Trends in Cancer

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Opinion

Connecting Timescales in Biology: Can Early Dynamical Measurements Predict Long-Term Outcomes?

Michael Tsabar,[1,2,3] Scott B. Lovitch,[4] Ashwini Jambhekar,[1] and Galit Lahav[1,*]

Prediction of long-term outcomes from short-term measurements remains a fundamental challenge. Quantitative assessment of signaling dynamics, and the resulting transcriptomic and proteomic responses, has yielded fundamental insights into cellular outcomes. However, the utility of these measurements is limited by their short timescale (hours to days), while the consequences of these events frequently unfold over longer timescales. Here, we discuss the predictive power of static and dynamic measurements, drawing examples from fields that have harnessed the predictive capabilities of such measurements. We then explore potential approaches to close this timescale gap using complementary measurements and computational approaches, focusing on the example of dynamic measurements of signaling factors and their impacts on cellular outcomes.

Highlights

Predicting long-term outcomes in cells based on short-term observations is critical for understanding how cells respond to drugs and other stimuli, and for developing prognostic biomarkers.

Monitoring signaling dynamics in single cells provides additional information beyond static measurements, but is insufficient to predict final outcomes due to limited timescales of measurement.

Combining dynamic measurement of signaling with complementary measurements, including transcriptomic and proteomic assessment, can improve predictive power, and is essential for bridging the timescale gap between short-term dynamics and long-term outcomes.

Ultimately, identifying a limited number of biomarkers that provide maximum predictive information will be essential to develop applications for future clinical use.

Introduction

Predicting the future based on past and current observations is a major goal across many disciplines and even in most aspects of daily life. The weather forecast, for example, is widely used from making everyday decisions to planning longer-term endeavors such as nautical voyages; yet its accuracy quickly declines the further into the future one looks. As for the weather, accurate predictions in most domains remain largely out of reach. Consider the following scenario – attending a baseball game in which your favorite team is leading at the bottom of the first inning. Is it possible to predict the long-term outcome (final score) based on the short-term observation (first inning)? Similarly, a major goal in science and medicine is to develop strong predictive power based on early events: can a physician determine the outcome of a treatment based on an early observation? Can a scientist determine whether a cell will die or survive based on the way it responds in the first few hours after a drug treatment? Which approaches will be most effective in bridging the gap between early observations and long-term outcomes in order to reach better and more accurate predictions?

Genomic Information Provides Critical Yet Limited Predictive Power

DNA sequencing and genomic technologies provide invaluable insights into predicting biological events and, in some cases, provide all necessary information. A glimpse at the genome of a human fetus reveals, with near-perfect accuracy, the sex of the fetus. In addition, DNA sequencing can predict whether a child will be born with any number of disorders rooted in genomic abnormalities, including Down syndrome, Fragile X disease, metabolic disorders, and even disorders for which the phenotype may not become apparent for many years such as Huntington's disease. Genomic information can also predict the development of certain cancers. Germline mutations in *APC*, *BRCA1*, or *Rb* exhibit extremely high correlations with the development of colon cancer, breast cancer, and retinoblastoma, respectively [1].

[1]Department of Systems Biology, Blavatnik Institute at Harvard Medical School, Boston, MA 02115, USA
[2]Laboratory of Systems Pharmacology, Blavatnik Institute at Harvard Medical School, Boston, MA 02115, USA
[3]Broad institute of Harvard and MIT, Cambridge, MA 02142, USA
[4]Department of Pathology, Brigham and Women's Hospital, Boston, MA 02115, USA

*Correspondence: galit@hms.harvard.edu (G. Lahav).

However, not all biological systems are so deterministic. Perhaps the most illustrative example of a nondeterministic biological process for which genomics offers no predictive value is differentiation. Because most cells in an organism carry the same genetic information, this information alone is insufficient for distinguishing one cell type from another. Therefore, while the sex of the developing human fetus can be determined accurately and early, determining the differentiation fate of a cell – for example, predicting whether it will develop into a skin cell or a blood cell – based only on its genetic signature is impossible.

Another situation in which genetic information is not sufficient to predict cellular outcomes is the response of cancer cells to treatment. While variation between cells in a tumor is largely attributed to genomic instability and the formation of resistant clones [2], recent studies showed the contribution of nongenetic heterogeneity in triggering variable outcomes [3–7]. Nongenetic variation can arise from both extracellular factors (e.g., the microenvironment of a cell) and intracellular factors (e.g., cell cycle phase, protein abundance, transcriptional noise, and basal DNA damage). Such sources of heterogeneity are difficult to track and pose a difficult challenge in predicting long-term responses based on short-term observations.

Dynamic Measurements Improve Intermediate, but not Long-Term, Outcome Predictions

Cells respond to their environment by activating signaling networks. These networks trigger a specific output in response to a given input, and are therefore central to understanding how cancer cells respond to drugs. Studies of signaling networks often focus on the transcription factors (TFs) central to guiding appropriate responses to stimuli. Under basal conditions, these TFs are either excluded from the nucleus (e.g., NF-κB), or expressed at low levels (e.g., p53); stimulation induces nuclear import and/or increases protein levels. Understanding the spatial and temporal regulation of signaling molecules is therefore critical to predicting the outcomes of activating signaling networks.

Biochemical assessment of changes in protein levels in response to a stimulus often relies on simple before and after snapshots – that is, measuring expression prior to and following drug administration. However, most such responses are nonlinear, and in these settings, relying on these two snapshots can provide an incomplete – if not erroneous – description of the response. For example, following induction of DNA double-stranded breaks by ionizing irradiation (IR), levels of the tumor suppressor p53 oscillate with fixed amplitude and frequency [8]. By contrast, accumulation of single-stranded DNA, for example, by exposure to UV light, induces sustained p53 expression [9]. Measuring p53 levels at the peak of oscillation following IR would therefore result in different conclusions from measuring at a trough, and neither would capture the dynamic nature of the response. Likewise, if p53 response following IR were only measured at a peak, one might erroneously conclude that IR and UV treatment induce similar p53 responses (Figure 1). The differences in p53 dynamics have significant bearing on cellular outcomes. Oscillatory dynamics following IR are associated with long-term cell survival, whereas sustained expression results in cell death or senescence [10]. In addition, heterogeneity in p53 dynamics in response to the chemotherapeutic drug cisplatin results in heterogeneous cellular outcomes: cells that rapidly accumulate p53 die, while those that accumulate p53 more slowly undergo cell cycle arrest [5]. These studies suggest that dynamical analysis provides greater predictive power than single snapshot measurements and, in some cases, can predict the cellular outcomes. Over the past two decades, study of these parameters, termed signaling dynamics, has become central to cancer biology.

Studies of signaling dynamics have improved our ability to make predictions from relatively short observations, as the influence of signaling dynamics on cellular outcomes can linger long after the

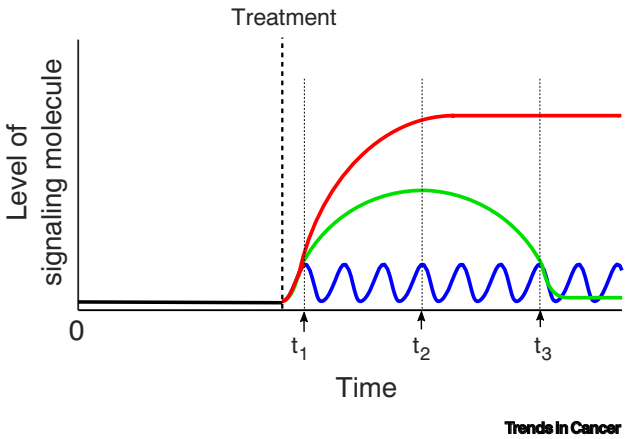

Figure 1. Snapshots of Complex Responses Can Mask Real Dynamical Behaviors. This schematic drawing shows three examples of dynamic responses of a signaling molecule: oscillatory (blue), single pulse (green), and sustained (red). If fixed measurements are taken in t1, the three dynamic behaviors appear similar. If fixed measurements are taken in t3, the green and blue dynamics appear similar. Only measurements in t2 allow distinguishing the three responses.

initial stimulus has ceased. For example, most cells remain arrested for many days after the initial exposure to IR. However, much like weather forecasts, the further we try to gaze into the cells' future using signaling dynamics, the poorer the accuracy of our predictions. For example, knowledge of the dynamical trajectory of p53 in a cell immediately after DNA damage does not guarantee its status several days afterwards. Imaging live single cells for several days following IR revealed that a fraction of cells escapes arrest, and that this escape results in a switch from oscillatory to sustained p53 dynamics [11,12]. In some cases, escape can arise from noise in the p53 amplitude, resulting in two or more low-amplitude p53 pulses. Thus, it may be possible to predict which cells will escape arrest. However, such prediction requires continuous monitoring of p53 levels, as escape could arise a few days or more after DNA damage. Although we have recently been able to follow cells under the microscope for 2 weeks [7], monitoring cells for this duration remains challenging and often final fates are executed even beyond this time frame. While the ultimate outcome of the escaper cells is still unclear, these observations complicate our ability to predict final cellular outcomes by analyzing early signaling dynamics, and provoke us to seek experimental insights from other fields.

How Do Other Fields Link Cellular Outcomes with Early Events?

Early Events in Immune Cells Lead to Commitment with Long-Term Outcomes

Immunology offers an example of a biological system in which critical early events profoundly influence, and can predict, long-term outcomes. The response of a naïve T cell to its initial encounter with antigen can determine the development of lasting immunity. If an antigenic peptide is present at sufficient dose and displayed by the appropriate MHC molecule in the presence of co-stimulatory signals, then the T cell will proliferate and initiate an immune response, generating lasting immunological memory. However, if the antigen dose is too low, or if it is presented in the absence of co-stimulation, the encounter instead induces a hyporesponsive state, anergy, in which the T cell becomes refractory to stimulation, even under conditions sufficient to induce activation on initial encounter [13–15]. These effects are largely irreversible and highly consequential – they could, for example, determine whether the organism survives infection with the same pathogen decades later – and are determined in the seconds and minutes following the initial encounter. On a longer timescale, chronic, repetitive antigen stimulation drives the T cell into a state of exhaustion in which the cell, while still responsive, fails to respond effectively upon subsequent antigen encounter [16–18]. Higher-resolution assessment of signaling dynamics in T cells, ideally at the single-cell level, could potentially allow accurate real-time prediction of T cell responses – a useful tool for fields ranging from vaccine development to cancer immunotherapy.

Differentiating Cells Traverse Multiple and Flexible Commitment Points

The above example illustrates a situation in which observation of early events can predict outcomes decades later. However, in many areas of biology, including cancer evolution, cells encounter multiple decision points that are not as well defined as those in T cells. Insights from the more probabilistic field of differentiation can elucidate how such decisions are made.

Early studies of cell fate determination have defined the process as a sequence of binary decision-making steps largely governed by master TFs [19] whose expression levels could predict cell fate. More recently, single-cell RNA sequencing (scRNA-seq) studies have questioned this binary decision-making model, and have instead suggested that cell-fate determination is a continuous and plastic process [20,21], with cells gradually committing to their final fates rather than making an instantaneous and irreversible decision [21]. Such a mechanism complicates predictions of ultimate cell fates based on early measurements of a small number of molecular markers. Nevertheless, these studies have suggested that combinations of markers measured at a few key timepoints during differentiation could hold predictive power. A set of differentially expressed genes during hematopoietic differentiation has greater predictive capacity than the traditionally used master TFs [22], such as PU.1 and CEBPα [23]. Transcriptional signatures defining developmental trajectories are also found in vertebrate development [20,22], raising the possibility that measuring sets of molecular markers at key timepoints could allow cell fate predictions. In addition to transcriptional profiles, predictive power is also found in determining the chromatin states of pluripotent cells during organ differentiation in mice [24], and in the dynamics of Erk signaling activity in the developing *Caenorhabditis elegans* germline [25]. These studies have suggested that initial investment in acquiring high-resolution data – whether transcriptional, epigenetic, or signaling dynamics –could help increase the efficiency of cell fate predictions by guiding selection of the best sets of factors and timepoints to use for measurement.

Similar to differentiating cells, cancers frequently arise from progenitor cells. Understanding the cell of origin (COO) of cancer cells, therefore, is critical for guiding cancer treatment. The identification of predictive markers in transcriptomic studies of normal development has led to significant progress in treatment of certain cancers, notably diffuse large B cell lymphoma (DLBCL). Gene expression profiling studies have led to recognition that this hematological malignancy, previously considered a monolithic entity, demonstrates two distinct profiles based on COOs [26], with one benefiting from more aggressive chemotherapy [27,28]. COO classification is now part of the standard of care for diagnostic workup of DLBCL [29].

Potential Approaches for Linking Short-Term Signaling Dynamics with Long-Term Outcomes

As seen in the studies above, initial signaling dynamics lead to the expression of quantifiable molecular indicators of cell state that, in the best-case scenarios, can predict a cell's phenotypic trajectory and its ultimate outcome. These indicators, which we term here intermediate markers may reflect any of various aspects of cell state, such as cellular identity during differentiation, metabolic status, DNA damage status, or motility. Note that in studying cancer initiation and progression, the markers of interest may be biomarkers that distinguish malignant from normal cells; therefore, monitoring or predicting their appearance will be important for understanding and halting tumor development.

Identification of Intermediate Transcriptional Markers

As differentiation studies have taught us, successful predictions require identification and generation of reliable intermediate transcriptional markers of cell state. For example, to know *a priori* which cell is going to escape irradiation-induced arrest, we need to determine what induces this escape and identify markers that can predict escape as early as possible.

Several studies have sought to link signaling dynamics with transcriptional outputs. Population measurements of transcription showed that oscillatory p53 dynamics promote pro-arrest and survival genes, while sustained p53 dynamics lead to upregulation of apoptosis and senescence genes [5,10,30,31]. However, heterogeneity in TF dynamics within a population limits the conclusions that can be drawn from population-level measurements. This limitation can be circumvented by measuring both TF dynamics and transcriptional output in the same cells at a single-cell level [32–34]. In the study that pioneered this approach, the researchers used live-cell imaging of cells trapped in a microfluidic chip to follow the dynamics of the TF NF-κB for 5 h following induction with lipopolysaccharide. The cells contained barcodes, which allowed their identification during imaging and subsequently after single-cell sorting in preparation of scRNA-seq, thus connecting, for the first time, TF dynamics to specific transcriptional outcomes [32]. This approach allowed the researchers to show that heterogeneity in NF-κB dynamics activates different transcriptional programs, demonstrating a functional importance of heterogeneity in dynamics. Such studies could reveal whether transcriptional changes precede, and could predict, later changes in signaling dynamics.

The developmental studies described above used transcriptional profiles to construct a developmental trajectory describing the progress of cells from their progenitor to final differentiated states [20–22]. This approach allows the identification of a smaller number of key transcriptional markers with high predictive capacity, suggesting that this approach could be useful in other systems to collapse transcriptomic signatures to one or a few key markers (Figure 2). However, this strategy requires knowledge of the transcriptional signature of the cellular outcome of interest, which is complicated because of the many possible outcomes and the multiple pathways of arriving at

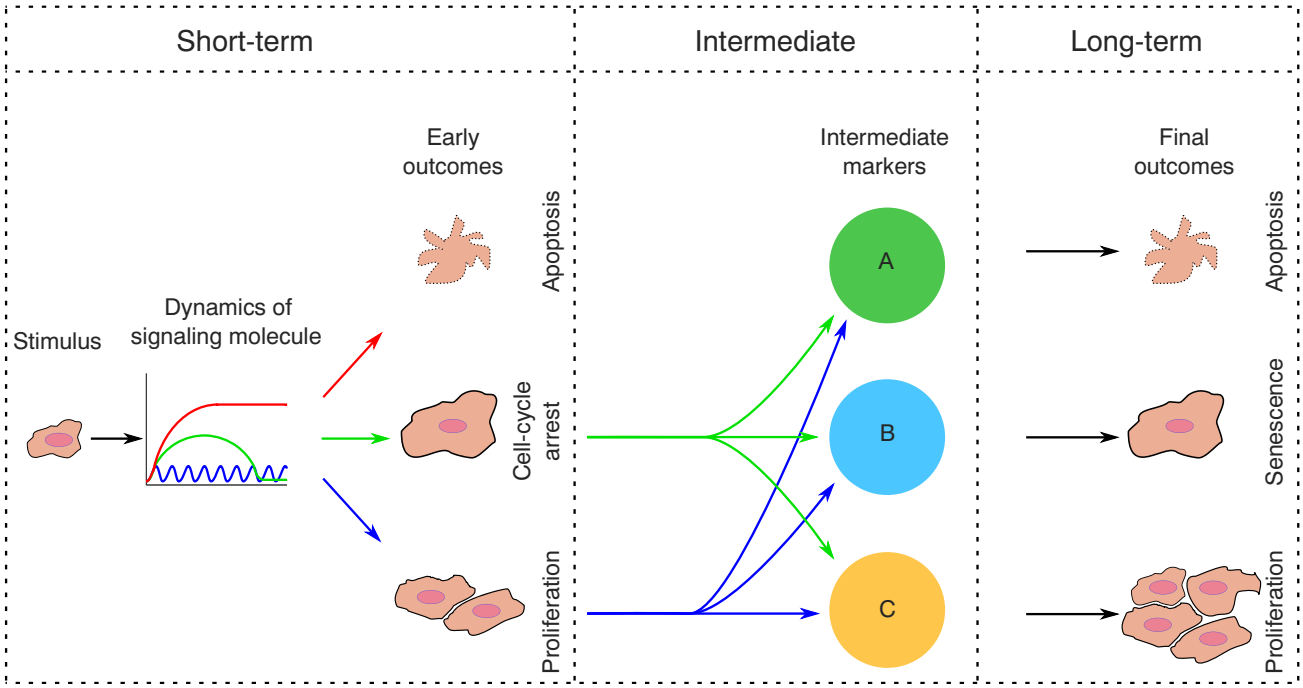

Figure 2. Connecting Short-Term Dynamic Measurements with Long-Term Outcomes Requires Identification of Intermediate Markers. In response to a stimulus, signaling dynamics of the responding transcription factors drive short-term outcomes. Three example outcomes are shown, but others are also possible. These early outcomes can also include terminal states such as apoptosis, but in other cases the initial state can evolve along different trajectories before cells arrive at their final outcomes (e.g., an arrested cell escapes the arrest and starts proliferating). Identifying and monitoring markers at intermediate stages (using transcriptomics, proteomics, epigenetics, and dynamics of additional signaling molecules) could allow predictions of long-term outcomes.

each one. A possible method to address this challenge would be to define intermediate stages for which the associated transcriptomes are highly informative (see Outstanding Questions). For example, we may be able to know *a priori* which cell would switch from oscillatory to sustained p53 dynamics by first defining the time points at which the transcriptomes of switching cells display predictive differences from nonswitchers, and then monitoring the key transcripts expressed by cells at these time points to determine which will switch in the long term. Such an approach would minimize the number and duration of measurements necessary for accurately predicting cellular outcomes.

Identification of Intermediate Protein Markers

Transcriptomics allow us to look at responses in single cells, tying TF dynamics to their immediate output – mRNA. However, in most cases the molecules that execute functions are proteins. To complicate matters further, the relationship between mRNA abundance and protein abundance in many systems was found to be complex and not linear [35,36]. The complex relationships are due in part to the resolution of measurements as well as to differences between the half-lives of mRNAs and their cognate proteins [35,36]. These caveats to transcriptomics make proteomics an attractive approach to understand how signaling dynamics control long-term fates, but this approach is limited by our current inability to analyze the proteome in single cells. Several methods aim to analyze numerous protein targets, and although they still require a more targeted candidate-based approach, they afford a comprehensive look at the proteomic response. Among them, time-of flight mass cytometry (CYTOF) and cyclic immunofluorescence (CYCIF) allow multiplex measurement of protein levels by mass spectrometry and imaging, respectively, although neither technique currently supports measurements of the entire proteome [37,38]. Two additional technologies, cellular indexing of transcriptomes and epitopes by sequencing (CITE-seq) and RNA expression and protein sequencing assay (REAP-seq), use antibodies conjugated to DNA barcodes that can be sequenced along with the transcriptome, thus allowing synchronous evaluation of proteomic output along with the transcriptome from the same single cell [39,40]. These approaches add an important dimension to the transcriptomic data, but are currently not compatible with dynamical measurements of cellular characteristics as well as lack the ability to assess the compete proteome. Development of single-cell whole proteome analysis, in concert with signaling dynamics, could, in the future, provide a more direct analysis of the functional output of the signal and thus improve our long-term predictions significantly. For the time being, scRNA-seq data could help identify fewer and potentially interesting protein targets, which can be analyzed using the multiplexed approached mentioned earlier.

Integration of Markers from Multiple Signaling Pathways

Another intriguing possibility is that the immediate response to a stimulus is governed by a single dominant signal, but the long-term outcomes require integration of two or more signals. Therefore, it is possible that to improve our prediction of cellular outcomes following treatment we will need to look beyond a single signal, with the hope of uncovering interactions between multiple signals. In *Arabidopsis thaliana,* integration of multiple internal and external signals controls various aspects of seed germination [41,42]. In *Saccharomyces cerevisiae*, the decision to undergo quiescence or senescence in response to glucose starvation occurs within the first 4 h and can be predicted to 88% accuracy by integrating information from at least five biomarkers [43]. A recent study showed that the memory of stress responses and mitogen signaling in previous cell cycles determines cell-cycle choices in subsequent cycles in human cells, demonstrating integration of information from two signaling pathways [44]. In the p53 network, it is possible that the initial response to IR is governed by a dominant signal (i.e., oscillations in p53 result in arrest), but the secondary decision point (escape or remain arrested) is controlled by a secondary signaling network. The next step in improving our predictions may therefore depend on our ability to integrate dynamics and local measurements from multiple signaling pathways.

Concluding Remarks and Future Perspectives

The goal of the approaches we propose in this article is to improve the prediction of outcomes using short term measurements in cells. While we believe that implementing these approaches in an experimental setting is, at least in part, already feasible, translation to the clinical setting is likely to be challenging for several reasons. First, some molecular markers used in the research setting are unsuited to clinical use (e.g., fluorescently tagged proteins). In addition, the predictive capacity of some markers in tissue culture may not be reproducible *in vivo*, or may not be suitable due to limited sample quantity or cost considerations. The overarching goal of the approaches we propose here should therefore be to collapse as much predictive power as possible into one or a few markers that can be easily assayed in a patient and retain predictive capacity, allowing physicians to decide which course of action is most likely to be effective and how an individual patient would respond to a treatment (see Outstanding Questions). Indeed, such strategy has already been successfully applied to DLBCL, as clinical implementation of COO classification (discussed earlier) initially proved difficult due to the high cost of transcriptomic analysis and the technical challenges of isolating and analyzing RNA from formalin-fixed biopsies. The identification of a minimal set of protein biomarkers, whose expression correlates closely with COO classification by gene expression but can be analyzed far more quickly and cheaply, proved a critical turning point in widespread adoption of COO classification as standard of care for DLBCL [45]. While universal development of minimal marker sets is clearly an ambitious goal, we are optimistic that intermediate transcriptomic and proteomic markers linking the dynamics of the response with cellular fates, and simultaneous measurement of markers from multiple signaling pathways will enable it in the not-too-distant future.

Outstanding Questions

Genetic, epigenetic, and environmental factors as well as temporal variations in protein expression or activity contribute to cellular responses to stimuli. Which variables will most enhance our ability to accurately predict cells' final outcomes following stimulation?

Cells frequently traverse through distinct physiological states before arriving at the final outcome. What are the intermediate states in this process that can predict the final outcomes?

Recent efforts have focused on integrating diverse types of data to obtain a more complete understanding of cellular responses to stimuli. Which markers best complement signaling dynamical information early in the response to improve the accuracy of outcome prediction?

Technological advances allow collection of multiple types of molecular data *in vitro*, but collecting a full set of data in patients remains challenging. What are the minimal sets of molecular markers that, in combination, can be used to predict patient responses to treatment?

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