Biophysical Journal Volume 107 December 2014 2492–2498

Biophysical Review

Using Fluctuation Analysis to Establish Causal Relations between Cellular Events without Experimental Perturbation

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ABSTRACT Experimental perturbations are commonly used to establish causal relationships between the molecular components of a pathway and their cellular functions; however, this approach suffers inherent limitations. Especially in pathways with a significant level of nonlinearity and redundancy among components, such perturbations induce compensatory responses that obscure the actual function of the targeted component in the unperturbed pathway. A complementary approach uses constitutive fluctuations in component activities to identify the hierarchy of information flow through pathways. Here, we review the motivation for using perturbation-free approaches and highlight recent advances made in using perturbation-free fluctuation analysis as a means to establish causality among cellular events.

THE IMPORTANCE OF ESTABLISHING CAUSALITY

A major goal of cell biology is to determine how a network of highly interconnected, context-dependent pathways connects the activity of specific molecules to cellular processes. The complexity of the pathway networks can make it difficult to determine the roles individual pathway components play: they may contribute to many different cell functions or they may have no obvious function at all. How can one then identify cause and effect in such a scenario?

The Merriam-Webster Dictionary (www.merriam-webster.com) defines causality as "the relationship between something that happens and the thing that causes it", but the accepted criteria for establishing causality in science varies across fields. For example, in epidemiological terms, causality is inferred by the percentage of a population that acquires a disease based on exposure, i.e., "smoking causes cancer in a fraction of patients". Fields such as physics define causality based on the fundamental laws of nature, i.e., if one event causes another, then the relationships between events must abide by the laws governing material transfer in this system. Causality in cellular systems is not as well defined. One particular difficulty is that we are interested in the causality of molecular events that are not necessarily connected by linear pathways but more complex topologies where cause-and-effect relations can be obscured by pathway features such as compensation and feedback (Fig. 1). Moreover, quite often we are not even concerned with cause-and-effect relations between pathway components, but instead in the specific contribution a pathway component makes to the cellular outputs conferred by the pathway. Given our growing appreciation for the complexity of cellular pathways, it is critical that we define causality in rigorous terms. Here, we define causality by the information transfer through pathways, i.e., as the hierarchy in which the activity of one pathway component influences the activity of another pathway component. The directness of influence depends on the time- and space-scales of the system and thus must be evaluated independently for each cellular system.

WHY WE NEED PERTURBATION-FREE EXPERIMENTS

Cell biologists tend to use inductive reasoning to build consensus of molecular function. We generate hypotheses and then seek evidence to disprove them, using growing congruent evidence to increase our confidence in the original hypothesis. However, it is critical that we test these hypotheses within the appropriate context of a cell's native state. The ability to overexpress, deplete, or functionally modify specific molecular components of pathways has generated countless molecule-function hypotheses that rely on the comparison of the perturbed cellular state (i.e., the perturbed phenotype) to the unperturbed state (i.e., the wild-type). Unfortunately, widespread and somewhat uncritical adoption of this approach belies several inherent limitations of such experimental pathway perturbations.

First and most importantly, the effects of a molecule's abundance on its function may be highly nonlinear. For example, nonlinear biochemical kinetics can cause the apparent function of a molecule to differ depending on its local abundance and the local abundance of its binding partners.

Second, the effect of complete elimination of a component may be fundamentally different from the effect of fractional changes in component abundance or activity. For example, if a protein binds two partners with different

Submitted August 8, 2014, and accepted for publication October 9, 2014.
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Editor: David Piston.

0006-3495/14/12/2492/7 $2.00
http://dx.doi.org/10.1016/j.bpj.2014.10.032

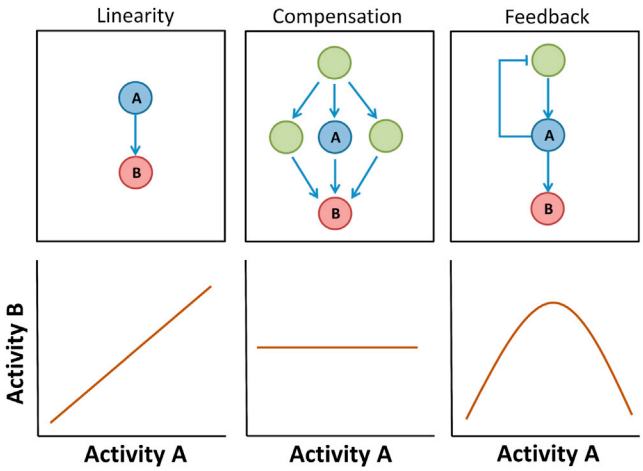

FIGURE 1 Pathway diagrams and corresponding cause-effect relations between upstream component activity *A* and downstream component activity *B* illustrate how increasing pathway complexity obscures the role of a particular component. To see this figure in color, go online.

affinities, then partial knockdown may affect the pathway mediated by lower-affinity interactions but not the pathway mediated by a higher-affinity interaction.

Third, chronic elimination of a component enables cellular adaptation. For example, cells can upregulate proteins or pathways that can take over the role of a perturbed pathway. Hence, the observed cellular phenotype reflects on the adjustment the system undergoes in absence of the targeted component but not on the component's function in the original system.

Fourth, pathway nonlinearity (including feedback) can obscure the effect of any specific perturbation. For example, a negative feedback motif can attenuate pathway activity, making it impossible to observe the effects of perturbation unless the sampling resolution is faster than the feedback.

Fifth, and finally, global perturbation obscures any spatial features, which might be critical for the pathway function. For example, although the role of spatial asymmetry in cellular functions such as polarity has been appreciated for quite some time (1), several recent studies have shown that spatial localization regulates cell functions not commonly associated with cell polarity (2,3). All of these limitations must be considered when drawing conclusions from studies of component perturbation. Acute perturbations to protein abundance or activity via inducible expression (4), or activation by light (5) or small molecules (6), prevent long-term cellular adaptation but likely still induce nonlinear pathway effects.

FLUCTUATION ANALYSIS AS A PERTURBATION-FREE METHOD

A complementary approach to experimental perturbation makes use of inherent fluctuations in component activity, enabling causality to be inferred from temporal covariation in the abundance or activity of pathway components. In principle, each change in component activity creates the opportunity to measure information transfer between pathway components. Provided that these fluctuations are not only due to measurement noise, they will propagate through a signaling pathway. Thus, consistent covariation between two pathway components signifies that one influences the other, i.e., there is a causal relation between them or that they are coregulated by a common input.

Historically, fluctuation analysis has proven useful in fields where perturbation is not feasible and where temporal data is available. One of the earliest applications of fluctuation analysis for inferring causality can be found in econometrics (7). Although the desire to predict influences among economic factors is obvious, the inability to perturb financial variables has forced economists to consider inherent fluctuations a source of microscopic perturbations that reveal how system components react to one another. To our knowledge, fluctuation analysis is the only method to determine causality in systems where perturbation is not possible.

Neurophysiology also employs perturbation-free fluctuation analysis. Much of what is known about information transfer in the human brain is based on dynamic neurophysiological measurements, where neuronal activation is monitored at high temporal and spatial resolution while a subject performs various tasks. The relationships between activity levels in groups of neurons are then used to infer connectivity between cortical sites (8). Initially based on concepts from econometrics, analytical methods have been developed to predict causality in cortical information flow (9,10).

FLUCTUATION ANALYSIS OF CELLULAR PATHWAYS

The intrinsic fluctuations that arise in cell biological systems can provide key insight into the native functions of pathway components. Cells operate far from equilibrium and thus pathways are constantly fluctuating. In certain pathways, fluctuations may also be associated with low numbers of interacting components or be induced by variations in the extracellular environment. Regardless of their origin, these small fluctuations in component activity can reveal cause-effect relations in pathways working at their normal (physiological) point of operation. Although there is no guarantee that these intrinsic fluctuations will lie within a linear region of the cause-effect relation, suitably small fluctuations may be studied analogously to the local derivative of a nonlinear function. Thus, measurement of small, intrinsic fluctuations allows analysis of component function within a range of activity that is most likely linear (Fig. 2).

A few experimental requirements must be met to enable a fluctuation analysis of cellular pathways.

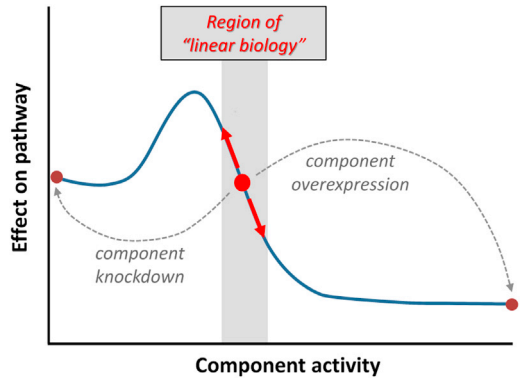

FIGURE 2 Fluctuation analysis enables study of protein function within the linear range. Due to the nonlinear effects, larger shifts due to component overexpression or knockdown may have unintelligible effects on pathway function. Naturally occurring fluctuations in component activity provide the opportunity to study slight changes to component activity, a regime that we term "linear biology". To see this figure in color, go online.

First, sampling frequencies in time and space must be higher than the frequencies of fluctuations that are transmitted by the pathway. Otherwise, it will be impossible to detect the coupling of components. Moreover, the sampling intervals must be shorter than the shortest delay between any two coupled pathway components. Otherwise, their activities will appear synchronous.

Additionally, the measurement noise must be unstructured in order not to appear as an intrinsic pathway fluctuation; and its magnitude must be low enough to allow algorithms that detect the coupling between component fluctuations to distinguish noise from propagated information. If spatial averaging of a component's activity must be performed, it must be done so carefully as to retain spatial relationships between fluctuations and so that highly localized signals are not obscured by lower signals in the surrounding regions.

Finally, the assays that measure component fluctuations such as the variation in protein activity must not significantly alter the pathway state (11).

Recent technological advances, mainly in imaging, make it possible to achieve the conditions for fluctuation analysis of subcellular events (12). Most importantly, advances in the design of biosensors enable instantaneous measurements of protein activity with minimal perturbation to the intracellular state (13). Such biosensors are designed to measure the abundance of a specific molecular target unique to the active protein, often the phosphorylated or GTP-bound form. Fluorescence resonance energy transfer (FRET) or shifts in fluorescence spectrum (14,15) are used to differentiate between active and inactive states of the biosensor. To normalize for the abundance of the biosensor, the signals of the active and inactive state are then usually divided by one another. Unfortunately, such ratiometric fluorescence signals have a narrow dynamic range, which can drastically limit the signal/noise. Accordingly, careful experimental and analytical steps must be taken to reduce the effect of spurious fluctuations (16).

An alternative to FRET-based biosensors involves protein localization, whereby a fluorescently tagged protein domain that binds the specifically localized protein products is used as a proxy for protein activity (17–19). This type of biosensor has the advantage of reduced background noise compared to ratiometric biosensors, but is only suitable when the active and inactive forms of a protein are spatially separated, e.g., cytosol versus plasma membrane. Additionally, the component translocation results in a time lag between activation and signal measurement, which must be accounted for during fluctuation analysis. In addition to advances in molecular measurements, advances in microscope sensitivity, speed, and resolution enable data sampling at the temporal and spatial scales necessary to quantify local, possibly short-lived fluctuations.

Computational and analytical innovations are also necessary to facilitate precise measurement of component activity and to quantify relationships between varying components. Thus far, cross-correlation is the most common method to identify covariation in component fluctuations in cells. Although it is the simplest method to quantify covariation in fluctuating time series, cross-correlation is capable of detecting structured variations between components even in the face of high-magnitude unstructured measurement noise. For example, Fig. 3 illustrates that cross-correlation analysis can be used to accurately measure the time shift between coupled data even as simulated measurement noise increases. Thus, an important feature of fluctuation analysis is that propagated fluctuations can be significantly smaller than noise, in fact so small that they cannot be discerned by eye. This favorable property originates in the analysis of a large number of random microperturbations of the pathway. Thus, instead of evaluating a single strong perturbation of the pathway in a perturbation experiment, fluctuations rely on accumulated evidence from hundreds to thousands of weak perturbations of the pathway.

CAUSALITY IN INTRINSIC PROTRUSION FLUCTUATIONS

To date, the application of fluctuation analysis in cell biology has mostly focused on the molecular dissection of protrusion and retraction cycles in motile cells. This process is indeed governed by numerous highly nonlinear and mutually redundant pathways. On the other hand, motile cell types exhibit sporadic protrusion and retraction events, whose frequency and magnitude are amenable to efficient sampling of fluctuation time series that indicate the coupling of membrane and cytoskeleton mechanics as well as of multiple interconnected chemical signaling activities. Perturbation of the core components of these pathways such as the regulatory GTPase signals produce dramatic phenotypes in terms of cytoskeleton organization and morphodynamics (20); but

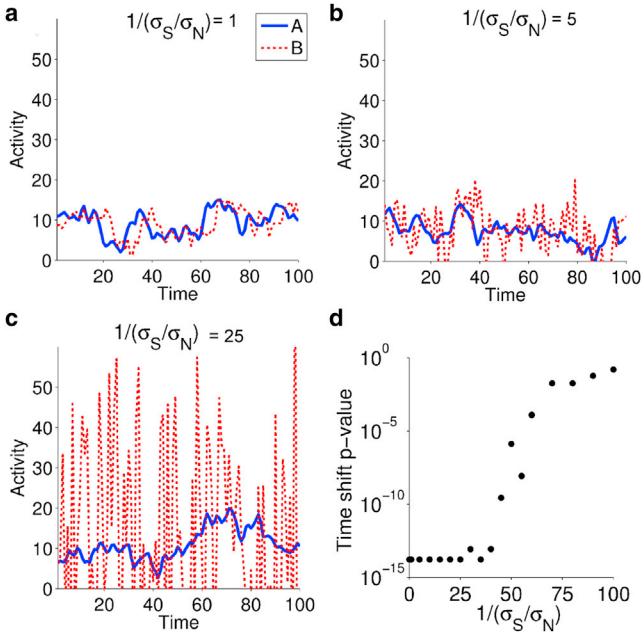

FIGURE 3 (*a–c*) Simulation of time-shifted data with varying noise. The upstream signal, component *A*, was simulated by an autoregressive moving average model with intrinsic fluctuations defined by σ-values. The downstream signal, component *B*, was calculated by time-shifting values of component *A* and adding different amounts of unstructured noise (σ_N). (*d*) The maximum of the mean Pearson's cross-correlation (30 independent simulations) was used to find the measured time shift, which was calculated for 30 independent replicates. The *p*-value indicates the level of confidence that the measured time shift is equal to the true shift, calculated using the nonparametric Wilcoxon rank sum test. To see this figure in color, go online.

these phenotypes have provided little insight into the coordination of all the factors necessary to control protrusion events. Thus, cell protrusion regulation is a particularly appropriate example to highlight the value of fluctuation analysis in reconstructing pathways while avoiding the system-breaking effect of experimental perturbations.

One of the earliest applications of cellular fluctuation analysis involved the relationship among actin polymerization, cell adhesion, and local force generation during protrusion (21). The measurement of these activities was enabled by several image analytical developments. Speckle microscopy was used to measure actin polymerization and depolymerization, as well as to infer the forces borne by the actin cytoskeleton. Local protrusions were quantified by cell-edge tracking algorithms designed to account for complex changes in cell shape. Assuming that the orientation of actin motion relative to force implies properties of external force, the authors inferred the contributions of cell membrane forces, intracellular contractility, and adhesion forces.

The fluctuation analysis performed by Ji et al. (21) offered biological insight that would not have been possible using conventional perturbation approaches. The study elucidated the timing of actin polymerization and cell adhesion during local protrusion. Initially, local increases in actin polymerization coincide with increases in local protrusion, but opposing forces applied by the membrane also increase. This increase in membrane force causes protrusion velocity to decrease even though actin polymerization continues increasing, peaking ~20 s after the maximum protrusion velocity. During this period of high actin polymerization, membrane force and adhesion force increase, reaching a maximum that is coincident with actin retrograde flow, ~20 s after maximum actin polymerization (40 s after maximum protrusion). The approach demonstrated by Ji et al. (21) was necessary because any perturbation to the system would destroy these complex spatiotemporal relationships. Specifically, the interactions among actin polymerization, actin flow, adhesion, and membrane forces form feedback loops whereby each variable influences the others (22,23). For example, adhesions form as a function of local cell protrusion rate (24), and these adhesions not only enable transfer of force to the substrate but also localize GEFs that can activate Rho family GTPases (25–29). Thus, depletion or genetic disruption of any one component would affect all other components. It would be impossible to assess how the activity of one pathway component influences the activity of another pathway component.

Further insight into the signaling of the same system was gained by applying FRET-based reporters of the three major Rho family GTPases Rac1, RhoA, and Cdc42 (30). Fluctuation analysis by cross-correlation of local time series of the signaling activities revealed that the relationship between GTPases and cell-edge movements was much more complex than was previously hypothesized based on perturbations of these molecules. Most surprisingly, RhoA was found to have increased activity at the cell edge coincident with local protrusion. Contrary to the ascribed role of Rac1 as a protrusion stimulator, this data showed that Rac1 activation rises after protrusion onset and peaks with a delay of ~40 s after maximal protrusion. Putting this data into the context of the results in Ji et al. (21), this suggests that in spontaneous cell migration Rac1's role is actually to reinforce actin polymerization as membrane tension increases, which is necessary for sustained edge advancement. Protrusion onset, however, is triggered by RhoA signaling. Intriguingly, the data also showed that Rac1 is not activated at the very edge but in an adhesion zone behind the edge. This led to the still untested hypothesis that the Rac1 activation is in fact responsive to the transient increase in membrane tension during protrusion, which is balanced in adhesions and possibly translated into GTPase signaling via mechanosensitive GEFs (31–33). Importantly, any experimental perturbation of this system would obscure these relations.

Fluctuation analysis depends on concurrent measurements of pathway components that exhibit hypothesized relationships with one another. Depending on microscope configurations and experimental parameters, the number of different component activities that can be captured in

one live-cell imaging experiment is limited to two or three (34). To address this limitation, Machacek et al. (30) demonstrated a computational multiplexing approach, whereby the spatiotemporal activity of components captured in different experiments could be compared by referencing them to a common standard, in this case cell-edge motion. Thus, fluctuation analysis also offers a framework for reconstruction of pathways with many more components than can be imaged at once.

In addition to random fluctuations in local protrusion, periodic fluctuations in cell-edge protrusion enable study of the causal relationships between antagonistic variables. For example, periodic fluctuations in *Xenopus* cell edges were tracked by active contours revealing protrusion and retraction events with a period 130–200 s (35). Surprisingly, increases in the localization of actin within a 5-μm region at the cell edge were out of phase with protrusion velocity. This observation led the authors to propose a computational model to simulate how interactions between an autocatalytic actin activator (possibly Arp2/3) and F-actin density, which inhibits the activator, might produce the observed periodic oscillations. The quantitative features of the observed protrusion events enabled estimation of free model parameters, highlighting how fluctuation analysis can directly inform computational models.

In addition to local cell protrusion, the mediators of local cell retraction have also been explored using fluctuation analysis (36). Specifically, local increases in calcium ions are inversely correlated with protrusion. The calcium spikes showed maximum correlation with edge retraction >6 μm from the cell edge, suggesting that they do not modulate protrusion via actin polymerization. Moreover, artificially induced local increases in Ca^{2+} caused an increase in cell-edge retraction. These observations led the authors to hypothesize that these calcium spikes increase cell contractility through myosin II; indeed, knockdown or inhibition of myosin II reduced-edge retraction and destroyed the correlation between Ca^{2+} spikes and edge retraction. Together, these results demonstrate how fluctuation analysis outlines the critical relationships between molecular events—here, Ca^{2+} spikes and cell-edge retraction— and generates predictions that can then be tested with specific perturbations, which are interpretable in the context of the unperturbed system.

COMBINING FLUCTUATION ANALYSIS WITH MOLECULAR PERTURBATIONS

Four recent studies exemplify further how fluctuation analysis can be used in combination with molecular perturbations to gain deeper understanding of causality in complex systems.

Spatial sampling and cross-correlation analysis provided causal evidence for the role of cyclic-AMP-activated protein kinase A (PKA) in local protrusion regulation (37). The observation that PKA signaling increased ~20 s after local protrusion, up to a distance of 4 μm from the cell edge, concurrent with a deactivation of RhoA signaling, suggested a causal relationship between PKA and RhoA. Precise perturbations were used to show that PKA-mediated phosphorylation of RhoA increased the interaction of RhoA with its inhibitor RhoGDI. Accordingly, inhibition of PKA prolonged RhoA-activated protrusions whereas overexpression of RhoGDI abrogated this effect. Furthermore, overexpression of a RhoA construct that was deficient in RhoGDI-binding also increased protrusion duration. Thus, in this work, fluctuation analysis generated insight into the timing between PKA and RhoA that led to the hypothesis regarding their molecular coupling via a negative feedback.

Fluctuation analysis and precise perturbations were also used to elucidate the role of phosphoinositide-3 kinase (PI3K) during cell reorientation (38). Local protrusion preceded increases in local PI3K activity by ~2 min, suggesting that like Rac1, PI3K reinforces local protrusion once it has begun. Acute, local stimulation of protrusion by focal activation of Rac1 also caused a delayed local increase in PI3K, providing further evidence for the causal relationship between protrusion and PI3K signaling.

Subsequent work showed that this temporal relationship between protrusion and PI3K activity holds when PI3K is stimulated globally (39). The timing placed PI3K potentially downstream of protrusion events, which called for a revision of the longstanding paradigm that PI3K operates as a driver of directed migration. Indeed, Welf et al. (38) showed that inhibition of PI3K signaling did not reduce cell migration speed but almost entirely eliminated cell reorientation events. Based on this evidence and observation of small unproductive protrusions when PI3K was inhibited, it was proposed that PI3K signaling creates a positive feedback that reinforces stochastic protrusions, enabling cells to maintain several divergent protrusive fronts that propagate to produce cell branching and reorientation.

Using similar strategies, Kunida et al. (40) added further insight to this feedback loop by inhibition of actin polymerization while performing autocorrelation analysis of Rac1 fluctuations and cross-correlation between fluctuations in Rac1 activity and in cell-edge movement (40).

DETERMINING CAUSALITY IN GENE EXPRESSION USING SYNTHETIC AND NATURAL GENE NETWORKS

Like the random cycles of protrusion and retraction in cell migration, variations in gene expression make fluctuation analysis a good approach for studying causality in gene regulation (41). An advantage of studying gene regulatory networks is the ability to create functional synthetic networks. Dunlop et al. (42) used this approach to evaluate a hypothetical model for causality among the bacteriophage λ CI, which represses production of a fluorescent reporter

under the control of a variant of the λ P_R promoter (42). The authors first simulated a model gene regulatory network to predict quantitative relationships between genes with a known causal relationship, and then created a synthetic experimental system to test the model. Causal relationships between genes were shown to fluctuate as predicted by the model. The authors went on to demonstrate the ability to predict causality in gene networks via perturbation of the endogenous *Escherichia coli* galactose metabolism.

OUTLOOK

Although its application is still limited to very few cellular systems and hypotheses, fluctuation analysis has great potential to become a tool for predicting causality among cellular events without the risks of misinterpretations of molecular function in grossly perturbed systems. These analyses become especially powerful when combined with precise and subtle perturbations that shift the relations between events within a linear range about the system's point of operation. Given the growing appreciation of adaptation in signaling networks with redundant and interconnected components, fluctuation analysis will be fundamental for developing initial predictions of causal relationships that can be tested further with experimental perturbations. Most importantly, as a community we must agree on what is required to establish causality and broaden our perspective of approaches we can use to achieve this goal. In particular, we must destigmatize the use of correlation between the covariation of two fluctuating events (43).

First, correlation is an excellent tool to test null hypotheses regarding causal relations between pathway components. Although indeed a correlation between two components does not establish causality, the absence of a correlation unambiguously signifies their functional independence.

Second, it must be recognized that outcome analysis of experimental perturbations is also correlative: A perturbation modulates the abundance or interaction strength of a pathway component to generate a variation in pathway response. Implicitly, this variation is then correlated with the input modulation.

Third, it must be recognized that the correlation of temporal variations in fluctuating events contains evidence of the directionality of information flow in a pathway and thus of causality. What happens first tends to be upstream of what happens next. This reasoning fails in cases of strong feedback; however, such feedbacks can be detected by nonlinear model-based correlation models.

Fourth, although a simple cross-correlation between two components with common input incorrectly suggests a coupling between them, a model-based correlation analysis that tests the statistical evidence for a direct versus indirect interaction between fluctuations can often pinpoint this scenario (43). Thus, there is nothing wrong with using correlation as one of the indicators of causation.

Going forward, advances in several directions will facilitate adoption of fluctuation analysis as a core technique for establishing causality. In particular, the increasing variety and precision of biosensors will enable direct and local observation of the fluctuation of ever closer pathway components, which will reduce the ambiguity of correlation. Moreover, adoption of sophisticated model-based correlation from fields that do not have the opportunity to intervene with the studied system will substantially increase the accuracy of distinguishing causal from coincidental covariations. The increasingly interdisciplinary nature of cell biology and biophysics, involving biology, chemistry, and computational analysis, should encourage these endeavors.

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