: the perturbations (uncertainty) are assumed to lie in the system parameters. These parameters typically consist of kinetic rate constants, transport coefficients, binding energies, *etc*. that correspond to the many cellular processes involved. Hence, the high or low sensitivities point to the cellular processes that are critical or inconsequential to the output, respectively. Though such information is certainly useful, the experimental validation proves to be a difficult task. For example, if the parametric analysis suggests the importance of the phosphorylation of a particular protein, the experimental validation will require modifying the corresponding kinase activity, which is not an easy task. This limitation motivates the development of a new robustness analysis which can give experimentally relevant sensitivities that are easy to validate.

In this article, we present a new sensitivity-based robustness analysis which can be directly validated in experiments. The analysis complements existing parametric sensitivity analysis in elucidating the robustness trade-off (robust-yet-fragile) property. In addition, this analysis can illustrate how a perturbation dynamically propagates through the system and highlight the key biological molecules that make signification contribution to the cellular phenotype. Such information can be used in the drug discovery research to understand drug effects, to optimize drug dosing and timing, and to identify potential molecules as drug targets. The analysis is applied to a model of cell death in human Jurkat cancer cells (Hua *et al.*, 2006) to illustrate the transient signalling of Fas-mediated cell death, to highlight the important biological molecules, and finally to identify potential drug targets in this cell line.

2. METHODOLOGY

The proposed robustness analysis will focus on biological networks that can be described by common ordinary differential equations (ODEs) given by:

$$\frac{d\mathbf{x}}{dt} = f(\mathbf{x},\mathbf{p}),\ \mathbf{x}(0) = \mathbf{x}_0 \tag{2}$$

where $\mathbf{x}\in\mathbf{R}^n$ is the state vector, $\mathbf{p}\in\mathbf{R}^p$ is the parameter vector, and $f(\mathbf{x},\mathbf{p})$ is a general vector-valued nonlinear function. The states in a cellular system typically describe the concentration of the molecules involved, such as proteins and mRNAs. The parameters are the relevant kinetics, energy, and/or transport coefficients that appear in the constitutive equations. This formulation is general enough to describe most systems of interest in biology, such as metabolic, signalling, and genetic regulatory models (Fell, 1992; Conrad and Tyson, 2006).

The new robustness analysis will be based on the concept of sensitivity, i.e. the change in the output relative to the perturbation. The novelty of this analysis is that these sensitivities are computed for uncertainties in the states rather than the usual parametric perturbations. The corresponding sensitivity coefficient is given by:

$$S^x_{i,j}(t,\tau) = \frac{\partial x_i(t)}{\partial x_j(\tau)} \text{ for } t \geq \tau, \tag{3}$$

which describes the relative change in the state x_i at time t due

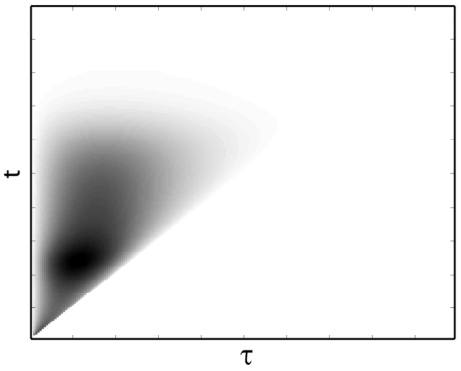

Fig. 1. Surface Contour Plot of $S^x_{i,j}(t,\tau)$. The τ-axis indicates the time of perturbations and the t-axis the time of observed change in the output. The darker region marks the high sensitivity magnitude for which the perturbation at time τ induces strong response from the output at time t.

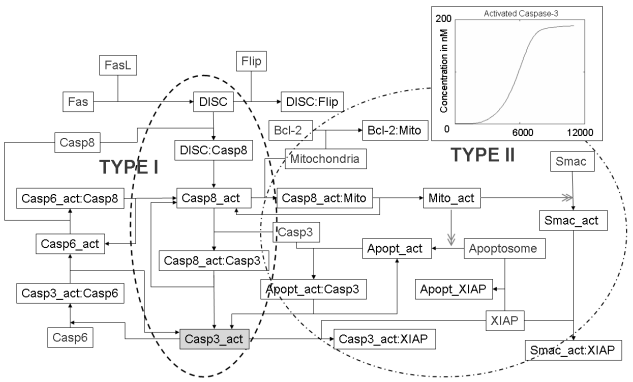

Fig. 2. A Model of FasL-induced Cell Death (Hua *et al.*, 2006). The response of caspase-3 activation is switch-like

to the perturbation in the state x_j at some previous time τ. The sensitivity analysis of the model given in (2) reduces to solving the following differential equation (see Appendix A for derivation)

$$\frac{d}{dt}\mathbf{S}^x(t,\tau) = \left(\nabla_{\mathbf{x}} f^T\right)^T \mathbf{S}^x(t,\tau) = \mathbf{J}(\mathbf{x})\mathbf{S}^x(t,\tau)$$
$$\mathbf{S}^x(\tau,\tau) = \mathbf{I} \qquad (4)$$

where $\mathbf{J}$ is commonly known as the Jacobian, $\mathbf{S}^x(t,\tau)$ denote the two-time sensitivity matrix with its (i,j)-th element given in (3), and $\mathbf{I}$ is the identity matrix. The sensitivities above will be solved simultaneously with (2) as the Jacobian $\mathbf{J}$ depends on the state vector. Unlike system parameters which are usually assumed constant with respect to time, the states are dynamical in nature, which motivates the formulation of the two-time (t,τ)-analysis.

The proposed method is closely related to the Lyapunov exponent, which characterizes the rate of separation among trajectories starting from infinitesimally nearby initial conditions. Recently, an analysis based on the Lyapunov exponent, called the direct Lyapunov exponent (DLE), was proposed to investigate regions of initial condition that give qualitatively different cellular behaviour (Aldridge et al., 2006). In this case, the DLE analysis is a special case of the sensitivity definition in (3) for which τ is equal to zero and the system change is measured as the spectrum of $\mathbf{S}^x(t,0)$ (i.e., maximum singular value of $\mathbf{S}^x(t,0)$). Furthermore, there also exists a relationship between the new sensitivity and the parametric sensitivity in (1). Both sensitivities are local in the context of the parameters $\mathbf{p}$ and initial conditions $\mathbf{x}_0$. In addition, the solution to (4) can be used to evaluate the parametric sensitivity following the Green's function method (Varma et al., 1999). However, $\mathbf{S}^x(t,\tau)$ contains much richer information than the parametric sensitivity as detailed in the next section.

The evaluation of the two-time sensitivity matrix $\mathbf{S}^x(t,\tau)$ is numerically challenging. The quickest method is to use a finite difference approximation according to:

$$S^x_{i,j}(t,\tau) \approx \frac{x_i\left(t;\Delta x_j(\tau)\right) - x_i\left(t;-\Delta x_j(\tau)\right)}{\Delta x_j(\tau)}, \qquad (5)$$

where the first (second) term in the denominator refers to the state x_i at time t after an additive perturbation Δx_j ($-\Delta x_j$) to the state x_j at time τ. The above formulation requires solving $2n$ ODEs to compute a second-order approximation for a given perturbation time τ, where n is the number of states in the system. However, this method may produce grossly inaccurate approximation as the accuracy depends on the size of the perturbation and the simulation tolerance. The most accurate method is to directly solve (4), which is discussed next.

For a given perturbation time τ, the coupled equations (2) and (4) comprise a total of (n^2+n) ODEs. Depending on the stiffness of the ODEs, this method can prove to be computationally expensive, especially if a large number of perturbation times are desired. Here, we make use of a chain rule in differentiation

$$\mathbf{S}^x(t_3,t_1) = \mathbf{S}^x(t_3,t_2)\mathbf{S}^x(t_2,t_1), \quad t_1 \le t_2 \le t_3 \qquad (6)$$

Hence, the two-time sensitivities are computed only for one time step, i.e. $\mathbf{S}^x(\tau+\Delta t,\tau)$, over $0 \le \tau \le t_{\text{end}}$, from which the sensitivities $\mathbf{S}^x(t,\tau)$ for $0 \le t \le t_{\text{end}}$ can be computed.

3. DATA ANALYSIS AND BIOLOGICAL RELEVANCE

The two-time sensitivity $\mathbf{S}^x(t,\tau)$ is an $n \times n$ matrix whose rows and columns correspond to the various outputs and perturbations in the system, respectively. Each (i,j)-th element can be presented in a surface contour plot as shown in Fig. 1. Such a plot illustrates two dynamical aspects of the perturbation-output relationship; the range(s) of time in τ that the perturbation may become significant and the range(s) of time in t that the corresponding output change appear. By analyzing either a selected perturbation (column of $\mathbf{S}^x(t,\tau)$) or a chosen output (row of $\mathbf{S}^x(t,\tau)$), one can obtain complementary information on the propagation of a perturbation signal through the system or the key molecules that take part in producing the observed output, respectively.

The sensitivities provided by the new robustness analysis are useful for the drug discovery research in two ways. First, as many new drugs target specific molecules in the cell, a drug action can therefore be treated as a state perturbation. The drug effect on the system can be analyzed using the proposed method by tracking the progression of such perturbation through the system, as described above (i.e., by analyzing the corresponding column of $\mathbf{S}^x(t,\tau)$). The drug efficacy, specificity, and toxicity are quantified by the sensitivities of some system outputs to the drug-induced perturbation, which are useful in the optimization of dosage and timing.

Second, the sensitivities of state(s) related to a disease phenotype (given by the row(s) of $\mathbf{S}^x(t,\tau)$), can provide a list of potential molecules for drug targeting. High sensitivities highlight the potential targets for the development of a new drug or the selection of drug combination in multicomponent

therapeutics. Further, a comparison of the analysis between healthy and disease state models can be used to screen the list for targets that are specific to diseased cells. As the sensitivities are computed for perturbations in the states, the predictions can be validated in relatively simple experiments. For example, if the system states correspond to proteins and/or mRNAs, the experiments may involve overexpressions or knock-outs of genes encoding the proteins or RNA interference.

4. EXAMPLE

The proposed analysis was applied to a model of the cell death regulation in human Jurkat T lymphocyte cell line (Hua et al., 2006). Fig. 2 summarizes the model which describes the cell death (apoptosis) signalling triggered by the death ligand FasL. The key molecule of interest is caspase-3, which is a protease that cleaves many protein substrates (Reed et al., 2004). The activation of caspase-3 by FasL follows a switch-like response as shown in Fig. 2 (see inset) by way of caspase-8 and caspase-6 dependent pathway (type-I) or mitochondria-dependent pathway (type-II).

The two-time sensitivities were computed as normalized values (i.e., percent change output over percent change perturbation), according to:

$$\bar{S}^{x}_{i,j}(t,\tau)=\frac{\partial x_i(t)}{\partial x_j(\tau)}\frac{x_j(\tau)}{x_i(t)}. \qquad (7)$$

The computation of the normalized sensitivity coefficient can also be formulated directly from (2) in the same manner as the derivation of (4) (see Appendix A). The magnitude of two-time sensitivities of activated caspase-3 (the variable Casp3_act in Fig. 2) to various state perturbations are presented in Fig. 3.

The results in Fig. 3 show the importance of selected molecules in the system at different times in the caspase-3 activation. As mentioned above, this corresponds to analyzing one particular row of $\mathbf{S}^{x}(t,\tau)$. In this case, the switch-like response of caspase-3 occurred at the time range between 4000 to 7000 seconds. By focusing on the output time axis (y-axis of Fig. 3) for this time span, one can identify the time sequence of key molecules that regulate the observed caspase-3 output. Figs. 3a–e suggest that Fas, caspase-8, XIAP, and mitochondria were among the early significant contributors. XIAP is an inhibitor of apoptosis, and thus negatively contributes to the activation of caspase-3. The switch can be largely attributed to the type-II mitochondria-dependent pathway as indicated by Figs. 3e–i, in the following sequence of events according to the location of the sensitivity peaks on τ-axis: the release of cytochrome-c from mitochondria (Mt-Activated), the formation of apoptosome, and finally the activation of caspase-3. Further, Fig. 3j points to the lack of type-I role in this cell line. Therefore, the results imply that the FasL-induced apoptosis in Jurkat cells depends mainly on the type-II pathway, in agreement with the literature (Scaffidi et al., 1998).

As a validation of the analysis, *in silico* impulse perturbation experiments were performed by increasing caspase-8 concentrations at two different times: τ = 0s and τ = 4000s. In comparison to the nominal trajectory, Fig.4 shows a significant increase in the caspase-3 activation when the initial available caspase-8 level was increased by 50%. The same increase at τ = 4000s however gave little observable change. This finding is in agreement with the high and low sensitivities of caspase-3 to caspase-8 perturbations (see Fig.3b) at τ = 0s and τ = 4000s, respectively, demonstrating the dynamic information that the proposed analysis can provide. The information from the above analysis can be used in the drug discovery research to identify potential molecules as drug targets or drug cocktail. Indeed, multicomponent therapy using combination of drugs has increasingly gained more attention with various reported successes, such as

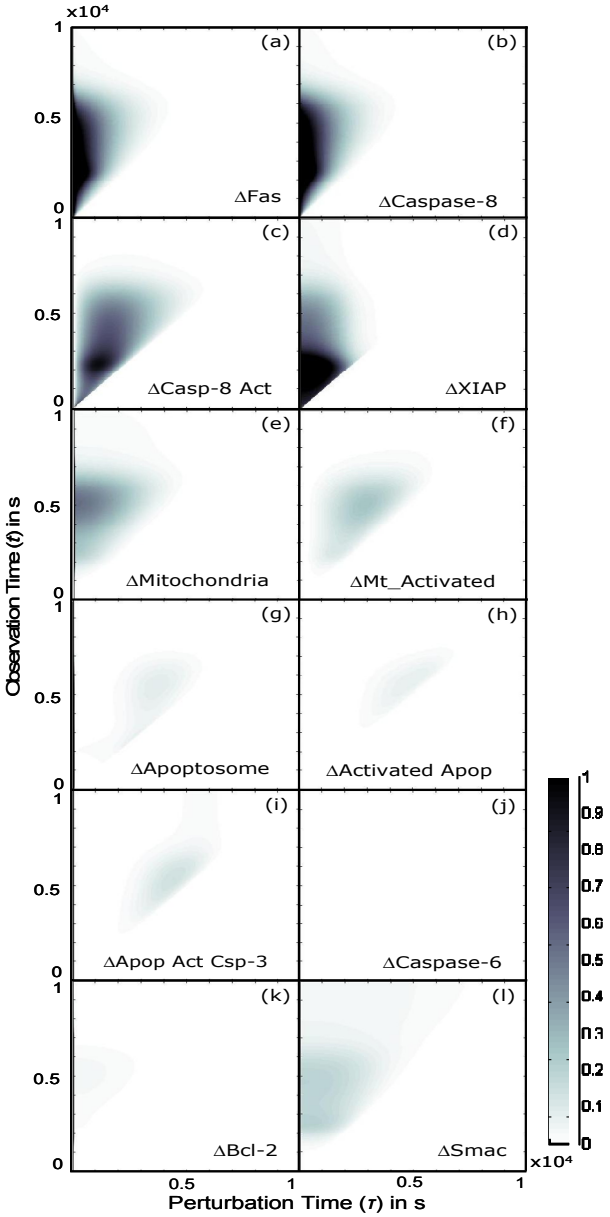

Fig. 3. Two-time Sensitivity Analysis of the Cell Death Signalling Model. Each subplot corresponds to the sensitivity of activated caspase-3 concentration with respect to the perturbation on the selected states.

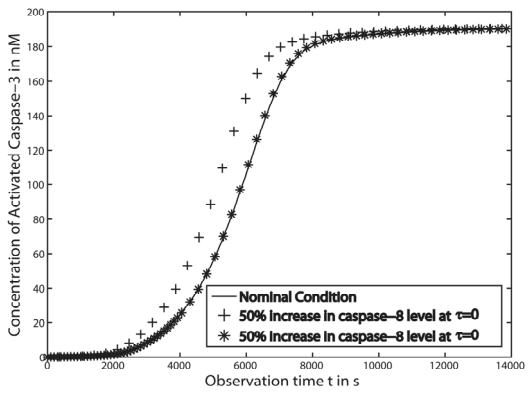

Fig. 4. *In Silico* validation of apoptosis analysis.

salmeterol-fluticasone in asthma (Advair - GlaxoSmithKline) (Nelson, 2001) and AZT-3TC in HIV infection (Combivir - GlaxoSmithKline) (Larder *et al.*, 1995). The selection of drug combination in a clinical setting however had been done through deliberate mixing by rational design or happenstance. Therefore, a systematic method in multicomponent therapeutics is highly desirable, either by a large scale screening (Borisy *et al.*, 2003) or by *in silico* network analysis as done here.

For example, in the present model, the cell-death is induced through Fas receptor, which belongs to a TNF (tumor necrosis factor) receptor family. In practice, several cancer therapeutic agents target TNF receptor to trigger apoptosis, such as Apo2L/TRAIL from Genentech and Amgen (Pollack *et al.*, 2001). According to the analysis, the effectiveness of cell death induction in Jurkat leukaemia cell line by such agent should depend on the type-II pathway. Therefore, the use of other drugs that increase the activity of type-II pathway should boost the sensitivity of Jurkat cancer cells to the apoptotic insult by a Fas-receptor ligand. A more detailed model of the cell death network will be needed to provide specific suggestions of the target molecules. In general, the high sensitivities in Fig. 3 imply the synergistic action between a Fas receptor drug and these molecules.

In summary, we have presented a new robustness analysis based on sensitivities to local perturbation of the molecular concentration, rather than the usual model parameters. The result of this analysis is experimentally relevant and offers dynamical and molecular information on how a phenotype is regulated in the cell. Such information can guide the drug discovery efforts by identifying potential drug targets and in the optimization of drug dosing and timing. The development of this method represents a concrete step toward robustness-based drug design in systems biology.

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Appendix A. DIRECT DIFFERENTIAL METHOD FOR TWO-TIME SENSITIVITY COMPUTATION

The two-time sensitivity equation in (4) is directly obtained by taking the derivative of (2) with respect to $x_j(\tau)$:

$$\frac{\partial}{\partial x_j(\tau)}\frac{d\mathbf{x}(t)}{dt} = \frac{d}{dt}\frac{\partial \mathbf{x}(t)}{\partial x_j(\tau)} = \dot{S}_j^x(t,\tau) = \frac{\partial}{\partial x_j(\tau)} f\left(\mathbf{x}(t),\mathbf{p}\right)$$

where S_j^x is the j-th column of $\mathrm{S}^x(t,\tau)$. Using chain-rule, the direct differential formulation of the sensitivities is given by

$$\dot{S}_j^x(t,\tau) = \left(\nabla_{\mathbf{x}} f\left(\mathbf{x}(t),\mathbf{p}\right)^T\right)^T \frac{\partial \mathbf{x}(t)}{\partial x_j(\tau)} = \mathbf{J}(\mathbf{x}) S_j^x(t,\tau)$$

Furthermore, the normalized sensitivity in (7) is equivalent to computing

$$\bar{S}_{i,j}^x(t,\tau) = \frac{\partial \log x_i(t)}{\partial \log x_j(\tau)} \tag{8}$$

To avoid division by zero in (7), the normalized sensitivity can be directly computed in terms of the logarithmic values. Here, we can rewrite (2) in term of normalized states:

$$\frac{1}{x_i}\frac{dx_i}{dt} = \frac{d\log x_i}{dt} = \frac{1}{x_i} f_i\left(\mathbf{x},\mathbf{p}\right)$$

By redefining $z_i = \log x_i$, the model equations can be expressed as

$$\frac{dz_i}{dt} = \frac{1}{e^{z_i}} f_i\left(\mathbf{e}^{\mathbf{z}},\mathbf{p}\right),\ z_i(0) = \log x_i(0),$$

where $\mathbf{e}^{\mathbf{z}}$ is the vector $\left[e^{z_1}, e^{z_2}, \ldots, e^{z_n}\right]^T$. As the states describe molecular concentrations, z_i is always defined except for $x_i = 0$ (a large negative number is used to approximate zero concentration). The normalized sensitivity coefficient is now restated as:

$$\bar{S}_{i,j}^x(t,\tau) = \frac{\partial z_i(t)}{\partial z_j(\tau)}$$

Following the direct differential approach, the computation of (8) can be derived from the normalized state model above.