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Validation and invalidation of systems biology models using robustness analysis

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Abstract: Robustness, the ability of a system to function correctly in the presence of both internal and external uncertainty, has emerged as a key organising principle in many biological systems. Biological robustness has thus become a major focus of research in Systems Biology, particularly on the engineering–biology interface, since the concept of robustness was first rigorously defined in the context of engineering control systems. This review focuses on one particularly important aspect of robustness in Systems Biology, that is, the use of robustness analysis methods for the validation or invalidation of models of biological systems. With the explosive growth in quantitative modelling brought about by Systems Biology, the problem of validating, invalidating and discriminating between competing models of a biological system has become an increasingly important one. In this review, the authors provide a comprehensive overview of the tools and methods that are available for this task, and illustrate the wide range of biological systems to which this approach has been successfully applied.

1 Introduction

Robustness, in both biological and engineering systems, may be defined as the ability of a system to function correctly in the presence of both internal and external uncertainty. The case for robustness being a key organising principle of biological systems was first made in an influential series of papers by J.C. Doyle and co-workers in the early 2000s [1, 2]. In these papers, the authors compare the robustness properties of biological and engineered systems, and suggest that the need for robustness is a key driver of complexity in both cases. They argue that radically simplified versions of both jet aircraft and bacteria (for example) could be conceived of that would function in highly controlled 'laboratory' conditions, but would lack the robustness properties necessary to function correctly in highly fluctuating real-world environments.

Somewhat paradoxically, the highly complex nature of these systems renders them 'robust yet fragile', that is, robust to types of uncertainty or variation that are common or anticipated, but potentially highly fragile to rare or unanticipated events. Biological organisms are usually highly robust to uncertainty in their environments and component parts but can be highly sensitive to minor genetic perturbations or to the presence of microscopic pathogens or toxins that disrupt structural elements or regulatory control networks. Modern aircraft are robust to atmospheric turbulence, changes in cargo loads and fuels, and defects in materials, but could be catastrophically affected by failures in a few computer chips or by software coding errors (in contrast to previous generations of much more simple 'mechanical' aircraft which had little or no reliance on computers). The similarities and differences between biological and engineering notions of robustness have since been further developed in a series of papers by Kitano and co-workers [3–7], and subsequently many researchers have sought to develop a general theory of biological robustness, with perhaps the most successful effort in this direction being the work of Wagner on mutational robustness [8].

In this review, we focus on one of the most practically useful ideas which has emerged from this sometimes rather philosophical line of enquiry. This idea was first made explicit in an article by Morohashi *et al.* [9], and is encapsulated in the title of their paper: Robustness as a measure of plausibility in models of biochemical networks. The idea is a logical consequence of the recognition of the robust nature of biological systems: if a particular feature of a system has been shown experimentally to be robust to a certain kind of perturbation or environmental disturbance, then any proposed model of this system should also demonstrate the same levels of robustness to simulated versions of the same perturbations or disturbances. For example, the period of oscillations in many biological systems (from circadian rhythms to cAMP oscillations in *Dictyostelium* cells) has been shown experimentally to be highly robust to variations across different cells and to changes in environmental conditions. Thus, valid models for these systems should not display large changes in the periods of their oscillations across ranges of parameter values corresponding to realistic levels of biological variation. The great advantage of this idea is that it provides a much more stringent test of a proposed model than the traditional approach of simply asking: does there exist a

biologically plausible set of model parameter values for which the model's outputs provide an acceptable match to experimental data? As the complexity of the quantitative models being developed in Systems Biology research continues to escalate, many conceptually quite different models may be proposed to explain the workings of a biological system, and each of these models will often have biologically reasonable sets of parameter values which allow the model to accurately reproduce the experimentally measured dynamics of the system. Since each of these models encapsulates a different hypothesis regarding the workings of the underlying biology, it is clear that further progress depends on the ability to reliably discriminate between different models, discarding some and focusing on others for further refinement, development and testing.

In this paper, we use the term 'model validation' to describe this process, although to be precise, as pointed out in [10], the complete validation of a particular model is never possible in practice, as it would require infinite amounts of both data and computational power. Usually, the best one can do is to proceed by a process of elimination, invalidating more and more competing models until a single un-invalidated model remains. This model then encapsulates our current level of understanding of the underlying biology, which may stand the test of time, or be subsequently refined in the light of new data. The evaluation of model robustness provides a powerful tool with which to achieve the goal of developing validated models of biological reality, and, as we shall show in Section 3 of this paper, this approach has now been used as an essential part of the model development process for a wide range of biological systems.

Before we review these successful applications, however, we must turn our attention to the challenges involved in reliably evaluating the robustness of complex models of biological systems. Indeed, the development and application of engineering robustness analysis tools to biological models has provided many challenges for control engineers working in the field of Systems Biology, and continues to be one of the key areas driving theoretical developments in control systems research. An overview of the tools and techniques which are available for robustness analysis, and which have been successfully applied in the context of biological systems, is given in Section 2 of this review. Finally, some conclusions and a discussion of the outlook for future research in this area is provided in Section 4.

2 Overview of methods for the robustness analysis of systems biology models

In this section, we provide a tutorial-style introduction to the range of tools and techniques that are available to evaluate the robustness of models of biological systems to various forms of uncertainty and variability. Many of these methods were first developed within the field of Control Engineering, where linear models, or models with particular forms of non-linearity, are typically used for the purposes of design and analysis. Biological systems, on the other hand, often display highly complex behaviour, including strong non-linearities, as well as oscillatory, time-varying, stochastic and/or hybrid discrete-continuous dynamics. Thus, the application of these methods in the context of Systems Biology is often far from straightforward, and care must often be exercised in interpreting the computed results. As shown below, however, careful analysis of Systems Biology models using these tools can often provide significant insight into both the validity of a particular model and the underlying biological mechanisms it represents.

2.1 Bifurcation diagrams

Biological systems typically operate in the neighbourhood of some nominal condition, for example, in biochemical networks the production and degradation rates of the biochemical compounds are often regulated so that the amounts of each species remain approximately constant at some levels. When such an 'equilibrium' is perturbed by an external event (e.g. by the presence of exogenous signalling molecules, like growth factors), a variety of different reactions may take place, which in general can lead the system either to operate at a different equilibrium point, or to tackle the cause of the perturbation in order to restore the nominal operative condition.

In mathematical terms, a point x_e in the state space of a generic non-linear system without exogenous inputs [In the following we make certain mild assumptions about the mathematical properties of f (e.g. it is autonomous, piecewise continuous and locally Lipschitz [11]), which are in practice true for the vast majority of models used in Systems Biology.]

$$\dot{x} = f(x) \tag{1}$$

is said to be an 'equilibrium point' if, whenever the state of the system starts at x_e, it will remain at x_e for all future time. The equilibrium points are the roots of the equation $f(x) = 0$. When the system has an exogenous input u, the generic model reads

$$\dot{x} = f(x, u) \tag{2}$$

and the pair (x_e, u_e) is an equilibrium point for the system if

$$f(x_e, u_e) = 0$$

One of the main differences between linear and non-linear systems is that the latter can exhibit zero, one or multiple isolated equilibria, which are in general different from the origin of the state space. In the linear case, where $\dot{x} = Ax$, the equation $Ax = 0$ admits only the trivial isolated solution $x = 0$, if $\det A \neq 0$, or a continuum of equilibrium points (e.g. a straight line in the state space of a second-order system) when A has one or more zero eigenvalues.

The above discussion relates to robustness due to the fact that the equilibrium points of a system, and their stability properties, depend not just on the structure of the equations, but also on the values of the parameters: in non-linear systems, even small changes in the value of a single parameter can significantly alter the map of equilibrium points, and thus the dynamic behaviour of the system. To see this, consider the following non-linear system

$$\frac{\mathrm{d}x}{\mathrm{d}t} = rx\left(1 - \frac{x}{q}\right) - \frac{x^2}{1 + x^2} = f(x) \tag{3}$$

The equilibrium points are solutions of the equation $f(x) = 0$ which implies

$$rx\left(1 - \frac{x}{q}\right) - \frac{x^2}{1 + x^2} = 0$$

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Obviously, $x = 0$ is an equilibrium point, but so are all the solutions to the equation

$$r\left(1 - \frac{x}{q}\right) = \frac{x}{1 + x^2} \tag{4}$$

These solutions can be easily visualised by plotting both sides of (4) as shown in Fig. 1: the intersections correspond to the equilibrium points of system (3). Note how both the location and the number of equilibrium points changes for different values of the parameter r.

From the above example, it is clear that non-linear systems can exhibit multiple equilibria, each one being (either simply or asymptotically) stable or unstable, and that the position of the equilibrium points, along with their stability properties and regions of attraction, can vary with the parameter values of the system. Therefore it comes as no surprise that the behaviour of a non-linear system might dramatically change when the value of some parameter varies, even by a small amount: this phenomenon is called a 'bifurcation'. Assume, for example, that the value of q in the above example is fixed at 20 and let r increase from 0.15 to 0.6. From Fig. 1, we can see that there will be two bifurcation points, where the number of equilibrium points changes from one (low value of r) to three and then back to one (high value of r). A stability analysis, via linearisation at the equilibrium points, reveals that the low- and high-valued equilibrium points are always asymptotically stable, whereas the middle-valued one, when it exists, is unstable.

The variations in the map of equilibrium points corresponding to changes of r can be effectively visualised by using a 'bifurcation diagram', in which the equilibrium values of some state variable are plotted against the bifurcation parameter. For example, the bifurcation diagram of system (3) is shown in Fig. 2, which shows the two bifurcation points where the number of equilibrium points changes from one to three (occurring at $r = 0.198$) and then back to one (occurring at $r = 0.528$). The solid lines represent the asymptotically stable equilibrium values, whereas the dashed line represents the unstable one. For intermediate values of r the system is bistable, and it can evolve to the upper or lower branch of the diagram,

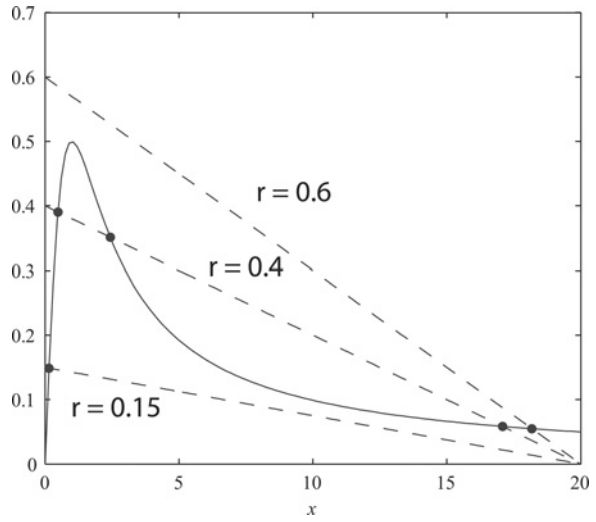

Fig. 1 *Intersections of $x/(1 + x^2)$ (solid line) and $r(1-x/q)$ (dashed lines) for $q = 20$ and $r = 0.15, 0.4, 0.6$*

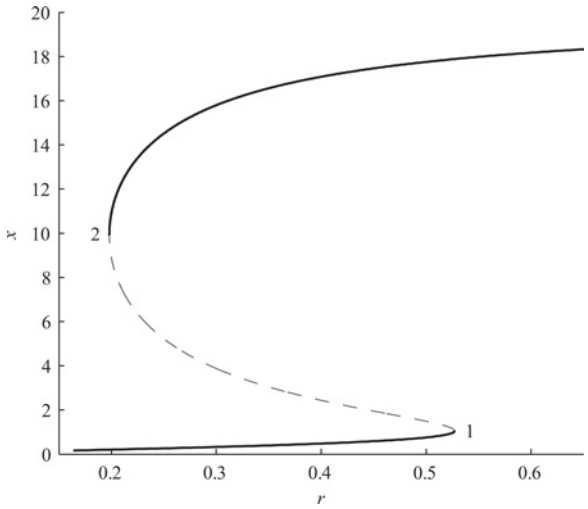

Fig. 2 *Bifurcation diagram of system (3)*

depending on whether the initial condition is above or below the middle branch, respectively.

The bifurcation diagram also informs us that there is a hysteresis-like behaviour in this system: when the system is at the lower stable equilibrium point and r is increased, the state jumps to the higher stable equilibrium point when r becomes greater than 0.528; however, to jump back to the lower equilibrium point, the value of r must drop below 0.198. Note that the presence of a hysteresis ensures a stable switching between the two operative conditions for the system; indeed, if the two thresholds were coincident, the system trajectories could constantly jump forth and back when the value of r is subject to stochastic variation around the bifurcation point. This robust bistable switch is a key mechanism in many biological regulatory systems.

Bifurcations are classified according to the type of modifications they produce in the map of equilibrium points and in their stability properties, some of the most common types are saddle-node, transcritical and pitchfork bifurcations, see [12] for more details.

Bifurcation diagrams are powerful tools for understanding how qualitative changes in the behaviour of non-linear Systems Biology models arise due to parametric uncertainty. As tools for measuring robustness, however, they suffer from two significant limitations, namely, that analytical solutions are available only for low-order models, and that they only provide information on the effects of varying one or two parameters at a time. (In principle, one could consider more parameters but the dynamic behaviour near bifurcations with codimension higher than three is usually so poorly understood that the computation of such points is not worthwhile.) Nonetheless, bifurcation analysis was the tool used in the first paper proposing the approach to model validation surveyed here. In [9], the authors represent a model of the biochemical oscillator underlying the Xenopus cell cycle as a mapping from parameter space to behaviour space, and utilise bifurcation analysis to study the robustness of each region of steady-state behaviour to parameter variations. The hypothesis that potential errors in models will result in parameter sensitivities was tested by analysis of the robustness of two different models of the biochemical oscillator. This analysis successfully identified known weaknesses in an older model and also correctly highlighted why the more recent model was more plausible. Also, in [13], the authors use a bifurcation analysis software

package named AUTO to examine the robustness of a model of cAMP oscillations in aggregating *Dictyostelium* cells to variations in each of the kinetic constants k_i in the model. In [14], the authors use bifurcation analysis to compare the validity of high- and low-order models describing regulation of the cyclin-dependent kinase that triggers DNA synthesis and mitosis in yeast. Finally, in [15], the authors introduce a novel robustness analysis method for oscillatory models, based on the combination of Hopf bifurcation analysis and the standard Routh–Hurwitz stability test from linear control theory.

2.2 Sensitivity analysis

Sensitivity analysis is a well-established technique for evaluating the relative sensitivity of the states or outputs of a model to changes in its parameters. In this sense, therefore, sensitivity may be interpreted as the inverse of robustness – parameter sensitivities yield a quantitative measure of the deviations in characteristic system properties resulting from perturbation of system parameters, and thus a higher (absolute) sensitivity of a parameter implies a lower robustness of the corresponding element of a model. The classical approach to sensitivity analysis considers small variations in a single parameter at a time. For the autonomous dynamical system described by the ordinary differential equation

$$\dot{x} = f(x(t), p, t) \tag{5}$$

with time $t \geq t_0$, the $n_S \times 1$ vector of state variables x, the $n_P \times 1$ vector of model parameters p, and initial conditions $x(t_0) = x_0$, parameter sensitivities with respect to the system's states along a specific trajectory $S(t)$ (the $n_S \times n_P$ matrix of state sensitivities) are defined by (Of course, analytical expressions for the relevant derivatives will rarely be available and thus numerical approximations will typically have to be employed)

$$S(t) = \frac{\delta x}{\delta p} \tag{6}$$

To allow for easier comparisons to be made between different models, these sensitivity of each parameter p_j may be integrated over discrete time points along the system's trajectory from T_0 to T_{n_T}, and normalised to relative sensitivity (log-gain sensitivity) to give the overall state sensitivity for parameter p_j

$$S_{Oj}(t) = \frac{1}{n_S} p_j \left(\sum_{k=1}^{n_T} \sum_{i=1}^{n_S} \left[\frac{1}{x_i} \frac{\delta x_i(t_k, t_0)}{\delta p_j} \right]^2 \right)^{1/2} \tag{7}$$

The sensitivity of each parameter with respect to any model output, or other characteristic, may be evaluated in the same way, for example, the sensitivity of the period and amplitude of an oscillatory system are evaluated, respectively, as

$$S_\tau = \frac{\delta \tau}{\delta p} \quad \text{and} \quad S_{A_i} = \frac{\delta A_i}{\delta p} \tag{8}$$

It is important to note that the above parameter sensitivities are only valid locally with respect to a particular point in the model's parameter space, that is, in a neighbourhood of a specific parameter set. They thus only provide information on the robustness of a particular parameterisation of a model, and care must be taken in interpreting their values globally. To derive global measures of parametric sensitivity [16], some kind of gridding or sampling strategy must be used, in order to evaluate the relative sensitivity of different parameters over the full range of their allowable values. Of course, this significantly increases the associated computational cost, and also makes the direct comparison of the sensitivity of different parameters more difficult (relative sensitivities may vary across different regions of parameter space). Nevertheless, in [17], F.J. Doyle and co-workers were able to use the above sensitivity metrics to investigate the specific structural characteristics that are responsible for robust performance in the genetic oscillator responsible for generating circadian rhythms in Drosophila. By systematically evaluating local sensitivities throughout the model's parameter space, global robustness properties linked to network structure could be derived. In particular, analysis of two mathematical models of moderate complexity showed that the trade-off between robustness and fragility was largely determined by the regulatory structure. An analysis of rank-ordered sensitivities allowed the correct identification of protein phosphorylation as an influential process determining the oscillator's period. Furthermore, sensitivity analysis confirmed the theoretical insight that hierarchical control might be important for achieving robustness. The complex feedback structures encountered in vivo were shown to confer robust precision and adjustability of the clock while avoiding catastrophic failure.

Another significant limitation of traditional sensitivity analysis methods is that they only consider the sensitivity of the model to variations in a single parameter at a time – in theory a model could display low sensitivity to such variations while being extremely sensitive to simultaneous variations in multiple parameters. Two recent papers have proposed some promising strategies for overcoming the local, one-parameter-at-a-time limitations of traditional approaches to sensitivity analysis. In [18], the authors used sensitivity analysis to validate a new computational model of signal transducer and activator of transcription-3 (Stat3) pathway kinetics, a signalling network involved in embryonic stem cell self-renewal. Transient pathway behaviour was simulated for a 40-fold range of values for each model parameter in order to generate Stat3 activation surfaces – by examining these surfaces for local minima and maxima, non-monotonic effects of individual parameters could be identified and isolated. This analysis provided a range of parameter variations over which Stat3 activation is monotonic, thus facilitating a global sensitivity analysis of parameter interactions. To do this, groups of parameters that had a similar impact on pathway output were clustered together, so that the effects of varying multiple parameters at a time could be analysed visually using a clustergram.

This analysis allowed the identification of groups of parameters that contribute to pathway activation or inhibition, as well as other interesting pathway interactions. For example, it was found that simultaneously changing the parameters determining the nuclear export rate of Stat3 and the rate of docking of Stat3 on activated receptors influenced Stat3 activation more significantly than either of these parameters in isolation or in combination with any other parameters. It was further demonstrated that nuclear phosphatase activity, inhibition of SOCS3 and Stat3 nuclear

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export most significantly influenced Stat3 activation. These results were unaffected by how much parameters were changed, and could be averaged over different fold changes in parameter values. The results of the sensitivity analysis were experimentally validated by using chemical inhibitors to specifically target different pathway activation steps and comparing the effects on the resultant Stat3 activation profiles with model predictions.

A different approach was adopted in [19], to produce what the authors refer to as a 'glocal' robustness analysis (see Fig. 3) of two competing models of the cyanobacterial circadian oscillator. The authors propose a two-stage approach where the first step involves the sampling of a large set of parameter combinations spanning several orders of magnitude for each parameter. From this sampling the authors select a subset of 'viable' parameter combinations which preserve the particular performance features of interest. Further sampling is conducted via an iterative scheme, where in each step the sampling distribution is adjusted based on a principle component analysis of the viable set of the previous step. After a Monte Carlo integration, the volume occupied by the set provides a first, crude characterisation of a model's robustness and can aid in model discrimination by proper normalisation. The second stage of the proposed approach defines a set of appropriate normalised local robustness metrics, for example, a measure of how fast the oscillator returns to its cycling behaviour when its trajectory is transiently perturbed with the use of Floquet multipliers, or the sensitivity of the period to perturbations in individual parameters or parameter vectors. These metrics are then evaluated for each viable parameter combination identified in the previous stage, and statistical tests are used to assess the analysis results.

Using this approach, the authors compared two models based on fundamentally different assumptions about the underlying mechanism of the cyanobacterial circadian oscillator, termed the 'autocatalytic' and 'two (phosphorylation)-sites' models, respectively. The results of their analysis showed that the two-sites model had significantly better global and overall local robustness properties than the other model, hence making the assumptions on which it is based a more plausible explanation of the underlying biological reality.

2.3 μ-analysis

In this section, we describe a tool for measuring the robustness of a model to 'simultaneous' variations in the values of several of its parameters. Since its introduction by J.C. Doyle [20, 21], in the early days of robust control theory, the structured singular value, or μ, has become the tool of choice among control engineers for the robustness analysis of complex uncertain systems [22].

It is generally possible to arrange any linear time invariant system, which is subject to some type of norm-bounded uncertainty in the form shown in Fig. 4, where M represents the known part of the system and Δ represents the uncertainty present in the system. Partitioning M compatibly with the Δ matrix, the relationship between the input and output signals of the closed-loop system shown in Fig. 4 is then given by the upper linear fractional transformation (LFT)

$$y = \mathcal{F}_u(M, \Delta)r = (M_{22} + M_{21}\Delta(\boldsymbol{I} - M_{11}\Delta)^{-1}M_{12})r \quad (9)$$

where $\boldsymbol{I}$ is the identity matrix. Now, assuming that the

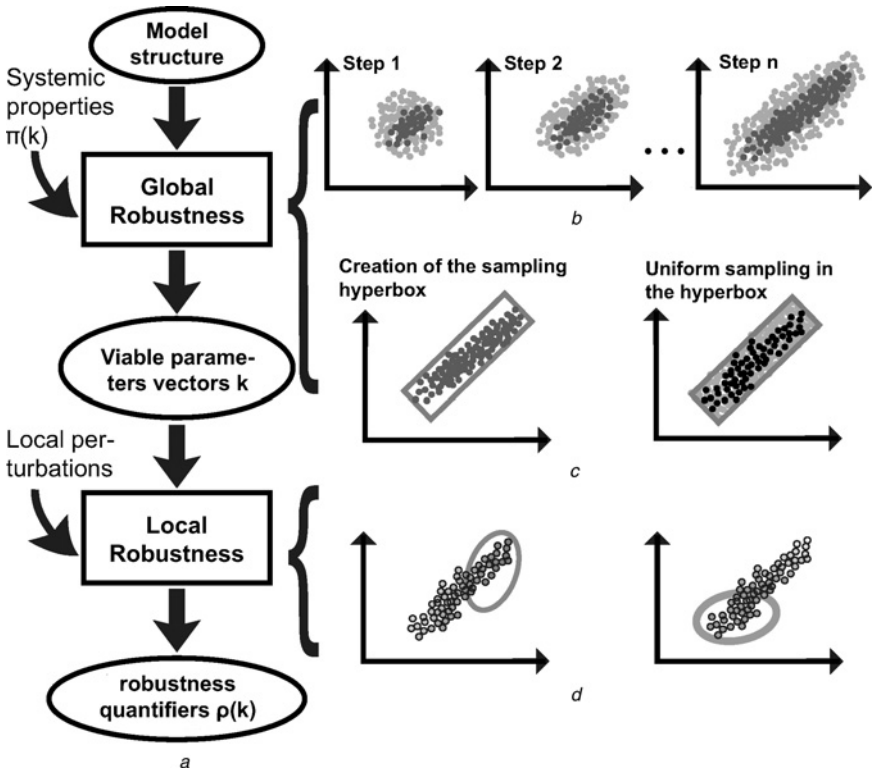

Fig. 3 *'Glocal' robustness analysis method [19]*

a 'Glocal' robustness analysis flow diagram
b Parameter search
c Monte Carlo integration
d Local analyses

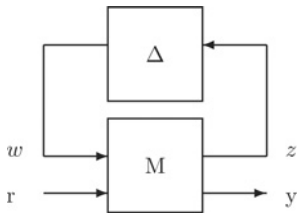

Fig. 4 *Upper LFT uncertainty description*

nominal system M in Fig. 4 is asymptotically stable and that Δ is a complex unstructured uncertainty matrix, the Small Gain Theorem [22], gives the following result: The closed-loop system in Fig. 4 is stable if

$$\overline{\sigma}(\Delta(j\omega)) < \frac{1}{\overline{\sigma}(M_{11}(j\omega))} \quad \forall\omega \tag{10}$$

where $\overline{\sigma}$ denotes the maximum singular value and ω is frequency. The above result defines a test for stability (and thus a robustness measure) for a system subject to 'unstructured uncertainty' in terms of the maximum 'singular value' of the matrix M_{11}.

Now, in cases where the uncertainty in the system also arises because of variations in specific parameters, the uncertainty matrix Δ will have a diagonal or block diagonal structure, that is

$$\Delta(j\omega) = \text{diag}(\Delta_1(j\omega), \ldots, \Delta_n(j\omega)), \quad \overline{\sigma}(\Delta_i(j\omega)) \leq k \quad \forall\omega \tag{11}$$

where, for example, certain Δ blocks could represent parametric uncertainty whereas others represent unmodelled dynamics because of structural changes in the system. Now again assume that the nominal closed-loop system is stable, and consider the question: What is the maximum value of k for which the closed-loop system will remain stable? We can still apply the Small Gain Theorem to the above problem, but the result will be conservative, since the block diagonal structure of the matrix Δ will not be taken into account. The Small Gain Theorem will in effect assume that all of the elements of the matrix Δ are allowed to be non-zero, when we know that many of the elements are in fact zero. Thus the SGT will consider a larger set of uncertainty than is in fact possible, and the resulting robustness measure will be conservative, that is, pessimistic.

In order to obtain a non-conservative solution to this problem, J.C. Doyle [20] introduced the structured singular value μ

$$\mu_\Delta(M_{11}) = \frac{1}{\min(k \text{ s.t. } \det(I - M_{11}\Delta) = 0)} \tag{12}$$

where $\mu_\Delta(M_{11}) = 0$ if there is no Δ which satisfies the determinant condition. The above result defines a test for stability (robustness measure) of a closed-loop system subject to 'structured uncertainty' in terms of the maximum 'structured singular value' of the matrix M_{11}. Singular value performance requirements can also be combined with stability robustness analysis in the μ framework to measure the 'robust performance' properties of the system.

An obvious limitation of the μ framework is that it can only be applied to linear systems. Since almost all biological systems are at least to some extent non-linear, this means that the system model must first be linearised, and hence the robustness measures provided by μ must be treated with caution when it comes to validating models of such systems: essentially μ provides local robustness guarantees about an equilibrium. A second complicating factor is that the computation of μ is an NP hard problem, that is, the computational burden of the algorithms that compute the exact value of μ is an exponential function of the size of the problem. It is consequently impossible to compute the exact value of μ for large dimensional problems, but an effective solution in this case is to compute upper and lower bounds on μ, and efficient routines for μ-bound computation are now widely available [23]. Note that to fully exploit the power of the structured singular value theory, tight upper and lower bounds on μ are required. The upper bound provides a sufficient condition for stability/performance in the presence of a specified level of structured uncertainty. The lower bound provides a sufficient condition for 'instability', and also returns a worst-case Δ, that is, a worst-case combination of uncertain parameters for the problem. The degree of difficulty involved in computing good bounds on μ depends on (a) the order of the Δ matrix, and (b) whether Δ is complex, real or mixed – see [23, 24] for a full discussion.

In [13], the authors employed μ-analysis to evaluate the robustness of a biochemical network model which had been proposed to explain the capability of aggregating *Dictyostelium* cells to produce stable oscillations in the concentrations of intra- and extra-cellular cAMP. Owing to the large number of uncertain parameters in the model, standard routines for computing lower bounds on μ failed for this problem, so that only an upper bound could be computed. Interestingly, and in contrast to the results of a parameter-at-a-time sensitivity analysis, this upper bound suggested a possible high degree of fragility in the model. This lack of robustness was subsequently confirmed by further analyses using a newly developed μ lower bound algorithm [25]. As shown in Fig. 5, simultaneous perturbations in the models kinetic parameters of $1/723 = 0.14\%$ are sufficient to destabilise the oscillations, in stark contrast to the original claims that variations in model parameters over several orders of magnitude had little effect on its dynamics.

μ-analysis was also successfully employed by Jacobsen and co-workers in [26, 27] to investigate the structural basis of robustness in the mammalian circadian clock. Systematic perturbations in the model structure were introduced, and the effects on the functionality of the model were quantified

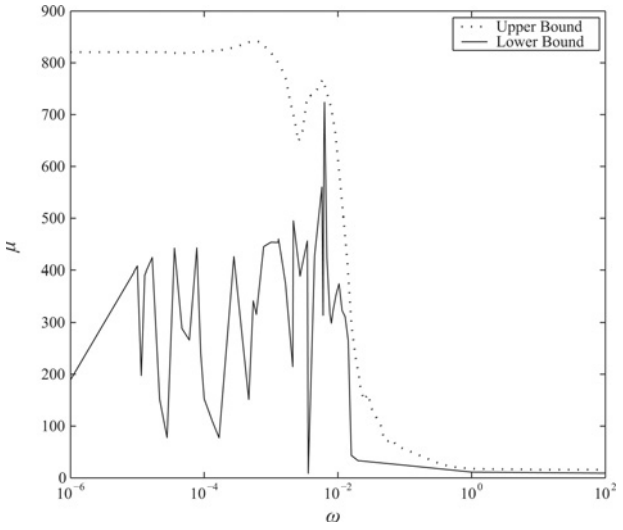

Fig. 5 *μ bounds for Dictyostelium network robustness analysis [25]*

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using the peak value of μ. Although in principle only one feedback loop involving the *Per* gene is required in the chosen clock model to generate oscillations, analysis using the structured singular value revealed that the presence of additional feedback loops involving the *Bmal1* and *Cry* genes significantly increases the robustness of the regulatory network. In [28] a similar approach was also used to validate models of oscillatory metabolism in activated neutrophils. Structural robustness analysis of biochemical and metabolic networks is also considered in [29, 30].

2.4 Optimisation-based robustness analysis

In robustness analysis, numerical optimisation algorithms can be used to search for particular combinations of parameters in the model's parameter space that maximise the deviation of the model's dynamic behaviour from experimental observations over a certain simulation time period. This type of search can be formulated as an optimisation problem of the form

$$\max_{p} c(x, p) \quad \text{subject to} \quad \underline{p} \le p \le \overline{p} \tag{13}$$

where x is a vector of model parameters with upper and lower bounds $\overline{p}$ and $\underline{p}$, respectively, and $c(x, p)$ is an 'objective function' or 'cost function' representing the difference between the simulated outputs of the model and one of more sets of corresponding experimental data [31]. By systematically varying the allowed level of uncertainty (defined by $\overline{p}$ and $\underline{p}$) in the model's parameters, and using the optimisation algorithm to compute the values of the model parameters which maximise this function, an accurate assessment of the model's robustness can be derived. A particular advantage of this approach is that it places little or no constraints on the form or complexity of the model – as long as it can be simulated with reasonable computational overheads, no additional modelling or analytical work is required to apply this approach. This is in sharp contrast to certain analytical approaches, such as μ-analysis or Sum-of-squares programming (see below) which require the model to be represented in a particular form before any analysis can be conducted.

Owing to the complex dynamics and large number of uncertain parameters in many Systems Biology models, the optimisation problems arising in the context of robustness analysis will generally be non-convex, and thus local optimisation methods, which can easily get locked into local optima in the case of multimodal search spaces, are often of limited use. Global optimisation methods, whether based on evolutionary principles [32], or deterministic heuristics [33], are usually much more effective, especially when coupled with local gradient-based algorithms via a hybrid switching strategy [34]. This was the approach adopted in [35], where numerical optimisation algorithms were applied directly to a non-linear biochemical network model to confirm an apparent lack of robustness indicated by a linear analysis using the structured singular value. Interestingly, it appears that the idea of using global optimisation to analyse the robustness and validity of complex simulation models was not first proposed in an engineering context, but by social scientists, who labelled the technique 'active non-linear tests (ANTs)' [36].

2.5 Sum-of-squares polynomials

Sum-of-squares (SOS) programming has recently been introduced in the Systems Biology literature as a powerful new framework for the analysis and validation of a wide class of models, including those with non-linear, continuous, discrete and hybrid dynamics, [37, 38]. A polynomial $p(y)$, with real coefficients, where $y \in R^n$, admits an SOS decomposition if there exist other polynomials $q_1, \ldots, q_m$ such that

$$p(y) = \sum_{i=1}^{m} q_i^2(y) \tag{14}$$

where the subscripts denote the index of the m polynomials. If $p(y)$ is SOS, it can be easily seen that $p(t) \ge 0$ for all y, which means that $p(y)$ is non-negative. Polynomial non-negativity is a very important property (as many problems in optimisation and systems theory can be reduced to it) which is however very difficult to test (it has been shown to be NP-hard for polynomials of degree greater than or equal to 4). The existence of a SOS decomposition is a powerful relaxation for non-negativity because it can be verified in polynomial time. The reason for this [39] is that $p(y)$ being SOS is equivalent to the existence of a positive semidefinite matrix Q (i.e. Q is symmetric and with non-negative eigenvalues) and a chosen vector of monomials $Z(y)$ such that

$$p(y) = Z^{\mathrm{T}}(y) Q Z(y) \tag{15}$$

This means that that the SOS decomposition of $p(y)$ can be efficiently computed using Semidefinite Programming [40] and software capable of formulating and solving these types of problems is now widely available [38]. To see how this framework can be applied to the problem of model validation (or more precisely, model 'invalidation') consider a model in the form of an autonomous, ordinary differential equation

$$\dot{x} = f(x, p) \tag{16}$$

where p is a vector in the allowable set of parameters $\mathcal{P}$ for the model and f satisfies appropriate smoothness conditions in order to ensure that given an initial condition there exists a locally unique solution. Now, for the system in question, assume that a set of experimental data $(t_i, \hat{x}_i)$ for $i = 1, \ldots, N$ exists, where the data points $\hat{x}_i \in \mathcal{X}_i$. Thus the sets $\mathcal{P}$ and $\mathcal{X}_i$ encode the uncertainty in the model parameters and the uncertainty in the data because of experimental error, respectively. We assume that these sets are 'semi-algebraic', that is, that they can be described by a finite set of polynomial inequalities. For example, if $\hat{x}_1^{(i)} \in [\underline{\hat{x}_1^{(i)}}, \overline{\hat{x}_1^{(i)}}]$ a for $i = 1, \ldots, n$, where $\hat{x}_1^{(i)}$ refers to the ith element of the experimental data taken at time t_1, then we obtain the n-dimensional hypercube

$$\mathcal{X}_1 = [\hat{x}_i \in \mathcal{R}^n | (\hat{x}_1^{(i)} - \underline{\hat{x}_1^{(i)}})(\hat{x}_1^{(i)} - \overline{\hat{x}_1^{(i)}}) \le 0, \quad i = 1, \ldots, n] \tag{17}$$

In this case there is a set of experimental time-course data points (due to significant levels of uncertainty arising from measurement noise etc.) and a set of models (due to uncertainty in the model's parameter values). A robust model would produce simulation results, for all models in

the given set, that match some data points in the set of experimental data. If however, we can show that no model from the set $\mathcal{P}$ matches even one data point in the set $\mathcal{X}_i$, then the model is clearly not robust and may be said to be invalidated. Note that in order to invalidate a model, one data point at $t = \mathcal{L}$ where $\mathcal{L} \in \{2, \ldots, N\}$, together with the initial time point t_1, is sufficient (usually the point with the largest residual between the nominal model and the data is selected).

The above problem can be solved using SOS programming via a method similar in concept to that of constructing a Lyapunov function to establish equilibrium stability. Lyapunov functions ensure the stability property of a system by guaranteeing that the state trajectories do not escape their sublevel sets. In [37], the related concept of barrier certificates is introduced. These are functions of state, parameter and time, whose existence proves that the candidate model is invalid given a parameter set and experimental data, by ensuring that the model behaviour does not intersect the set of experimental data. Consider a system of the form given in (16), and assume that $x \in \mathcal{X} \in \mathcal{R}^n$. Given this information, if it can be shown that for all possible system parameters $p \in \mathcal{P}$ the model cannot produce a trajectory $x(t)$ such that $x(t_1) \in \mathcal{X}_1, x(t_\mathcal{L}) \in \mathcal{X}_\mathcal{L}$ and $x(t) \in \mathcal{X}$ for all $t \in [t_1, t_\mathcal{L}]$, then the model and parameter set are invalidated by $\mathcal{X}_1, \mathcal{X}_\mathcal{L}, \mathcal{X}$. This idea is formalised in the following theorem [37]:

Theorem 1: Given the candidate model (16) and the sets $\mathcal{X}_1, \mathcal{X}_\mathcal{L}, \mathcal{X}, \mathcal{P}$, suppose there exists a real valued function $B(x, p, t)$ that is differentiable with respect to x and t such that

$$B(x_\mathcal{L}, p, t_\mathcal{L}) - B(x_1, p, t_1) > 0, \quad \forall (x_\mathcal{L}, x_1, p) \in \mathcal{X}_\mathcal{L} \times \mathcal{X}_1 \times \mathcal{P}$$

$$\frac{\delta B(x,p,t)}{\delta x} f(x,p) + \frac{\delta B(x,p,t)}{\delta t} \leq 0, \quad \forall (x,p,t) \in \mathcal{X} \times \mathcal{P} \times [t_1, t_\mathcal{L}]$$

Then the model is invalidated by $\mathcal{X}_1, \mathcal{X}_\mathcal{L}, \mathcal{X}$ and the function $B(x, p, t)$ is called a barrier certificate.

A key advantage of SOS programming is that these barrier certificates can be constructed algorithmically using Semidefinite Programming and SOSTOOLS software. Using this approach, Papachristodoulou and co-workers showed in [10] how a barrier certificate could be constructed for a simple generic biochemical network model, hence invalidating the model over a certain range of its parameters for a given set of time-course data, whereas in [41] they showed how the same approach could be used to test a model of G-protein signalling in yeast. In [42] they demonstrated the use of SOS tools for the design of input experiments which maximise the difference between the outputs of two alternative models of bacterial chemotaxis. This approach can be used to design experiments to produce data that are most likely to invalidate incorrect model structures. Other recent papers that have exploited semidefinite programming include [43], which develops tools for quantifying the robust stability of uncertain genetic networks with sum regulatory functions, and [44], which develops a test for model validity based on approximating the set of parameters for which model trajectories are consistent with the available experimental data.

The main advantages of the SOS approach is that it can be applied to non-linear models and that it is simulation-free, that is, the results are analytical and thus provide guaranteed results. This is in contrast to simulation-based approaches which, for example, can never 'prove' that a model with a given set of uncertain parameters will not enter a defined region of state space (although of course in practice one can obtain answers to such questions with arbitrarily high statistical confidence if one is prepared to run enough simulations – see below). The main limitation of SoS techniques, aside from certain restrictions they place on the form of the model equations, is due to the computational limitations of the semidefinite programming software, which currently prohibits their application to high-order models.

2.6 Monte Carlo simulation

Monte Carlo simulation has for many years been the method of choice in the engineering industry for examining the effects of uncertainty on complex simulation models. The method is extremely simple, and relies on repeated simulation of the system over a random sampling of points in the model's parameter space. The sampling of the system's parameter space is usually carried out according to a particular probability distribution, for example, if there are reasons to believe that it is more likely for the system's actual parameter values to be near the nominal model values than to be near their uncertainty bounds, then a normal distribution may be used, whereas if no such information is available a uniform distribution may be chosen. For a given number of samples of a system's parameter space, statistical results can be derived which may be used to evaluate the effects of uncertainty on the system's behaviour. For the purposes of robustness analysis, these results provide probabilistic confidence levels that the extremal behaviour found among the Monte Carlo simulations is within some distance of the true 'worst-case' behaviour of the system.

The numbers of Monte Carlo simulations required to achieve various levels of estimation uncertainty with different confidence levels were calculated using the Chebyshev inequality and central limit theorem in [45] and are reproduced here in Table 1. Alternatively, if we use the well-known Chernoff bound [46, 47] to estimate the number of simulations required, the numbers are as shown in Table 2. Note that in both cases it is clear that the number of samples required to produce a given set of statistical results is 'independent' of the number of

Table 1 Numbers of simulations for various confidence and accuracy levels (derived using the Chebyshev inequality and central limit theorem [45])

Percent of estimation uncertainty	20%	15%	10%	5%	1%
Uncertainty probability range					
0.750 → 0.954	25	45	100	400	10 000
0.890 → 0.997	57	100	225	900	22 500
0.940 → 0.999	100	178	400	1600	40 000

Table 2 Numbers of Monte Carlo simulations required for various confidence and accuracy levels (derived using the Chernoff bound [46])

%, Confidence	Accuracy level ε	No. of simulations
99	0.05	1060
99.9	0.01	27 081
99.9	0.005	108 070

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uncertain parameters in the model, and this, together with the absence of any requirements on the form of the model, represents the main advantage of Monte Carlo simulation for robustness analysis. The key disadvantage of the approach, however, is also readily apparent from the tables, namely, the exponential growth in the number of simulations with respect to the statistical confidence and accuracy levels required – typically at least 1000 simulations would be required in engineering applications before the statistical performance guarantees would be considered reliable.

Although the statistical nature of the results generated using Monte Carlo simulation can sometimes hinder the comparison of the robustness properties of different models, one very useful capability of this approach is that it allows the characterisation of the size and shape of robust or non-robust regions of parameter space. This is often an important issue in robustness analysis, since it is clear that a model which fails a robustness test because of a single (perhaps biologically unrealistic) parameter combination should not be considered equivalent to a model which contains a large region of points which fail the same test. For example, in [35], Monte Carlo simulation was used to establish that the loss of oscillatory behaviour of a biochemical network model was not due to a single point but to a significant region in its parameter space. In [48], Bullinger and co-workers evaluate the robustness of models of the direct signal transduction pathway of receptor-induced apoptosis via Monte Carlo simulation. By analysing the topology of robust regions of parameter space, the authors were able to evaluate the robustness of the bistable threshold between cell reproduction and death, and hence discriminate between competing models of the network.

3 Biological case studies

In this section, we describe a number of different biological systems for which the use of robustness analysis has been an integral part of the model development and validation process.

3.1 P53-Mdm2 system

The negative feedback loop between the tumor suppressor p53 and the oncogene *MDM2* is by now one of the best-studied protein circuits in human cells [49]. Cells that experience stresses such as DNA damage, hypoxia and abnormal oncogene signals activate an array of internal self-defense mechanisms. One of the most important of these is the activation of the tumor suppressor protein p53, which transcribes genes that induce cell cycle arrest, DNA repair and apoptosis. p53 transcriptionally activates the Mdm2 protein which, in turn, negatively regulates p53 by both inhibiting its activity as a transcription factor and by enhancing its degradation rate, see Fig. 6. Many additional proteins interact with p53 and Mdm2, so that the negative feedback loop is embedded inside a network of additional interactions, many of which are not fully characterised. The negative feedback loop formed by p53 and Mdm2 also includes significant time delays arising from transcriptional and translational processes, and as a result can produce complex oscillatory dynamics. Oscillations of p53 and Mdm2 protein levels in response to ionising radiation (IR)-induced DNA damage appear to be damped in assays that measure averages over population of cells. Recent *in vivo*

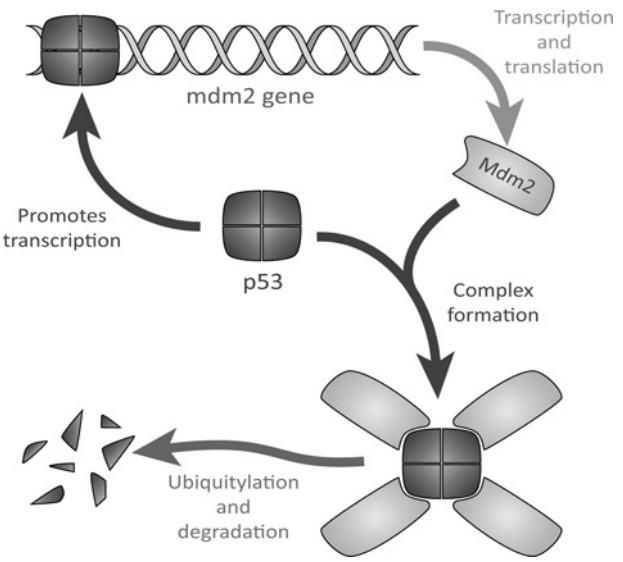

Fig. 6 *p53-Mdm2 system*

fluorescence measurements in individual cells, however, have shown undamped oscillations of p53 and Mdm2 lasting for at least 3 days. Although the oscillations are initially synchronised to the gamma irradiation signal, small variations in the timing of these oscillations inevitably arise because of stochastic variations across individual cells, causing the peaks to eventually go out of phase and thus the p53 and Mdm2 dynamics to appear as damped oscillations in assays over cell populations [50].

Intriguingly, single-cell measurements in experiments with varying levels of IR have also revealed that increased DNA damage produces (on average) a greater number of oscillations, but has no effect on their average amplitude or period. The precise biological purpose of this 'digital' type of response still remains to be fully elucidated, but one theory is that the oscillations of p53 may act as a timer for downstream events – genes inducing growth arrest (e.g. p21) are rapidly expressed during the first oscillation of p53, whereas proapoptotic p53 target genes such as *NOXA, PUMA* or *BAX* are gradually integrated over multiple cycles of p53 pulses, ratcheting up at each pulse until they reach a certain threshold value that activates apoptosis [51].

Several recent studies have attempted to develop computational models of the complex dynamics of this system. In [51], Wagner and co-workers developed a model in which ATM, a protein that senses DNA damage, activates p53 by phosphorylation. Activated p53 is modelled as having a decreased degradation rate and an enhanced transactivation of Mdm2. The model includes two explicit time delays, the first representing the processes (primarily, elongation and splicing) underlying the transcriptional production of mature, nuclear Mdm2 mRNA, and the second representing Mdm2 transport to the cytosol, translation to protein and transport of Mdm2 protein into the nucleus. As part of the model development process, the authors examined a large number of variations in their model to evaluate its ability to robustly generate oscillations in the presence of significant levels of model uncertainty. For example, they explored other kinetics for ATM activation of p53 and Mdm2 ubiquitination of p53 and considered the effects of adding both Mdm2-dependent and Mdm2-independent ubiquitination of active p53. In all cases, the model was shown to be robust to such changes, and the conclusions arising from its analysis did not

change. An investigation of the effects of varying different model parameters was carried out using bifurcation analysis, and this analysis produced new predictions regarding the source of robustness in the oscillatory dynamics. For example, with activated ATM-stimulated Mdm2 degradation, sustained oscillations occurred in the model if the total time delay is more than a 16-min threshold. When the activated ATM-dependent degradation of Mdm2 was removed, however, while keeping the rest of the model parameters at their nominal values, then there are no sustained oscillations regardless of how high the time delay and the DNA damage is. Thus, the mechanism of activated ATM-dependent degradation of Mdm2 appears to be a key factor in ensuring oscillatory robustness in this system.

In another recent study of the p53 system, Alon and co-workers considered six different mathematical models of the p53–Mdm2 system [50]. All of the models include the negative feedback loop in which p53, denoted by x, transcriptionally activates Mdm2, denoted by y, and active Mdm2 increases the degradation rate of p53. Three of the models were delay oscillators: Model I includes an Mdm2 precursor representing, for example, Mdm2 mRNA, and the action of y on x is described by first-order kinetics in both x and y. In model IV, the action of y on x is non-linear, and described by a saturating Michaelis–Menten function. In model III, the Mdm2 precursor is replaced by a stiff delay term, which makes the production rate of Mdm2 depend directly on the concentration of p53 at an earlier time. Note that the model of Wagner and Ma described above combines features of models III and IV. In addition to the three delay oscillators, the authors also considered two relaxation oscillators (II and V) in which the negative feedback loop is supplemented by a positive feedback loop on p53. This positive feedback loop might represent in a simplified manner the action of additional p53 system components, which have a total upregulating effect on p53. These models include both linear positive regulation (model V) and non-linear regulation based on a saturating function (model II). Models (I–V), although differing in detail, all rely on a single negative feedback loop. The last model (VI) considered in the study proposes a novel checkpoint mechanism, which uses two negative feedback loops, one direct feedback and one longer loop that impinges on an upstream regulator of p53. In this model, a protein downstream of p53 inhibits a signaling protein that is upstream of p53.

In order to discriminate between these six different models of the p53 system, the authors numerically solved all six models for a wide range of parameter values and evaluated the ability of the different model structures to robustly generate stable undamped oscillations. Models I–III were shown to be incapable of robustly producing stable undamped oscillations, whereas, in contrast, models IV–VI could generate sustained or weakly damped oscillations over a broad range of parameter values. Interestingly, most of the parameters shared by these three models showed very similar best-fit values, indicating that these models may provide estimates of the effective biochemical parameters such as production rates and degradation times of p53 and Mdm2. When low-frequency multiplicative noise was added to the protein production terms in the model to take account of stochasticity in protein production rates, all models showed qualitatively similar dynamics to those found in experiments, including occasional loss of a peak. However, only model VI was able to reproduce the authors' experimental observations that p53 and Mdm2 peak amplitudes had only a weak correlation (all other models had a strong coupling in the variations of the peaks of these two proteins).

Finally, a recent study of the robustness of the p53 protein-interaction network [52] shows that the idea of robustness analysis can also be usefully applied at the topological network level. By subjecting the model to both random and directed perturbations representing stochastic gene knockouts from mutation during tumourigenesis, the p53 cell cycle and apoptosis control network could be shown to be inherently robust to random knockouts of its genes. Importantly, this robustness against mutational perturbation was seen to be provided by the structure of the network itself. This robustness against mutations, however, also implies a certain fragility, as the reliance on highly connected nodes makes it vulnerable to the loss of its hubs. Evolution has produced organisms that exploit this very weakness in order to disrupt the cell cycle and apoptosis system for their own ends: tumour-inducing viruses (TIVs) target specific proteins to disrupt the p53 network, and this study identified these same proteins as the network hubs. Although TIVs had previously been likened to 'biological hackers', this study showed why the TIV attack is so effective: TIVs target a specific vulnerability of the network that can be explained by analysing the robustness of the network architecture.

3.2 Bacterial chemotaxis

A key requirement of many biological sensing devices is the ability to adapt to a persistent input stimulus, thereby increasing the range of sensitivity of the sensor. This capability is particularly apparent in the signaling apparatus mediating bacterial chemotaxis, which exhibits perfect adaptation to chemoattractants: the output is reset exactly to the prestimulus value so that the steady-state behaviour of the system is independent of the concentration of a homogeneous distribution of the attractant [53]. Bacteria traverse gradients of chemoeffectors by engaging in a biased random walk consisting of alternating periods of smooth runs and random tumbles. Detecting higher levels of a chemoattractant decreases the probability of a tumble, thus propelling the bacteria in the favourable direction. This control over the length of runs is mediated by a signal transduction pathway consisting of transmembrane receptors (methyl-accepting proteins) and the products of six Che genes: cheA, cheB, cheR, cheW, cheY and cheZ.

In [54], Alon and co-workers provided strong experimental evidence that the precision of adaptation in bacterial chemotaxis in *Escherechia coli* is robust to dramatic changes in the levels of the chemotactic proteins making up the signalling network. Based on this demonstrated level of robustness in the real system, J.C. Doyle and co-workers considered the plausibility of two different mathematical models of the underlying signalling network [53]. Using a simple type of bifurcation analysis, the authors were able to demonstrate that only one of the two models was consistent with the observed robustness of the system, and that this robustness derived from a particular type of feedback control mechanism which is widely used in engineered industrial control systems, that is, integral feedback control.

Finally, in [42], Papachristodoulou and co-workers applied a novel approach for iteratively invalidating models to the chemotaxis pathway of the bacterium *Rhodobacter sphaeroides*. The approach allows the systematic design of

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in silico experiments to determine the inputs and model parameter perturbations that will differentiate best between model outputs and experimental observations. The designed experiments were then performed on live cells and the resulting data used to invalidate all but one of a set of competing candidate models.

3.3 Circadian clocks

In addition to the studies by the groups of F.J. Doyle and Jacobsen cited previously, several other authors have successfully applied robustness analysis to gain an improved understanding of the design principles underlying circadian clocks.

In [55], Millar and co-workers used an iterative cycle of experiment and mathematical analysis to extend a model of the clock network in the higher plant *Arabidopsis thaliana*. The model comprises interlocking feedback loops comparable to those identified experimentally in other circadian systems. Validation of the model proceeded by finding optimal parameter sets to fit the experimental data and then checking the robustness of the model to parameter variations. For example, changes in the period and amplitude of TOC1 RNA oscillation under light–light cycles were examined after a 5% increase or decrease of each model parameter value in turn. A key finding validating the proposed multiple feedback loop model was that its period and amplitude were much less sensitive to parameter changes than a previously developed single-loop LHY/CCA1–TOC1 model. Similar results validating an interlocked feedback loop model of the network generating circadian rhythms in Drosophila were derived in [56].

An evolutionary perspective on the generation of robust network topologies is provided by Wagner in [57], where the author investigated in silico several hundred different topologies for a simple biochemical model of circadian oscillations. This study found that the distribution of robustness among different network topologies was highly skewed, with most showing low robustness, and a very few topologies (involving the regulatory interlocking of several oscillating gene products) being highly robust. To address the question of how robust network topologies could have evolved, the author defines a topology graph, each of whose nodes corresponds to one circuit topology that shows circadian oscillations. Two nodes in this graph are connected if they differ by only one regulatory interaction within the circuit. For the circadian oscillator under consideration, it could be shown that most topologies are connected in this graph, thus facilitating evolutionary transitions from low to high robustness. Interestingly, other studies of the evolution of robustness in biological macromolecules have generated similar results, suggesting that the same principles may govern the evolution of robustness on different levels of biological organisation.

Finally, a recent paper by Akman and co-workers investigated the notion of 'flexibility' as an important counterpoint to robustness in circadian clocks [58]. Flexibility measures how readily the rhythmic profiles of all the molecular clock components can be altered by modifying the biochemical parameters or environmental inputs of the clock circuit. Robustness, on the other hand, describes how well a biological function, such as the phase of a particular clock component, is maintained under varying conditions. As noted in [58], the relationship between these two high-level properties can be a rather complex one, depending on the particular properties of the system of interest. This is because, although flexibility might be assumed to imply decreased robustness by increasing sensitivity to perturbations, in certain cases it can also yield greater robustness by enhancing the ability of the network to tune key environmental responses. This somewhat paradoxical result was nicely illustrated in the authors' analysis of a model of the fungal circadian clock, which is based on the core FRQ-WC oscillator that incorporates both negative frq and positive wc-1 loops, as well as part of the light-signalling pathway. By introducing a simple measure of the flexibility of the network, based on quantifying how outputs of the entrained clock vary under parameter perturbations achievable by evolutionary processes, the authors demonstrate that the inclusion of the positive wc-1 feedback loop yields a more flexible clock. This increased flexibility is shown to be primarily characterised by a greater flexibility in entrained phase, leading to 'enhanced' robustness against photoperiod fluctuations.

3.4 Mitogen-activated protein kinase pathway

The mitogen-activated protein kinase (MAPK) cascade is a highly conserved signal transduction pathway found in organisms of complexity spanning from yeast to humans. This signal transduction pathway has drawn much interest from systems biologists in recent years, and several computationally intensive models have been developed which have been shown to display levels of parametric robustness corresponding to experimentally measured data [59, 60]. In many mammalian tissue types, this pathway can correctly transduce signals from different extracellular messengers, leading to specific and often mutually exclusive cellular responses. The transduced signal is tuned by a set of positive and negative feedback control mechanisms and fed into a downstream gene expression network. A key question which arises in the study of this system is the nature of the relationship between these regulatory mechanisms and the specificity (the total amount of proper pathway output divided by the spurious pathway output, for a given input) of the pathway. In [61], Thalhauser and Komarova addressed this question by formulating a new and interesting definition of robustness, that is, 'robust specificity': the ability of a signal transduction network to cope with variations in input signal profiles so that it can properly interpret wide ranges of input signals into the proper temporal output. By analysing a number of different models, the authors showed that the complicated nature of the feedback controls involved in the mammalian MAPK pathway confers robust specificity, thus allowing the cell to identify and transduce the proper signal without having to invest in two completely separate signal cascades.

3.5 cAMP oscillations in Dictyostelium cells

A series of recent papers have used robustness analysis to interrogate and extend a model, originally proposed in [62], of the biochemical network underlying stable oscillations in cAMP in aggregating *Dictyostelium* cells. In this network model, shown in Fig. 7, cAMP is produced inside the cell when adenylyl cyclase is activated after the binding of extracellular cAMP to the surface receptor CAR1. Ligand-bound CAR1 activates the mitogen-activated protein kinase (ERK2), which in turn inhibits the cAMP phosphodiesterase RegA by phosphorylating it. When

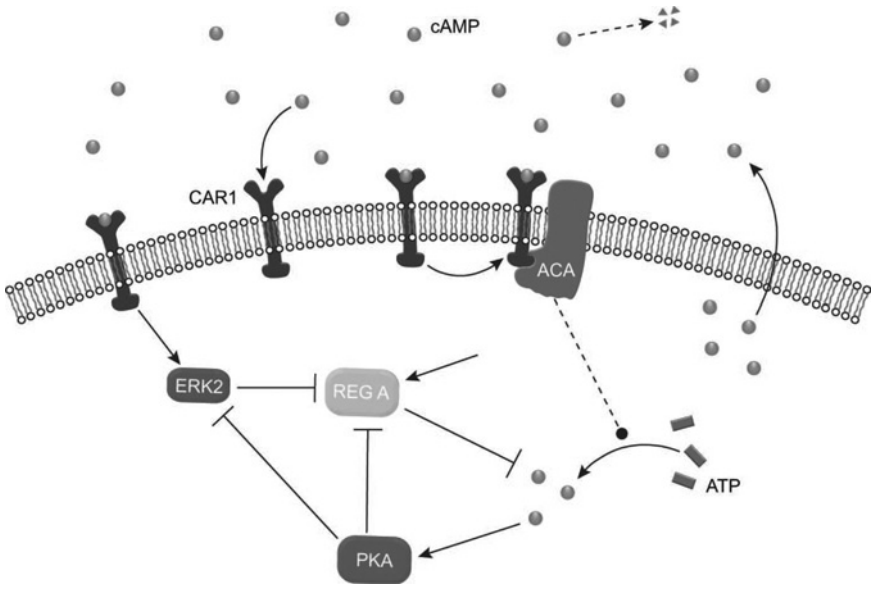

Fig. 7 *Model of [62] for the network underlying cAMP oscillations in Dictyostelium*
Normal arrows and the broken arrows represent activation and self-degradation, respectively. The bar arrows represent inhibition

cAMP accumulates internally, it activates the protein kinase PKA by binding to the regulatory subunit of PKA. ERK2 is inactivated by PKA and hence can no longer inhibit RegA by phosphorylating it. A protein phosphatase activates RegA such that RegA can hydrolyse internal cAMP. Either directly or indirectly, CAR1 is phosphorylated when PKA is activated, leading to loss-of-ligand binding. When the internal cAMP is hydrolysed by RegA, PKA activity is inhibited by its regulatory subunit, and protein phosphatase(s) returns CAR1 to its high-affinity state. Secreted cAMP diffuses between cells before being degraded by the secreted phosphodiesterase. The dynamics of the network shown in Fig. 7 can be expressed as a set of non-linear differential equations with kinetic constants k_{1-14}. The activity of each of the seven components in the network is determined by the balance between activating and inactivating enzymes which is then reflected in the equations in the form of an activating and deactivating term. The model thus consists of a set of non-linear differential equations in the following form

$$\begin{aligned}
\frac{\mathrm{d}\ \mathrm{ACA}}{\mathrm{d}t} &= k_1\,\mathrm{CAR1} - k_2\,\mathrm{ACA}\,\mathrm{PKA}\\
\frac{\mathrm{d}\ \mathrm{PKA}}{\mathrm{d}t} &= k_3\,\mathrm{cAMPi} - k_4\,\mathrm{PKA}\\
\frac{\mathrm{d}\ \mathrm{ERK2}}{\mathrm{d}t} &= k_5\,\mathrm{CAR1} - k_6\,\mathrm{PKA}\,\mathrm{ERK2}\\
\frac{\mathrm{d}\ \mathrm{Re}\,gA}{\mathrm{d}t} &= k_7 - k_8\,\mathrm{ERK2}\ \mathrm{RegA} \qquad (18)\\
\frac{\mathrm{d}\ \mathrm{cAMPi}}{\mathrm{d}t} &= k_9\,\mathrm{ACA} - k_{10}\,\mathrm{RegA}\ \mathrm{cAMPi}\\
\frac{\mathrm{d}\ \mathrm{cAMPe}}{\mathrm{d}t} &= k_{11}\,\mathrm{ACA} - k_{12}\,\mathrm{cAMPe}\\
\frac{\mathrm{d}\ \mathrm{CAR1}}{\mathrm{d}t} &= k_{13}\,cAMPe - k_{14}\,\mathrm{CAR1}
\end{aligned}$$

where cAMPi and cAMPe are internal and external cAMP, respectively. The dynamics of this model were shown in [62] to closely match experimental data for the period, relative amplitudes and phase relationships of the oscillations in the concentrations of the molecular species involved in the network. Based on ad-hoc simulations, the model was also claimed to be robust (in terms of the period and amplitude of its oscillations) to very large changes in the values of its kinetic parameters, and this robustness was cited as a key advantage of the model over previously published models in the literature. However, a formal analysis of the robustness of the model to simultaneous variations in the values of its kinetic constants, using the structured singular value μ and global non-linear optimisation, revealed extremely poor robustness characteristics [35] as shown in Fig. 5. This rather surprising result merited further investigation in a number of follow-up studies, since the experimental justification for the proposed network structure appeared sound. The first of these studies [63] used Monte Carlo simulation to evaluate the effects of intrinsic stochastic noise, as well as the effects of synchronisation between individual *Dictyostelium* cells, on the robustness of the resulting cAMP oscillations. Interestingly, the effect of intrinsic noise was to 'enhance' the robustness of cAMP oscillations to variations between cells, whereas synchronisation of oscillations between cells via a shared pool of external cAMP also significantly improved the robustness of the system. Two further studies suggested a significant role for other subnetworks involving calcium and IP3 in generating robust oscillations [64, 65]. Using a combination of structural robustness analysis [64] and biophysical modelling [65], an extended model including these subnetworks (Fig. 8) was constructed which exhibited significantly higher robustness than the original model, as shown in Fig. 9. The results of these studies clearly illustrate the power of robustness analysis techniques to analyse, develop and refine computational models of biochemical networks.

3.6 Physiological simulation models

As shown above, the analysis of model robustness has now been used as an integral part of the model development

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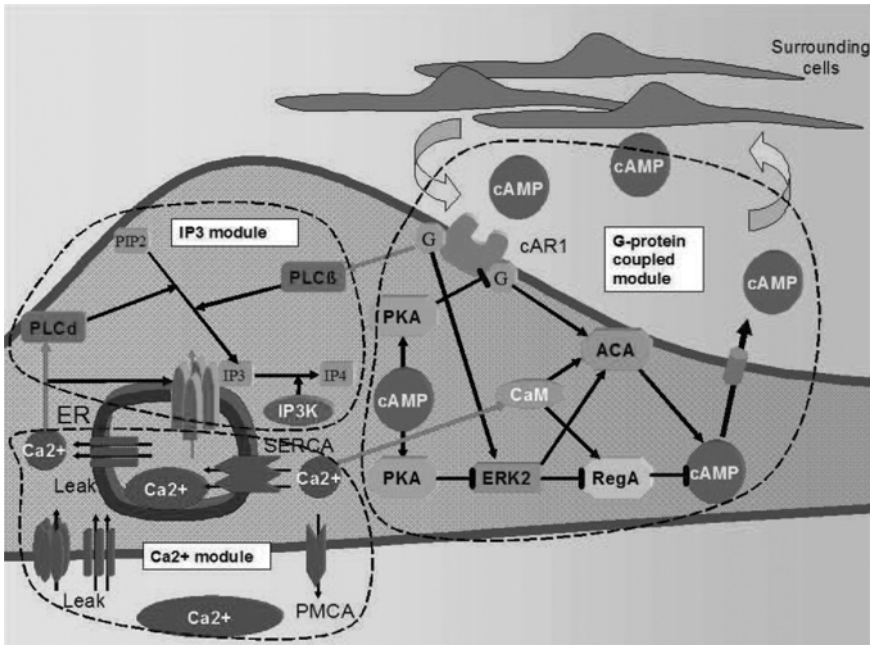

Fig. 8 *Extended model of the Dictyostelium cAMP oscillatory network incorporating coupled subnetworks involving Ca^{2+} and IP3 [65]*

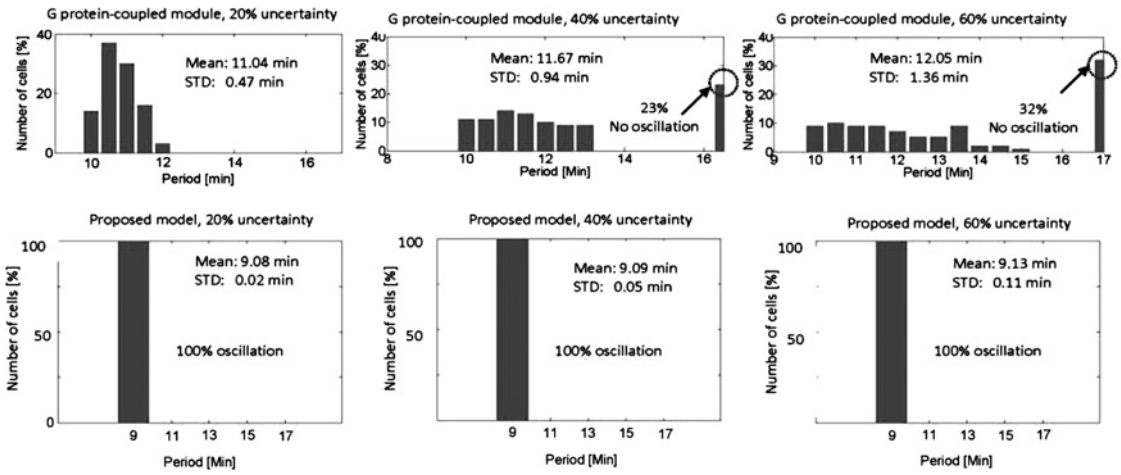

Fig. 9 *Comparison of the robustness of the original and extended model to variations in four kinetic parameters common to both models*
Analysis conducted using Monte Carlo simulations with three different levels of parametric uncertainty [64]

process for a wide range of biological systems. To date, this approach has been almost completely confined to studies of systems at the molecular level, but in principle there is no reason why it could not also be applied to the development of Systems Biology models at the organ, organism or even ecological scales. A first step in this direction was recently made in a paper by Hardman and co-workers [66], who used an optimisation-based analysis framework, Fig. 10, to investigate the robustness of a pulmonary physiology simulator representing a dynamic in-vivo cardio-pulmonary state iterating through a mass-conserving set of equations based on established physiological principles. Physiological simulation models that are intended for use in clinical environments face harsh expectations from medical practitioners; they must cope with significant levels of uncertainty arising from non-measurable parameters, population heterogeneity and disease heterogeneity, and their validation must provide watertight proof of their applicability and reliability in the clinical arena. In exactly the same way that various kinetic parameters in molecular-level models will be inherently uncertain, many parameters in physiological simulators will also contain significant levels of uncertainty – in this case variations in parameters representing the haemoglobin level (Hb), cardiac output (CO), oxygen consumption (VO_2), respiratory quotient (RQ) and the core body temperature (T) are considered. By

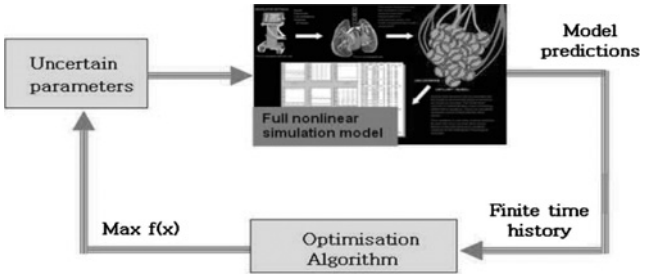

Fig. 10 *Optimisation-based model validation framework for a pulmonary physiology simulator [66]*

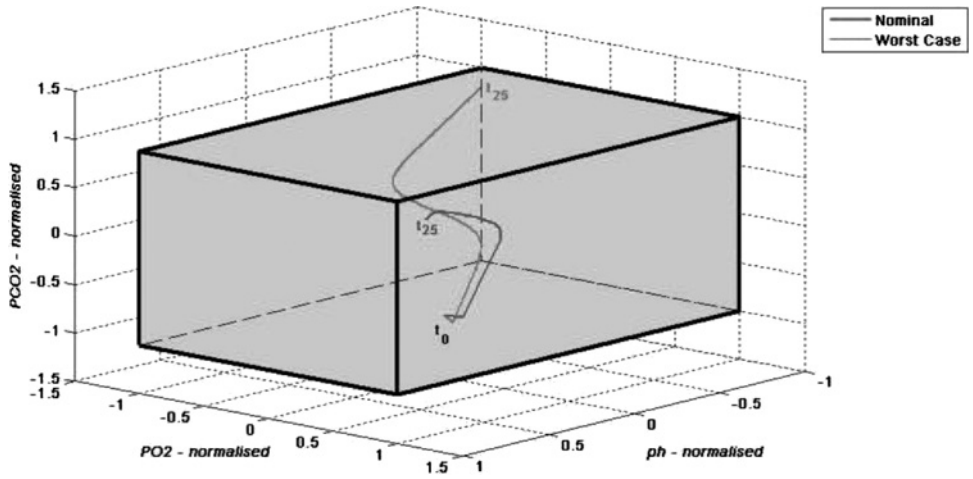

Fig. 11 *Nominal and worst-case model predictions for normalised PO_2, PCO_2 and pH*
t_0 – simulation starting time, t_{25} – simulation end time, box – allowable values [66]

combining explicit modelling of uncertainty/variability with advanced global optimisation methods, the authors demonstrate that the model predictions for the partial pressures of oxygen, carbon dioxide and blood pH never deviate from physiologically plausible values for all realistic levels of parametric uncertainty – see Fig. 11.

4 Conclusions and outlook for future research

The growth in interest in the notion of robustness in Systems Biology research over the last decade has been remarkable, and must represent one of the most striking examples of the wholesale transfer of an idea from the field of engineering to the life sciences. Along with this interest in biological robustness per se, has come the recognition that many of the tools and methods that have been developed within engineering to analyse the robustness of complex systems can be usefully employed by Systems Biologists in their efforts to develop and validate computational models. In a pleasing example of interdisciplinary feedback, this interest has recently spurred the development of several new analysis techniques which are specifically oriented towards the analysis of biological systems.

In [67], for example, Kwon and Cho used a computational approach to investigate generic topological properties leading to robustness and fragility in large-scale biomolecular networks. This study found that networks with a larger number of positive feedback loops and a smaller number of negative feedback loops are likely to be more robust against perturbations. Moreover, the nodes of a robust network subject to perturbations are mostly involved with a smaller number of feedback loops compared with the other nodes not usually subject to perturbations. This topological characteristic could eventually make the robust network fragile against unexpected mutations at the nodes which had not previously been exposed to perturbations. In [68, 69], meanwhile, Chaves and Sontag propose novel analytical approaches for estimating the size and shape of robust regions in parameter space, which could provide useful complements or alternatives to traditional Monte Carlo analysis. Another fundamental topic in Systems Biology is the effect of intrinsic stochastic noise on the stability of biological network models. Promising initial adaptations of traditional control engineering analysis techniques to address this issue were recently reported in [70, 71], and there is clearly tremendous scope for extending these results to deal with related robustness analysis problems.

The outlook for future research in this area is clearly very positive, as the range of biological systems to which the approach to model validation outlined in this paper is applied will no doubt continue to grow. This process will necessitate the development of new robustness analysis tools, which can handle models that do not fall into the traditional category of differential equation-based systems, for example, Boolean network models, Bayesian networks, hybrid dynamical systems etc. As usual, progress is likely to be most rapid on the interface between traditionally separate domains of expertise, for example, statistics and dynamical systems [72] or evolutionary theory and control theory [73, 74].

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