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Review

Computational analysis of signaling patterns in single cells

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ABSTRACT

Signaling proteins are flexible in both form and function. They can bind to multiple molecular partners and integrate diverse types of cellular information. When imaged by time-lapse microscopy, many signaling proteins show complex patterns of activity or localization that vary from cell to cell. This heterogeneity is so prevalent that it has spurred the development of new computational strategies to analyze single-cell signaling patterns. A collective observation from these analyses is that cells appear less heterogeneous when their responses are normalized to, or synchronized with, other single-cell measurements. In many cases, these transformed signaling patterns show distinct dynamical trends that correspond with predictable phenotypic outcomes. When signaling mechanisms are unclear, computational models can suggest putative molecular interactions that are experimentally testable. Thus, computational analysis of single-cell signaling has not only provided new ways to quantify the responses of individual cells, but has helped resolve longstanding questions surrounding many well-studied human signaling proteins including NF-κB, p53, ERK1/2, and CDK2. A number of specific challenges lie ahead for single-cell analysis such as quantifying the contribution of non-cell autonomous signaling as well as the characterization of protein signaling dynamics *in vivo*.

Contents

1. Introduction

The ability to visualize signaling proteins in real time and at single-cell resolution has revealed a staggering picture of complexity in cellular signal transduction. Genetically identical cells can show vastly different signaling patterns—even under basal conditions or in response to the same stimulus. In fact, if there has been one lesson learned from single-cell dynamics, it is that variability from cell to cell is the rule rather than the exception. Cells in the same culture dish can show patterns of gene or protein expression that vary over several orders of magnitude [1–3], and signaling patterns measured in real time are noisy and asynchronous [4,5]. These observations present major challenges for understanding single-cell signaling: How much of the observed heterogeneity from cell to cell is meaningful? Are the observed patterns variations of a single signaling response or are there multiple responses? If there

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are multiple responses, how can we distinguish among different signaling patterns in individual cells?

The idea that a single signaling protein can display multiple behaviors is already well appreciated in the field of intrinsically disordered proteins (IDPs), the focus of this special issue. It has long been observed that certain proteins associated with signal transduction have unusually high levels of disordered regions in their peptide sequence [6–8]. This trend may reflect the tendency for signaling proteins to have multiple binding partners as well as multiple functionalities in the cell [6,8,9]. It is our observation that many IDPs (or those involved in signaling complexes with disordered proteins) have been examined by fluorescence time-lapse microscopy. Often, these proteins display rich and complex signaling dynamics in single cells (Fig. 1). This is yet another indication that signaling proteins are structurally pliable molecules capable of sophisticated information processing [10]. As such, the variability of single-cell protein dynamics of IDPs adds additional challenges and opportunities for understanding the role of these proteins in cell signaling and disease.

In this review, we examine how signaling patterns among individual cells have been resolved through the use of computational analyses. We first introduce some of the different patterns of signaling observed in individual cells across different cellular pathways. Since this topic has been reviewed elsewhere in depth [11,12], we provide a brief overview of the variety of signaling patterns observed among mammalian proteins and highlight the insight gained from an exciting set of recent studies [1,13–18]. These studies are drawn together by a common set of computational approaches used to analyze signaling patterns in individual cells. We find that these approaches are essentially variations of existing methods for normalizing and comparing biological data. However, they are specifically tailored toward the heterogeneous temporal data gathered from single cells. In many cases these approaches not only resolve, but also make use of, cell-to-cell heterogeneity by relating variation in signaling to differences in downstream behaviors. We further show how single-cell signaling has been modeled computationally to predict cellular behaviors and suggest new mechanistic interactions. Finally, we discuss specific challenges for understanding single-cell signaling responses that must build on existing work in the field.

2. Single-cell dynamics of human signaling proteins

An increasing number of human signaling proteins have been characterized in living cells including several proteins with significantly disordered protein structures (Fig. 1). These studies typically quantify protein abundance over time using a fluorescent reporter protein that is covalently linked to the coding region of the protein of interest [19,20]. In cases where the enzymatic activity of the protein is more biologically relevant than its expression level, it is necessary to use a genetically encoded biosensor that exhibits conformational changes that reflect changes in the enzymatic activity of interest [21]. These biosensors include, for example, fluorescent substrates that mimic endogenous cleavage sites used to measure protease activity [22]. Once the fluorescent reporter is stably expressed, cells are cultured directly on the microscope, relevant perturbations are performed, and images are acquired periodically at a time scale that is appropriate for the biological process under investigation [11]. Segmentation borders are then drawn to separate neighboring cells. The resulting data set is a time series of fluorescence intensity values that reveal how the protein, or enzyme activity, changes over time in individual cells.

Human signaling proteins show a wide range of dynamical behaviors including pulses [13,18,23], bursts [14], oscillations [24], switches [25], and decays [26]. One of the best-studied proteins in single cells is the stress response factor NF-κB. NF-κB is a transcription factor that responds to cytokines, inflammation, and other cellular stresses. Upon activation with inflammatory stimuli such as tumor necrosis factor α (TNFα), NF-κB localizes to the nucleus and promotes transcription of IκBα, an inhibitor that binds to NF-κB and triggers export of NF-κB to the cytoplasm. Activation of NF-κB and subsequent expression of IκBα leads to multiples cycles of NF-κB nucleo-cytoplasmic shuttling. Live-cell imaging of NF-κB localization has revealed that, after stimulation with TNFα, NF-κB shows a prominent first pulse of activity followed by a series of long-term pulses [24]. At low doses of TNFα, activation of NF-κB is highly heterogeneous with the majority of cells showing all-or-nothing activity [27].

Additional signaling proteins in the immune response have been characterized in individual cells. Notably, two isoforms of the nuclear factor of activated T-cells (NFAT1 and NFAT4) show dramatically different nuclear localization dynamics in response to calcium stimulation [14]. NFAT1 responds slowly to stimulation, showing prolonged occupation of the nucleus over several hours. In contrast, NFAT4 shows rapid and repeated bursts of nuclear localization that last between 5 and 10 min. Although it is premature to make any firm conclusions, it appears that a large proportion of signaling proteins that have been measured in live cells show some form of pulsatile signaling. Whether "frequency modulated" signaling is a pervasive theme in biology remains to be determined [11,12,28]. If so, it would suggest that the temporal pattern of protein signaling may be as relevant as its absolute abundance. Such a finding expands our notion of good indicators of functional relevance to include both expression levels and dynamical patterns of activity.

Another well-characterized protein in live cells is the tumor suppressor p53 [23,29]. Following DNA damage, p53 undergoes posttranslational modification that frees it from Mdm2, an E3 ubiquitin ligase that promotes rapid degradation of the p53 protein. However, because Mdm2 is also a target gene product of p53, the induced elevation of p53 eventually promotes its own degradation, leading to periodic accumulation of nuclear p53. p53 dynamics were originally predicted to be damped oscillations based on population measurements of p53 and Mdm2 by Western blot [30]. When imaged in single cells, however, p53 signaling was shown to occur in a series of pulses with uniform width and height [23,31]. Rather than increasing the absolute levels of p53, larger doses of DNA damage increases the number of consecutive pulses.

As a well-recognized intrinsically disordered protein, p53 has also been examined at the single-molecule level to understand its binding and oligomerization properties [32]. Following initial work to characterize the binding affinity of p53 to DNA using ensemble methods such as analytical ultracentrifugation [33], fluorescence correlation spectroscopy was used to determine the precise kinetics of p53 oligomerization [34]. More recently, analysis of p53 oligomerization dynamics in single cells confirmed that dimers are the predominant form under basal conditions. Interestingly, after DNA damage, formation of p53 tetramers precedes increases in p53 protein levels suggesting that p53 oligomerization is dynamically regulated in response to genotoxic stress [35]. These examples show how the structural disorder of a signaling molecule may affect its cellular function and regulation.

Additional components of the DNA damage response were recently characterized in single human cells. Two members of the hypoxia-inducible factor family of transcription factors (HIF-1α and -2α), which can bind both Mdm2 [36] and p53 [37] to alter cellular stress responses, show a single 3 h pulse that is rapidly terminated under continuous hypoxic conditions [13]. The p53 binding protein 1 (53BP1), which localizes to double-strand DNA breaks, shows exponential decay kinetics that reflect the rate of DNA repair in individual cells. When combined with a