Structural Identification of Nonlinear Dynamic Biomolecular Feedback and Feedforward Loops

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Abstract: Many biomolecular networks are still widely unknown. With high structural uncertainties, bottom-up identification based on model discrimination tends to fail because of combinatorially many possible model structures. Top-down approaches, in contrast, describe general signal processing properties, rather than specific mechanisms. Most of these approaches originated in engineering disciplines where linearity is commonly (approximately) given, but naturally evolved networks exhibit a rather high degree of nonlinearity. Here, we present a top-down structural identification method that is not only applicable to nonlinear biomolecular networks, but indeed requires the network under study to be nonlinear. Similar to traditional frequency domain analysis, we apply an oscillatory input signal to a biomolecular network. However, instead of varying the frequency, we vary the mean value of the oscillatory signal to detect, discriminate, and partly characterize single feedback and feedforward loops in nonlinear networks.

Keywords: Non-parametric identification; Nonlinear systems; Feedback; Feedforward.

1. INTRODUCTION

The underlying signal transduction and processing mechanisms of many biomolecular networks are still only poorly characterized. Often, whole sub-networks are unknown and the roles of key players are not understood. Typically, identification in systems biology uses bottom-up approaches, for example by large-scale experimental measurements and detailed mechanistic modeling. However, current experimental technologies do not allow quantification of all relevant components and–even if this was the case–properties emerging by their interplay have to be understood and characterized *a posteriori*. In contrast, in top-down approaches one conducts systematic experiments to identify structural properties of the network *prior* to the construction of detailed models. System behaviors such as multi-stability or autonomous oscillations can be directly identified by specific experiments; they significantly reduce the space of possible model structures by requiring certain topological network features (e.g., positive feedback for multi-stability), and do not require specific prior knowledge of the respective network.

To generalize such concepts, we here present a novel structural identification approach based on experimentally observable input-output relations of an inducible biomolecular system that can deduce the existence of nonlinear dynamic feedback or feedforward loops without requiring these loops to lead to oscillations or multi-stability. Importantly, our method can distinguish between feedforward and feedback loops, and it provides information on their (relative) strengths.

We utilize an approach similar to traditional frequency domain analysis in which the amplification and the phase delay of an oscillatory signal transmitted through a dynamic system are measured for different frequencies of the input signal [Lipan and Wong, 2005, Mettetal et al., 2008, Bennett et al., 2008, Hersen et al., 2008]. Based on this data, a (minimal) state space representation of the system can be reconstructed. However, frequency domain analysis is essentially a linear method; it requires the linearization of typically nonlinear biological networks [see Schoukens et al., 1998, on how to discriminate nonlinearities from unmodeled linear dynamics]. Furthermore, typically the frequency of the input signal has to be changed over several orders of magnitude, and the output oscillations have to be distinguishable from stochastic noise even for very low amplifications. Limitations in biological experiments and high levels of stochastic noise often imply that these requirements cannot be fulfilled in biomolecular research [Lipan and Wong, 2005, Tan et al., 2007]. Finally, the mapping from a transfer function to a state space representation is not unique. In particular, feedback and feedforward loops are often difficult or impossible to distinguish using such a linear approach (see examples below).

Instead of varying the frequency of the oscillatory input signal, we vary its mean value, while keeping the frequency fixed close to the network's bandwidth. For different mean values, we measure amplification and phase delay of the oscillations of the output, as well as its mean value. Amplification and phase delay of linear systems are independent of the mean input level, whereas the mean value of the output is linear in the mean value of the input. However, in nonlinear systems, these measures co-vary: amplifica-

tion and phase delay can change depending on the mean input level. We show that these variations have specific, distinguishable properties if a nonlinear dynamic feedback or a feedforward loop exists in the network, which can be used for structural systems identification.

Here, we represent the differential equations describing a biological system as higher-order, possibly parallel, cascades of block-oriented Wiener-Hammerstein (LNL) systems including feedbacks. Around the 1980's, several methods were developed to identify mainly small cascades of LNL systems using white and colored noise, or sinusoidal inputs [Giannakis and Serpedin, 2001, Haber and Unbehauen, 1990]. However, only few methods can deal with non-cascade models. Billings and Fakhouri [1978, 1982] analyzed unity feedback LNL models, Baumgartner and Rugh [1975] parallel LNL systems with distinct integer power nonlinearities (S_M systems), Billings and Fakhouri [1979] and Pawlak and Hasiewicz [1998] model classes including Hammerstein systems in parallel with linear dynamics, and Chen [1994] parallel LNL, as well as LNL models with static nonlinear feedbacks. These methods typically require *a priori* that the system under study belongs to a specific class of networks (cascade, feedforward, unity feedback; an exception is the method of Chen [1994]), and often additional information such as the order of the linear dynamics has to be specified [Baumgartner and Rugh, 1975]. However, note that these methods can usually detect if a network belongs to a certain sub-class; a method applicable for LNL systems might detect if a network is a Hammerstein, Wiener, or linear network.

Our approach is based on the work of Singh and Subramanian [1980], who analyzed general block-oriented systems with exactly one odd nonlinearity. Using zero mean sinusoidal input signals with varying amplitudes and the describing function of the nonlinearity, Singh and Subramanian [1980] showed that the complex gain of the output oscillations with respect to the input oscillations qualitatively differs for LNL systems with a linear feedback, a linear feedforward, both, or none. Here, we extend this idea to a method applicable in the context of biomolecular research. Specifically, we allow for more than one mixed (odd and even) nonlinearity. We change the mean value of the input oscillations rather than the amplitude to effectively decouple the effect of the linear dynamics from the nonlinearities, and to enable the application to biological systems with typically only non-negative inputs. Finally, we express the complex gain in relation to the derivative of the static input/output function to discriminate between networks with a nonlinear-dynamic (ND) feedback, a ND-feedforward, a combination thereof, none of both, and linear systems. The ability to reliably detect these topological network features promises to be especially useful in the context of naturally evolved biomolecular networks, where–typically nonlinear–feedback [Brandman and Meyer, 2008] and feedforward [Mangan and Alon, 2003] loops are abundant.
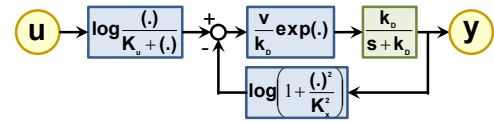

Fig. 1. Example WH-graph of the transcriptional network with non-competitive self-inhibition as described by (1). Blue vertices represent static nonlinearities, green linear dynamics, circles junctions, and yellow the input and the output. The symbol (.) denotes the respective input of the static nonlinearities.

2. RESULTS

2.1 Network Representation

We utilize that the ordinary differential equations (ODEs) of most biomolecular systems can be expressed as block oriented models. Here, we describe a block-oriented model as a Wiener-Hammerstein graph (WH-graph; see Fig. 1 for an example), a directed graph with vertices representing single-input, single-output (SISO) static nonlinearities, linear dynamics, and additive junctions. Static nonlinearities are single-valued differentiable functions $M : \mathbb{R} \to \mathbb{R}$, $z = M(v)$ without memory, that is, $\forall t_1, t_2 : v(t_1) = v(t_2) \Rightarrow z(t_1) = z(t_2)$. Linear dynamics are sets of exponentially stable linear ODEs with one input and one output. The input-output relationship of linear dynamics can be expressed–after decay of the initial conditions–in the Laplace space as $Z(s) = G(s)V(s)$, with $V(s) = \mathfrak{L}(v(t))$, $Z(s) = \mathfrak{L}(z(t))$, $\mathfrak{L}(.)$ the Laplace operator, $s = j\omega$ the Laplace variable, and $G(s)$ the transfer function in the Laplace space. In the following, we assume that the system under study does not contain any linear high-pass filters ($G(0) \neq 0$), which is given for most biomolecular networks. Junctions are the only vertices that have an indegree larger than one: $J : \mathbb{R}^k \to \mathbb{R}$, $z = J(v_1, \ldots, v_k) = \sum_{j=1}^{k} \pm v_j$. Finally, two vertices represent the input (indegree zero) and the output (outdegree zero) of the network, respectively.

The requirement that the junctions of a WH graph have to be additive might seem as a strong limitation since many reactions in biomolecular networks have reaction rates including multiplicative terms. However, multiplications become additions in log-space; they can be expressed as an additive junction with static nonlinearities at the inputs and at the output. For example, the ODE for a transcriptional network with non-competitive feedback

$$\frac{d}{dt}x = v \frac{u}{u + K_u} \frac{K_x^2}{x^2 + K_x^2} - k_D x \qquad (1)$$

can be rewritten as

$$\underbrace{\left(\frac{1}{k_D}\frac{d}{dt} + 1\right)}_{=:G^{-1}} x = \underbrace{\frac{v}{k_D} \exp}_{:=M_3} \left[\overbrace{\underbrace{\log \frac{u}{u + K_u}}_{=:M_1} - \underbrace{\log\left(1 + \frac{x^2}{K_x^2}\right)}_{=:M_2}}^{=:J}\right]$$

and represented as a WH graph (Fig. 1).

A feedback is a simple directed cycle in a WH-graph. A feedback is static if it only contains static nonlinearities, linear if it only contains linear dynamics, and ND if it contains both and is not reducible to a static or linear feedback. A feedback is simple if it does not contain any

additional junction. Similarly, a feedforward is a set of two distinct directed paths starting at the same vertex and ending at the same junction. It is simple if the two paths do not contain any additional junctions, and the other classifications for feedback apply accordingly.

We define the operations on a WH-graph that do not change the input-output dynamics of a network as shown in Fig. 2. Thus, the WH-graphs before and after such an operation are not distinguishable in terms of structural systems identification. Similar to the operations in Figs. 2e and 2f, it is also possible to join a static or a linear feedforward into one vertex, or to join successive junctions (not shown).
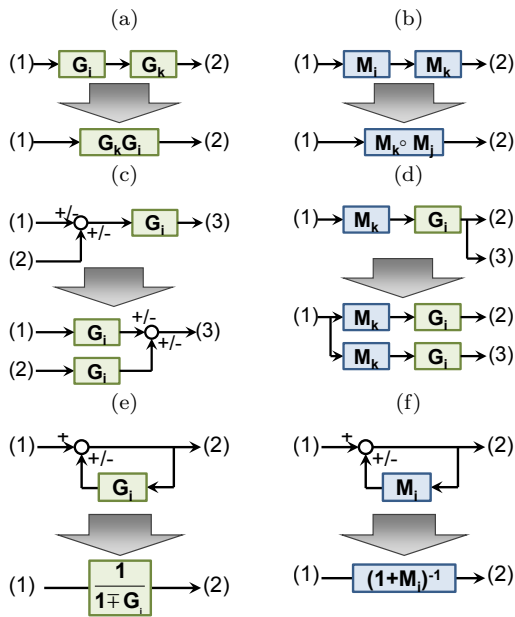

Fig. 2. Elementary operations on a WH-graph. (a) Merging two successive linear dynamics or (b) static nonlinearities; (c) pushing a junction to the right of a linear dynamic by using the distributive law; (d) copying linear dynamics or static nonlinearities to move splits of the path upstream; and (e) transformation of a simple linear feedback into a linear dynamic, or (f) a simple static feedback into a static nonlinearity.

These elementary operations can be applied successively to convert a WH-graph into its normal form (Fig. 3). A WH-graph is in its normal form if all feedbacks and feedforwards are nonlinear-dynamic, feedforwards are not reducible to be static or linear, no two directly connected vertices are both static nonlinearities or both linear dynamics, all vertices with an outdegree greater or equal to two are either junctions or the input vertex, and every junction is directly connected to at least one static nonlinearity or the output vertex. Furthermore, the transfer functions of all linear dynamics have to be one for zero frequency ($G_i(0) = 1$).

In the following, we apply system inputs of the type

$$u(t) = u_0 + u_1 \cos(\omega t), \tag{2}$$

with a static part u_0 overlayed with a harmonic oscillation with period $T = \frac{2\pi}{\omega}$ and amplitude u_1, and we require $u_0, u_1 > 0$, $u_0 \geq u_1$, $u_1 \ll 1$, and $\omega > 0$. With u_1 small,
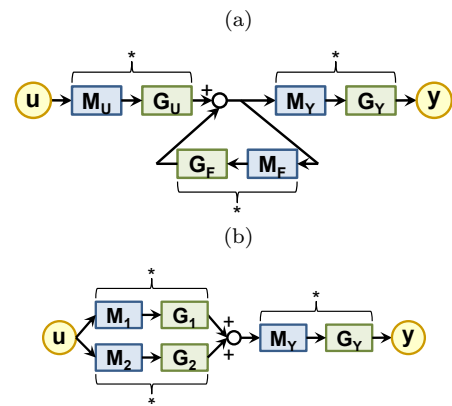

Fig. 3. Normal forms of a WH-graph containing (a) only one ND-feedback, or (b) only one ND-feedforward. Vertices denoted by a star can occur in arbitrarily long alternating cascades. Combining the networks by using the output of the feedforward model as the input of the feedback model results in another WH-graph in normal form, but vice versa this holds only if the set of vertices G_Y and M_Y of the feedback network is empty.

the oscillations can be considered as a small perturbation. The output y of the system will then have the form

$$y(t) = F_0(u_0) + F_1(u_0, \omega) u_1 \cos(\omega t + \phi_1(\omega)) + O(u_1^2). \tag{3}$$

In traditional frequency domain analysis, the same setup is used to generate Bode diagrams or Nyquist plots. However, there the frequency ω is typically varied while keeping u_0 and u_1 constant, thus effectively linearizing the network around the steady state set by u_0 and determining the linear transfer function. Here, we will instead keep the frequency ω constant while varying u_0 to determine structural network properties.

2.2 Systems with one ND-Feedback

In this section, we assume to have a WH-graph in its normal form with exactly one ND-feedback and no ND-feedforward (Fig. 3a). The network consists of greater or equal to zero alternating linear dynamics and static nonlinearities before (G_U, M_U) and after (G_Y, M_Y) the feedback, and greater or equal to one alternating static nonlinearities and linear dynamics in the feedback. For inputs of type (2), the relationship H between the input $u(t)$ and the output $y(t)$ is given by

$$y = \prod_{i=n_Y}^{1} (G_{Y,i} \circ M_{Y,i} \circ) \left[1 + \prod_{i=n_F}^{1} (G_{F,i} \circ M_{F,i} \circ) \right]^{-1}$$
$$\prod_{i=n_U}^{1} (G_{U,i} \circ M_{U,i} \circ) u,$$

with $\circ$ denoting the non-commutative application of the linear transfer functions $G_{X,i}$, respectively the nonlinear static functions $M_{X,i}$ on the element to the right.

In the following, we will refer to the function

$$H_\omega(u_0) = \mathfrak{L}\left(F_1(u_0,\omega)e^{\phi_1 j}\right), \tag{4a}$$

$$= \frac{\prod_{i=1}^{n_Y} G_{Y,i} N_{Y,i}(u_0) \prod_{i=1}^{n_U} (G_{U,i} N_{U,i}(u_0))}{1 + \prod_{i=1}^{n_F} (G_{F,i} N_{F,i}(u_0))} \tag{4b}$$

$$= N_Y(u_0) G_Y \frac{1}{1 + N_F(u_0) G_F} G_U N_U(u_0), \tag{4c}$$

with $j = \sqrt{-1}$, as the amplitude-transfer function for a given frequency ω. Since $u_1 \ll 1$, the derivatives

$$N_{Y,i}(u_0) = \left.\frac{d}{dv} M_{Y,i}(v)\right|_{v=v_0(u_0)} \tag{5}$$

of the static nonlinearities $M_{Y,i}(v)$ with respect to their input v, evaluated at the steady state $v_0(u_0)$ corresponding to a constant input $u(t) = u_0$ approximate the amplification of the oscillations by the static nonlinearities. For a given value of u_0, these amplifications are constant (albeit unknown), such that the order of the static nonlinearities and the linear dynamics can be switched, leading to (4c) with generalized static nonlinearities N_X and generalized linear dynamics G_X, $X \in \{U,Y,F\}$, at the input ($X = U$), output ($X = Y$), and feedback ($X = F$) defined by

$$N_X(u_0) = \prod_{i=1}^{n_X} N_{X,i}(u_0) \tag{6a}$$

$$G_X(j\omega) = \prod_{i=1}^{n_X} G_{X,i}(j\omega). \tag{6b}$$

For a linear system, the amplitude-transfer function is equivalent to the linear transfer function $G(s = j\omega)$, evaluated at a specific value of the Laplace variable s. The phase delay of the output vanishes for small frequencies, and the amplitude-transfer function becomes the first derivative of the static input-output function $F_0(u_0)$ with respect to u_0 ($H_0(u_0) = \frac{d}{du_0} F_0(u_0)$).

The inverse of the transfer functions H_0^{-1} for frequencies close to zero, and H_ω^{-1} for larger frequencies ω can then be written as

$$H_0^{-1} = N_U^{-1} N_Y^{-1} + N_F N_U^{-1} N_Y^{-1}$$
$$= N_\eta^{-1}[1 + N_\delta] \tag{7a}$$
$$H_\omega^{-1} = N_U^{-1} N_Y^{-1} G_U^{-1} G_Y^{-1} + N_F N_U^{-1} N_Y^{-1} G_F G_U^{-1} G_Y^{-1}$$
$$= N_\eta^{-1} G_\eta^{-1}[1 + N_\delta + N_\delta(G_\delta - 1)]. \tag{7b}$$

The transformations are based on arbitrarily selecting a reference input $u_{\text{ref}} = const$, leading to a reference strength of the nonlinearity in the feedback $N_{\text{ref}} = N_F(u_{\text{ref}})$. The relative dynamics and nonlinearities of the system are then expressed by

$$G_\eta := \frac{1 + N_{\text{ref}}}{1 + N_{\text{ref}} G_F} G_U G_Y \tag{8a}$$

$$N_\eta(u_0) := \frac{N_U N_Y}{1 + N_{\text{ref}}} \tag{8b}$$

$$G_\delta := \frac{1 + N_{\text{ref}}}{1 + N_{\text{ref}} G_F} G_F \tag{8c}$$

$$N_\delta(u_0) := \frac{N_F - N_{\text{ref}}}{1 + N_{\text{ref}}}. \tag{8d}$$

These substitutions correspond to a rearrangement of the system as shown in Fig. 4.

$H_0(u_{\text{ref}})$ and $H_\omega(u_{\text{ref}})$ can be measured directly, and the value of G_η is given by
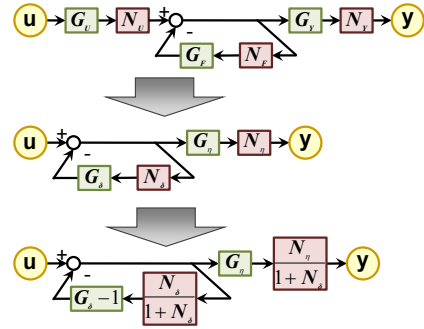

Fig. 4. Visualization of the definitions of G_η, N_η, G_δ, and N_δ (8a–8d), and of the terms appearing in the definitions of T_ω and V_ω (10a–10b). Note that N_δ is zero for the reference input u_{ref}, and $G_\delta - 1$ is zero for frequency zero (H_0).

$$G_\eta = \frac{H_\omega(u_{\text{ref}})}{H_0(u_{\text{ref}})}. \tag{9}$$

Thus, for a nonlinear-dynamic feedback the following equalities hold:

$$T_\omega(u_0) := H_\omega^{-1} G_\eta - H_0^{-1} = \frac{N_\delta}{N_\eta}[G_\delta - 1] \tag{10a}$$

$$V_\omega(u_0) := \frac{H_0 G_\eta}{H_\omega} - 1 = \frac{N_\delta}{1 + N_\delta}[G_\delta - 1], \tag{10b}$$

If the feedback is nonlinear ($N_\delta(u_0) \neq 0$) and dynamic ($\angle G_\delta(u_0) \neq 0$), the functions $T_\omega(u_0)$ and $V_\omega(u_0)$ correspond to lines in the complex space through the origin ($T_\omega(u_{\text{ref}}) = V_\omega(u_{\text{ref}}) = 0$, $\angle T = \angle V = const$). In contrast, without a ND-feedback, $T_\omega(u_0)$ and $V_\omega(u_0)$ are zero, and for more than one feedback or a feedforward (or a combination thereof), the phase of $T_\omega(u_0)$ and $V_\omega(u_0)$ will not be constant in general.

Since $T_\omega(u_0)$ can be obtained from measurement data alone without knowledge of the underlying network, it can be used as an indicator if the network contains a ND-feedback; the absolute values of $T_\omega(u_0)$ and $V_\omega(u_0)$ indicate the change of the strength of the feedback (V_ω), respectively the change of the strength relative to the open-loop system (T_ω).

2.3 Systems with one ND-Feedforward

Similar to Section 2.2, the amplitude transfer functions for a WH-graph in normal form with the only loop being a ND-feedforward (Fig. 3b) can be written as

$$H_0 = N_Y(N_1 + N_2) \tag{11a}$$
$$H_\omega = N_Y G_Y(N_1 G_1 + N_2 G_2)$$
$$= N_Y(N_1 + N_2) G_\eta \left[1 + \frac{N_\delta}{N_1 + N_2}(G_\delta - 1)\right], \tag{11b}$$

with

$$G_\eta := G_Y \frac{N_{\text{ref},1} G_1 + N_{\text{ref},2} G_2}{N_{\text{ref},1} + N_{\text{ref},2}} \tag{12a}$$

$$N_\delta := N_1 - N_2 \frac{N_{\text{ref},1}}{N_{\text{ref},2}} \tag{12b}$$

$$G_\delta := \frac{(N_{\text{ref},1} + N_{\text{ref},2}) G_1}{N_{\text{ref},1} G_1 + N_{\text{ref},2} G_2}. \tag{12c}$$
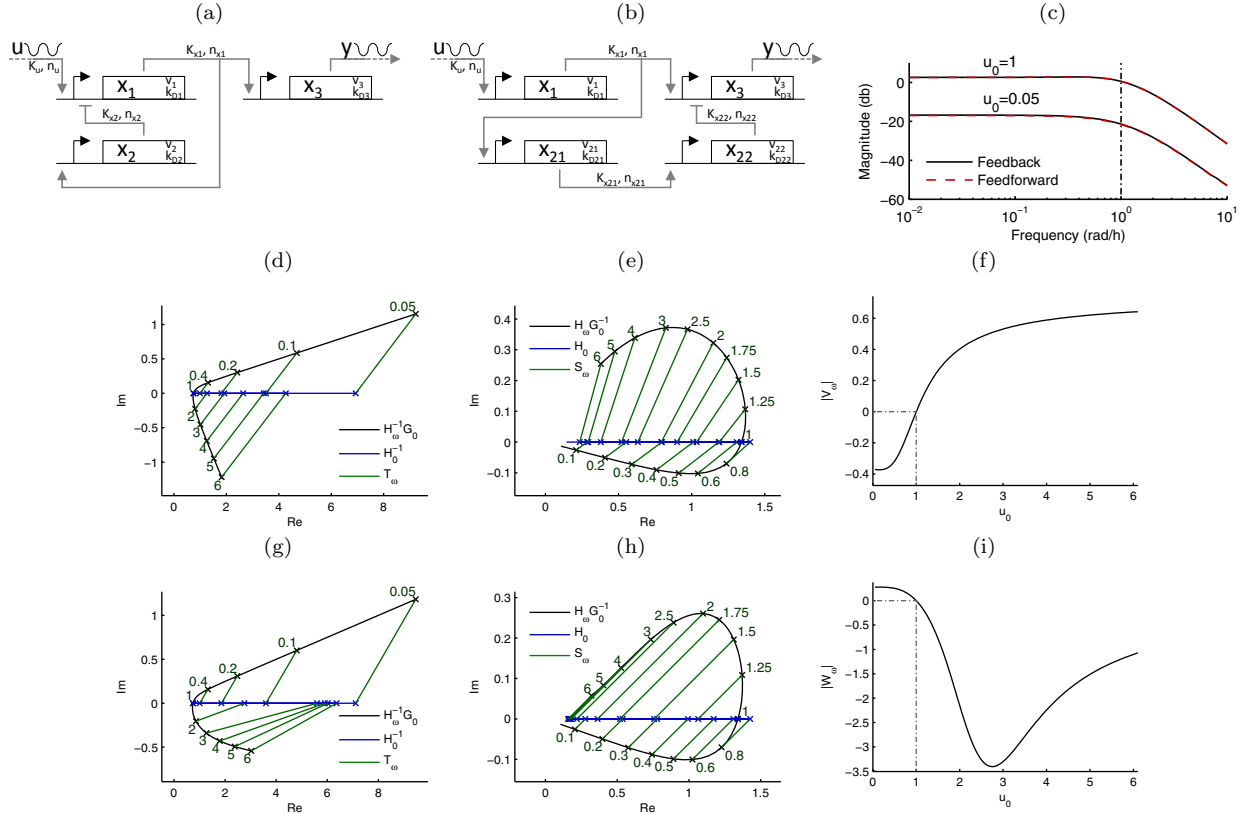
Fig. 5. Example feedback (a,d,e,f) and feedforward (b,g,h,i) networks and their dynamic responses. (a,b) Network structures in standard notation, with degradation rate constants k_{DX}, maximal production rates v_X, dissociation constants K_x, and Hill coefficients n_x. The coregulation of the expression of x_1 (feedback) and x_3 (feedforward) is non-competitive. (c) Bode magnitude plots for input oscillations with constant mean ($u_0 = 0.05\mu M$ or $1\mu M$) and amplitude ($u_1 = 0.1u_0$), and varying frequency ($\omega = 10^{-2}$–$10^2 rad/h$). (d,g) $H_\omega^{-1}G_0$ (black), H_0^{-1} (blue), T_ω (green) using simulated measurement data (10a). (e,h) $H_\omega G_0^{-1}$ (black), H_0 (blue), S_ω (green) for the same data (13a). (f) $|V_\omega|$ for the feedback and (i) $|W_\omega|$ for the feedforward system (sign corrected for $180°$ phase shift at $u_0 = u_{ref}$). Parameters feedback (concentration units in μM, time units in h): $K_u = 10$, $n_u = 1$, $v_1 = 18.03$, $k_{D1} = 1.5$, $K_{x1} = 8$, $n_{x1} = 2$, $v_2 = 32.5$, $k_{D2} = 0.5$, $K_{x2} = 3.29$, $n_{x2} = 2$, $v_3 = 65$, and $k_{D3} = 1$; parameters feedforward: $K_u = 10$, $n_u = 1$, $v_1 = 16.5$, $k_{D1} = 1.5$, $K_{x1} = 8$, $n_{x1} = 2$, $v_{21} = 65$, $k_{D21} = 1$, $K_{x21} = 8$, $n_{x21} = 1$, $v_{22} = 9$, $k_{D22} = 1$, $K_{x22} = 2.47$, $n_{x22} = 2$, $v_3 = 75.64$, and $k_{D3} = 1$.

For this transformation, we arbitrarily select a reference input u_{ref} for which the strengths of the nonlinearities in the two branches of the feedforward become $N_{ref,1} := N_1(u_{ref})$ and $N_{ref,2} := N_2(u_{ref})$. Note that $N_\delta(u_{ref}) = 0$, and that G_η can be measured directly (9).

Thus, for a ND-feedforward the following equations have to hold:

$$S_\omega := H_\omega G_\eta^{-1} - H_0 \tag{13a}$$
$$= N_Y N_\delta (G_\delta - 1) \tag{13b}$$
$$W_\omega := \frac{H_\omega}{H_0 G_\eta} - 1 \tag{13c}$$
$$= \frac{N_\delta}{N_1 + N_2}(G_\delta - 1). \tag{13d}$$

If the nonlinearities ($N_\delta(u_0) \neq 0$) and the linear dynamics ($\angle G_\delta \neq 0$) in the branches of the feedforward loop are different, the functions $S_\omega(u_0)$ and $W_\omega(u_0)$ correspond to lines through the origin in the complex space ($S_\omega(u_{ref}) = W_\omega(u_{ref}) = 0$, $\angle S_\omega = \angle W_\omega = const$). Without any nonlinear-dynamic loop, $S_\omega(u_0)$ and $W_\omega(u_0)$ become zero for all u_0, and for more than one feedforward or a feedback

(or a combination thereof), generally the phase of $S_\omega(u_0)$ and $W_\omega(u_0)$ will be non-constant.

3. EXAMPLES

To exemplify our approach, we consider a transcriptional feedback (Fig. 5a) and a transcriptional feedforward (Fig. 5b) network. Traditional linear frequency domain analysis using oscillatory inputs with constant mean and amplitude, and varying frequency cannot distinguish these networks because the state space representation of linear transfer functions is not unique (Fig. 5c). Here, we created artificial measurement data for both networks by simulating their respective ODE models. The mean of the input oscillations (frequency $\omega = 1h^{-1}$) was slowly increased from $0.05\mu M$ to $6.1\mu M$, and the amplitude was set to 10% of the mean. The mean, the amplitude, and the phase delay of the output were measured by fitting the time-series data to a sine curve. H_0 was derived from the mean values of y by central derivatives, and H_ω by utilizing (4a). For both networks, we used the reference input $u_{ref} = 1\mu M$.

We then used (10a,13a) to calculate the curves T_ω and S_ω (Figs. 5d, 5e, 5g, and 5h) for both networks. From these measurement data alone, we conclude the following on the network structures: (i) H_0 and H_ω are not constant, hence, both systems are not linear; (ii) with non-constant S_ω and T_ω, both networks clearly contain at least one feedback or feedforward loop; (iii) only the phase of T_ω (S_ω) for the feedback (feedforward) model is approximately constant, whereas it varies for the feedforward (feedback) model. Since a ND-feedback (ND-feedforward) implies a constant phase of T_ω (S_ω), the respective types of loops in both models as identified by the measurement data alone are consistent with the known underlying network structures, demonstrating that our method is able to correctly identify nonlinear-dynamic feedforward and feedback loops in biomolecular networks.

It is possible to further qualitatively characterize the respective networks using our method. The magnitude of V_ω (W_ω) provides information about the relative strength of a ND-feedback (ND-feedforward). For our negative feedback circuit, the magnitude of V_ω increases for increasing means u_0 of the input (Fig. 5f). Since the strength N_F of a ND-feedback increases monotonously with increasing magnitudes of V_ω (10b), this indicates either a negative ND-feedback with increasing, or a positive ND-feedback with decreasing strength, but approximately constant strength–probably zero–at low mean input concentrations. This agrees with the known model structure, in which the negative feedback decreases expression of species x_1 non-competetively with a Hill coefficient of $n_{x2} = 2$. Note that the two alternatives (decreasing positive or increasing negative ND-feedback) are in general not uniquely distinguishable, albeit additional analysis might render one explanation more likely than the other.

The magnitude of W_ω of the positive ND-feedforward example (Fig. 5i) is also approximately constant for low mean inputs u_0; it decreases with u_0 for median mean inputs, before increasing again. As above, this either indicates an incoherent feedforward with a maximal relative strength at around $u_0 = 2.8\mu M$, or a coherent feedforward with a minimal relative strength at the same mean input concentration. This result is consistent with the known model structure, an incoherent feedforward inhibiting non-competetively x_3 with a Hill coefficient of $n_{x22} = 2$. The decreasing relative strength above $2.8\mu M$ of the ND-feedforward results from saturation of the negative branch for high input concentrations, while the positive branch of the feedforward is not yet saturated.

4. CONCLUSION

We developed a simple structural identification method for biomolecular networks with which one can distinguish between networks with a ND-feedback, a ND-feedforward, none of both, or a combination of both. Different to traditional frequency domain analysis, our approach does not imply a linearization of the underlying network. It *only* works for nonlinear networks, utilizing that phase delay and amplification of oscillations of the output in response to oscillations of the input not only depend on the frequency–as for linear systems–but also on the mean value of the input.
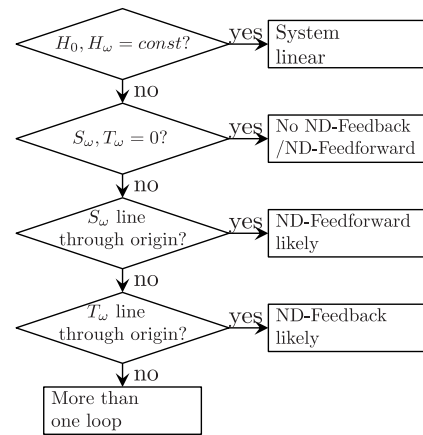

Fig. 6. Decision diagram to classify an unknown biomolecular network based on its nonlinear dynamics. Note, that all conclusions depend on the input range $[u_{min}, u_{max}] \ni u_0$, and on the tested time-scale given by $2\pi\omega^{-1}$. Thus, a system classified as linear might show nonlinear behavior for input concentrations outside the tested range, and might have feedbacks or feedforward loops with significantly different time constants.

We consider this a first step toward an exhaustive methodology with which a large class of nonlinear networks can be structurally identified. The approach currently only applies to distinguishing networks that include only one ND-feedback or one ND-feedforward from networks with more than one or no loop (see decision diagram in Fig. 6). Because large biomolecular networks usually contain several loops, the current method might be most helpful to structurally identify smaller gaps in well-characterized networks, or to analyze larger networks with only few loops that operate at different timescales.

Several future extensions are possible: one could measure several amplitude-transfer functions at various frequencies to extend the method's applicability to networks with more loops. Combinations with traditional linear frequency domain methods to further specify the dynamics in the loops, or with Volterra kernels and similar approaches [Billings, 1980, Haber and Unbehauen, 1990] to specify the order of the nonlinearities and linear dynamics in the loops appear possible. Finally, our method should be evaluated on real *in vivo* experimental measurement data, for example using synthetic networks with *a priori* known structure and microfluidics [Mettetal et al., 2008] or light-induced transcription factors [Lipan and Wong, 2005] to generate the required dynamic inputs.

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