



# Integrated Strategies to Gain a Systems-Level View of Dynamic Signaling Networks

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## Abstract

In order to survive and function properly in the face of an ever changing environment, cells must be able to sense changes in their surroundings and respond accordingly. Cells process information about their environment through complex signaling networks composed of many discrete signaling molecules. Individual pathways within these networks are often tightly integrated and highly dynamic, allowing cells to respond to a given stimulus (or, as is typically the case under physiological conditions, a combination of stimuli) in a specific and appropriate manner. However, due to the size and complexity of many cellular signaling networks, it is often difficult to predict how cellular signaling networks will respond under a particular set of conditions. Indeed, crosstalk between individual signaling pathways may lead to responses that

are nonintuitive (or even counterintuitive) based on examination of the individual pathways in isolation. Therefore, to gain a more comprehensive view of cell signaling processes, it is important to understand how signaling networks behave at the systems level. This requires integrated strategies that combine quantitative experimental data with computational models. In this chapter, we first examine some of the progress that has recently been made toward understanding the systems-level regulation of cellular signaling networks, with a particular emphasis on phosphorylation-dependent signaling networks. We then discuss how genetically targetable fluorescent biosensors are being used together with computational models to gain unique insights into the spatiotemporal regulation of signaling networks within single, living cells.



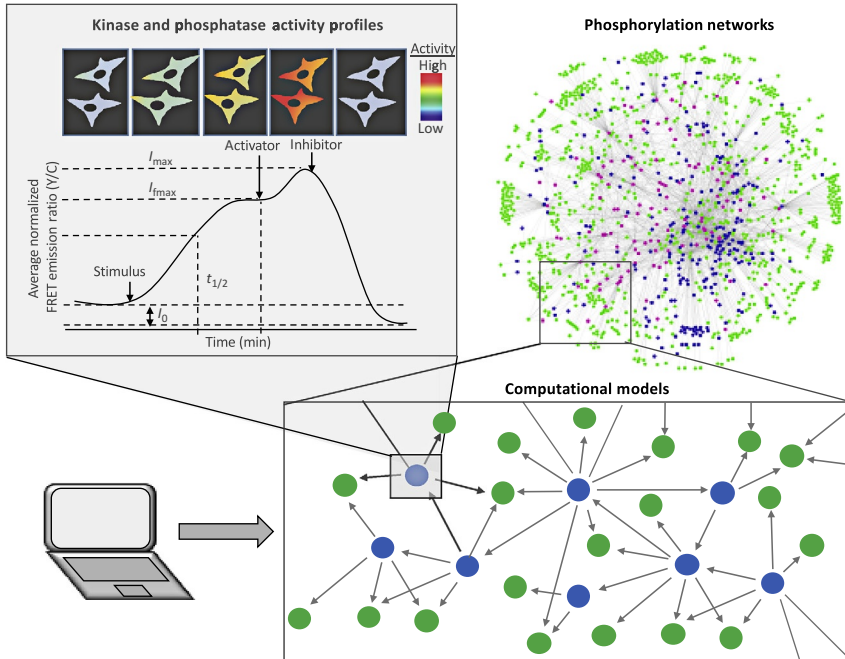
## 1. INTRODUCTION

The ability of cells to receive and rapidly process information about their environment is critical, both for their survival and for their proper function. Indeed, the disruption of cellular signaling mechanisms is believed to underlie a wide range of pervasive diseases, including cancer, diabetes, heart disease, and various autoimmune disorders (Guo, 2014; Kooij et al., 2014; Ortsater, Grankvist, Honkanen, & Sjöholm, 2014; Roskoski, 2016; Ruprecht & Lemeer, 2014; Salzer, Santos-Valente, Keller, Warnatz, & Boztug, 2016; Yang & Yang, 2016). Cellular information processing is achieved through the coordinated action of a diverse collection of signaling enzymes and small molecule second messengers that form extensive, highly integrated signaling networks. At the heart of most eukaryotic signaling networks are protein kinases and phosphatases, whose opposing activities are believed to govern the phosphorylation status of ~30%–40% of cellular proteins (Cohen, 2000; Hu, Neiswinger, Zhang, Zhu, & Qian, 2015).

Inside the cell, protein kinases, phosphatases, and their respective substrates are organized into complex phosphorylation networks that must be precisely regulated in cellular space and time. In recent years, tremendous progress has been made in identifying the structure and organization of cellular phosphorylation networks. As will be discussed in Section 2, this has been accomplished using both “top-down” approaches, such as tandem mass spectrometry (MS/MS) and mass cytometry, that start at the cellular level and “work their way down” to the protein level, and “bottom-up” approaches, such as functional protein microarrays, that start at the protein

level and “work their way up” to the cellular level (Newman, Zhang, & Zhu, 2014). Together, these approaches provide complementary information about specific sites of phosphorylation and the corresponding kinase(s) mediating the phosphorylation event, respectively (Newman et al., 2014). This information can be integrated into detailed maps of kinase–substrate relationships (KSRs) that describe which sites can be phosphorylated by a given kinase (or dephosphorylated by a given phosphatase). However, while such maps provide valuable insights about the global organization of cellular phosphorylation networks, in order to better understand how modulation of these networks correlates with a particular physiological response, it is also important to know when and where these phosphorylation events are likely to occur inside the cell. Indeed, the tight spatial and temporal regulation of cellular phosphorylation networks is believed to underlie the specificity of cellular signal transduction. Therefore, in order to gain a more comprehensive, systems-level understanding of dynamic phosphorylation networks, the static maps described earlier must be supplemented with information about the spatial and temporal regulation of the signaling enzymes within these networks. This requires an integrated approach that combines information from large-scale phosphoproteomics experiments with computational models and kinetic data from live-cell imaging experiments designed to measure the activity profiles of specific pools of kinases and phosphatases within intact networks (Fig. 1).

In this chapter, we first examine several emerging high-throughput technologies that are being used to gain insights into the organization and regulation of cellular phosphorylation networks at the systems level. We then describe how this information is being integrated into computational models of signaling networks to provide a conceptual framework for studying complex interactions between components within the networks. Finally, we turn our attention to the application of genetically targetable fluorescent biosensors to the study of endogenous signaling networks, highlighting how these molecular tools are being used in conjunction with computational models to provide unique insights into the spatiotemporal regulation of dynamic signaling networks at the single-cell level. Throughout the chapter, we will highlight both the advantages and the limitations of the various approaches and, importantly, describe how they are being used together to gain a more comprehensive understanding of dynamic cellular signaling networks.



**Fig. 1** Integrated strategies to study dynamic phosphorylation networks. Information about the topology of intracellular phosphorylation networks obtained from large-scale phosphoproteomics studies (*upper right*) can be combined with live-cell imaging data describing the spatiotemporal regulation of key signaling enzymes in the network, such as protein kinases and phosphatases (*upper left*), to generate computational models (*bottom*) that can be used to predict the systems-level properties of dynamic phosphorylation networks. Importantly, the resulting computational models can be used to generate testable hypotheses that serve to further refine model parameters.



## 2. STRATEGIES TO CHARACTERIZE GLOBAL CHANGES IN THE PHOSPHORYLATION STATUS OF CELLULAR PROTEINS

To date, over 270,000 nonredundant phosphorylation sites (also called phosphosites) have been identified, primarily from humans and mice (PhosphositePlus database <http://www.phosphosite.org> as of 9/2/16) (Hornbeck et al., 2015). However, information about how the majority of these sites are modulated in response to various cellular stimuli is not currently available. Likewise, in many cellular contexts, it is unclear to what extent crosstalk and other systems-level regulatory mechanisms impact

phosphorylation dynamics at these sites. Finally, for the vast majority of sites identified *in vivo*, the identity of the cognate kinase(s) mediating a given phosphorylation event is not currently known. Answers to these questions require systems-level analyses of cellular phosphorylation networks.

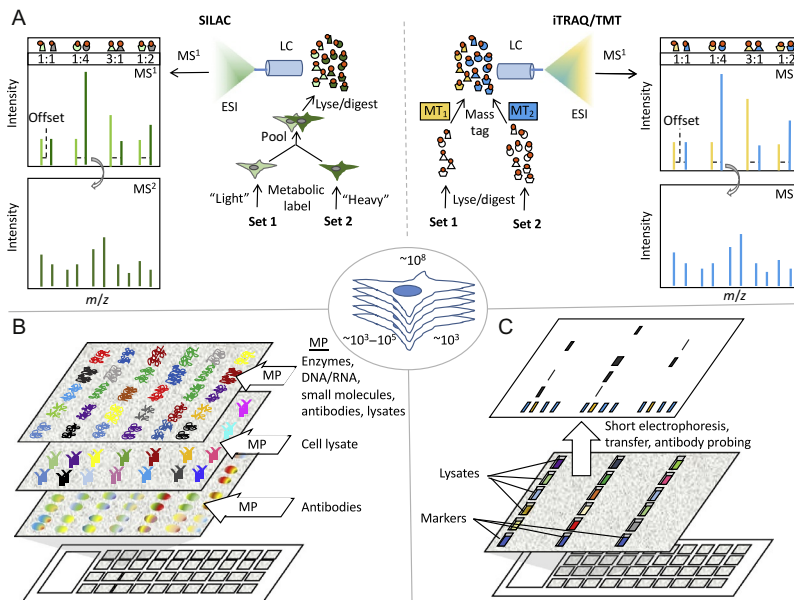
Traditionally, changes in the phosphorylation status of cellular proteins have been measured using standard immunological methods, such as Western blotting, enzyme-linked immunosorbent assays, and immunofluorescence (IF). Indeed, due to their specificity and sensitivity, these “*tried and true*” techniques have been, and will continue to be, staples within the cell signaling community. However, these techniques are only able to provide detailed information about one (or, at most, several) molecular species of interest during a given experiment. As a consequence, even with the recent development of enabling technologies that dramatically reduce the time needed to conduct an experiment using these techniques, their throughput still remains too low to realistically analyze the hundreds, or even thousands, of phosphoproteins required to gain a truly systems-level view of cellular phosphorylation networks. Therefore, to complement and extend these traditional approaches, several high-throughput methods have recently been developed that are able to examine global changes in the phosphorylation status of many cellular proteins simultaneously. These techniques, which can be used to simultaneously monitor time- and/or context-dependent changes in the phosphorylation status of hundreds to thousands of different molecular species, include several ensemble-based approaches, such as quantitative MS/MS (Olsen & Mann, 2013; von Stechow, Francavilla, & Olsen, 2015), protein microarrays (Sutandy, Qian, Chen, & Zhu, 2013; Zhang & Pelech, 2012), and microwestern arrays (Ciaccio, Wagner, Chuu, Lauffenburger, & Jones, 2010), as well as a number of emerging technologies, such as mass cytometry (Bodenmiller et al., 2012), single-cell western arrays (Hughes et al., 2014), and multiplexed cyclic IF (Lin, Fallahi-Sichani, & Sorger, 2015) that provide insights into the regulation of phosphorylation dynamics at single-cell resolution. As described later, together these approaches are providing important information about both the organization and the global regulation of cellular phosphorylation networks. We and others have recently reviewed this topic in detail (Day, Sosale, & Lazzara, 2016; Di Palma & Bodenmiller, 2015; Gingras & Wong, 2016; Grecco, Imtiaz, & Zamir, 2016; Landry, Clarke, & Lee, 2015; Newman et al., 2014; Sutandy et al., 2013; Tape, 2016; Terfve & Saez-Rodriguez, 2012); therefore, here, we will only provide a brief overview of select quantitative phosphoproteomics approaches

and highlight how they are being used to gain insights into the global changes in the phosphorylation status of cellular proteins under various cellular conditions. We begin by examining several ensemble methods designed to analyze cellular material (e.g., cell lysates) that has been pooled from a population of cells. We then discuss several recently developed technologies that are providing new information about phosphorylation networks within individual cells.

## 2.1 Ensemble Approaches Based on Quantitative Mass Spectrometry

As alluded to earlier, the past decade has witnessed an explosion in the number of phosphosites that have been identified *in vivo* and *in situ*. The vast majority of these newly identified phosphosites have been characterized using MS/MS-based approaches. This revolution in the field of phosphoproteomics has been fueled by several factors, including the development of upfront enrichment methods designed to isolate relatively rare phosphoproteins; the introduction of novel fragmentation protocols, such as higher-energy collision dissociation (HCD) (Olsen et al., 2007) that preserve phosphate monoester bonds during the ionization/fragmentation step, improvements in the sensitivity and accuracy of mass analyzers, and the development of sophisticated software packages for the analysis of mass spectra (Newman et al., 2014). However, though standard MS/MS protocols have become well suited for identifying and annotating phosphopeptides from complex cellular mixtures, it is often difficult to monitor *changes* in the phosphorylation status of cellular proteins using traditional MS/MS workflows. This can be attributed to many factors, including the complexity of biological samples, low fractional stoichiometries of many phosphosites inside the cell, and run-to-run variations that can occur at several steps during phosphopeptide enrichment protocols. To overcome these challenges, researchers have developed several quantitative MS methods, such as stable isotope labeling of amino acids in cell culture (SILAC) (Ong, 2012) and isobaric tags for relative and absolute quantitation (iTRAQ) (Evans et al., 2012) that make it possible to directly compare phosphorylation profiles of multiple samples in a single experiment (Fig. 2A). These approaches, which rely on isotopic labeling of protein and peptide fragments, respectively, have quickly become cornerstones in the field of phosphoproteomics.

During a SILAC experiment, cellular proteins are first metabolically labeled by growing the cells in culture media containing either a stable isotope of a particular “heavy” amino acid(s) (usually  $^{13}\text{C}$ -Arg and/or  $^{13}\text{C}$ -Lys)



**Fig. 2** Ensemble methods to study global changes in the phosphorylation status of cellular proteins. (A) Quantitative mass spectrometry (MS) approaches, such as SILAC (left) and iTRAQ/TMT (right), allow changes in the relative levels of thousands of phosphoproteins to be measured in a single experiment. In a SILAC experiment, cellular proteins are differentially labeled by growing cells in the presence of either a “heavy” isotope of a particular amino acid (dark green) or its naturally occurring “light” counterpart (light green). Cells are then pooled, lysed, and digested before being subjected to phosphoenrichment and liquid chromatography (LC). Following chromatographic separation, fragments are ionized via electron spray ionization (ESI) and analyzed by tandem MS. During the first stage of mass analysis (MS<sup>1</sup>), the relative abundance of each phosphoprotein is determined based on peak intensities. Peaks containing heavy and light isomers of a given fragment are offset by a known amount, depending on the mass difference between the amino acid isotopes used for metabolic labeling. Finally, the identity of each fragment is determined during the second stage mass analysis (MS<sup>2</sup>). The workflow for iTRAQ/TMT (right) is similar to that of SILAC, except proteolytic fragments are not labeled with isobaric tags (MT<sub>1</sub> and MT<sub>2</sub>) until after cells have been lysed and subject to proteolysis. Once labeled, the proteolytic fragments are pooled, enriched, and analyzed by LC-MS/MS, as described for SILAC. (B) Approaches based on protein microarrays. Functional protein microarrays (top) are composed of purified proteins or protein domains immobilized on a functionalized glass surface in a spatially defined manner. Typically, individual proteins are printed in duplicate or triplicate on the arrays. Functional protein microarrays can be used to study interactions between the proteins immobilized on their surfaces and a variety of biomolecules in the mobile phase (MP), including active enzymes (to study global enzyme–substrate relationships), DNA/RNA (to assess the DNA/RNA binding properties of proteins), small molecules

*Continued*

or its more common, “light” counterpart (Fig. 2A, left). Following lysis, the lysates from each set of cells are mixed together and subjected to identical downstream processing steps, including proteolytic digestion, liquid chromatography (LC)/gel-based separation and phosphopeptide enrichment, followed by MS/MS-based detection. Similarly, iTRAQ and related techniques, such as tandem mass tagging (TMT) (Dayon & Sanchez, 2012; Jia, Andaya, & Leary, 2012), use isotopic labeling to distinguish the cellular contents derived from different samples during analysis (Fig. 2A, right). However, as opposed to SILAC, the isotopic labeling step during iTRAQ and TMT experiments occurs after the cells have been lysed and their lysates subjected to protease digestion. Though this increases the potential for side reactions that can complicate analysis, it also makes these approaches amenable to analysis of primary cells (in addition to cultured cells). Once labeled, the fragments are pooled, processed, and analyzed in a manner similar to SILAC.

The key to isotopic labeling approaches is that, because the isotopic isomers derived from differentially labeled cells are chemically identical, they behave identically during upfront enrichment steps and LC-MS/MS analysis. However, importantly, the mass difference between the isotopic isomers leads to a measurable difference in their respective mass-to-charge ( $m/z$ ) ratios, allowing them to be distinguished from one another during mass analysis. As a consequence, relative changes in the phosphorylation status at a given site can be determined based on the ratio of the intensities between the heavy and light isotopes of fragments containing that site. Thus, quantitative MS approaches represent powerful tools for tracking system-wide changes in phosphorylation networks in a variety of cellular contexts.

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**Fig. 2—cont’d** (protein–small molecule interactions), antibodies (antibody recognition), and whole-cell lysates. Meanwhile, analytical protein microarrays (*middle*) contain a series of antibodies immobilized on their surface. These arrays are treated with cell lysates in the MP in order to measure the relative abundance of various proteins under a given condition. Finally, reverse phase protein arrays (RPPAs; *bottom*) are composed of a small amount of cell lysate obtained from cells under different conditions and/or from different patients. Each RPPA is treated with a specific antibody in the MP. (C) Microwestern arrays are similar to RPPAs except the proteins in each lysate can be resolved from one another during a short electrophoresis step. Following electrophoresis, the proteins are transferred to a nitrocellulose membrane and probed with various antibodies. *Center*. The approximate number of cells required per assay is shown (e.g., MS/MS-based analysis typically requires  $\sim 10^8$  cells/experiment while protein microarrays typically use between  $10^5$  cells (analytical microarrays) and  $10^3$  cells (RPPA) per assay).

For instance, Humphrey et al. recently used SILAC to generate dynamic maps of the insulin-sensitive phosphoproteome in 3T3-L1 adipocytes (Humphrey et al., 2013). To this end, the authors employed a multiplexed SILAC strategy to identify those phosphosites whose levels were altered at various times after insulin stimulation. In total, these studies identified over 37,200 phosphosites on 5705 distinct proteins, 15% of which were regulated in an insulin-dependent manner. Moreover, by combining the observed phosphorylation profiles at each time point with a network of well-characterized phosphorylation sites, these studies uncovered functionally linked temporal clusters within the network. Interestingly, all of the well-characterized substrates of some kinases (e.g., Akt) were found in one temporal cluster while all those of other kinases (e.g., p70S6K) were found in another cluster. This suggests that, in some contexts, kinase activation alone may be the rate-limiting step in substrate phosphorylation. As we will see in [Section 3](#), this has potentially important implications for systems-level modeling of dynamic phosphorylation networks using kinetic data obtained using FRET-based kinase activity reporters.

Though temporally resolved phosphoproteomes have the potential to offer important insights into the global regulation of phosphorylation networks, until recently, dynamic profiling of phosphoproteomes has been restricted to relatively few time points and/or biological replicates. This is due, in large part, to the requirement for time-consuming fractionation steps prior to mass analysis. Therefore, to improve the temporal resolution afforded by MS/MS studies, the Mann laboratory recently developed EasyPhos, a streamlined MS/MS-based phosphoproteomics workflow that is optimized for phosphoprotein digestion and enrichment while eliminating labor-intensive desalting steps (Humphrey, Azimifar, & Mann, 2015). Consequently, EasyPhos, which is designed to be compatible with parallel 96-well plate formats, dramatically increases the throughput of MS/MS-based phosphoproteomic analyses. For instance, using this approach, the authors were able to analyze 91 biologically distinct liver tissue phosphoproteomes in parallel. These analyses, which consisted of between 6 and 10 replicates at each of 10 time points after insulin stimulation (including 4 early time points at 5, 10, 15, and 30 s), revealed functionally linked temporal nodes similar to those observed in 3T3-L1 adipocytes. They also uncovered points of signal integration with the fibroblast growth factor receptor (FGFR) signaling pathway and a novel phosphorylation site on Akt2 S478. The latter, which is likely phosphorylated by mammalian target of rapamycin complex 2 (mTORC2) based on consensus site information,

was proposed to play a role in a feed-forward mechanism that could lead to Akt2 ultrasensitivity during insulin signaling.

By providing a wealth of information about the specific phosphosites present under different cellular conditions, large-scale MS-based studies such as those described earlier are helping to define the dynamic phosphoproteome. These data promise to provide important insights into the cellular targets of protein kinases and phosphatases during both physiological and pathological signaling processes. However, due to the high cost of instrumentation and the high level of technical expertise required to conduct MS/MS experiments, advanced MS/MS approaches often require substantial upfront investment and/or dedicated core facilities that are not available to many researchers. Moreover, compared to many of the other phosphoproteomics techniques outlined later, MS/MS-based strategies require a relatively large number of cells for analysis (e.g., a typical experiment requires  $\sim 10^8$  cells per lysate). Finally, and perhaps most importantly, like most other “top-down” approaches, MS/MS-based experiments are unable to definitively match a given phosphorylation event with the upstream kinase catalyzing the phosphorylation. This KSR information is critical for the construction of functional phosphorylation networks. Therefore, to complement and extend the information provided by MS/MS-based studies, other high-throughput platforms have recently been developed that are amenable to the study of phosphorylation networks.

## **2.2 Ensemble Approaches Based on Protein Microarrays**

Recently, several approaches based on protein microarray technology have emerged as powerful tools for probing the phosphorylation states of a large number of cellular proteins simultaneously. Briefly, protein microarrays are composed of a collection of proteins immobilized on a functionalized glass surface in a spatially defined manner. Currently, three main classes of protein microarrays are broadly available—functional protein microarrays, analytical microarrays, and reverse phase protein microarrays (RPPAs)—and all three have been useful for studying cellular phosphorylation networks (Fig. 2B) (Sutandy et al., 2013; Zhang & Pelech, 2012).

### **2.2.1 Functional Protein Microarrays**

We will begin by examining functional protein microarrays, which are composed of purified full-length proteins (and/or protein domains) immobilized on their surfaces (Fig. 2B, top). These arrays have proven to be highly versatile tools for studying signaling processes on a global scale. For instance,

functional protein microarrays have been used to study a variety of biochemical processes related to signal transduction, including protein–protein interactions (Fasolo, Im, & Snyder, 2015; Popescu et al., 2007; Zhu et al., 2001), protein–nucleic acid interactions (Hu, Xie, Blackshaw, Qian, & Zhu, 2011; Hu et al., 2009; Sutandy, Hsiao, & Chen, 2016), and protein small-molecule binding (Huang et al., 2004; Kung et al., 2009). Importantly, because they are also amenable to activity assays designed to determine enzyme–substrate relationships, functional protein microarrays have been particularly useful for determining the global substrate specificity of key signaling enzymes (Cox et al., 2015; Jeong, Rho, & Zhu, 2011; Lu, Lin, Boeke, & Zhu, 2013; Sun et al., 2016). For example, to gain a better understanding of the organization of human phosphorylation networks, we recently examined the global substrate profiles of 289 unique human kinases using functional protein microarrays composed of ~4200 full-length human proteins (Newman et al., 2013). These studies, which used in-depth bioinformatics analysis to identify 3656 physiologically likely KSRs from among the over 24,000 raw KSRs identified based solely on signal intensity, more than doubled the number of KSRs that had been identified to that point (Hu et al., 2014; Newman et al., 2013). Moreover, by combining our KSR dataset with information both about *in vivo* phosphorylation sites determined by MS/MS and consensus phosphorylation motifs for each of the kinases in the network, we were able to construct a high-resolution map of phosphorylation networks that connects 230 kinases to 2591 *in vivo* phosphorylation sites on 652 substrates (Newman et al., 2013). This information will be particularly useful for understanding the functional consequences of phosphorylation and for developing new tools, such as phospho-specific antibodies and novel FRET-based kinase activity reporters, used to study dynamic phosphorylation networks. Moreover, the generalizability of this approach suggests that it will be useful for examining the global substrate profiles of enzymes involved in SUMOylation (Cox et al., 2015), ubiquitylation (Jeong et al., 2011), acetylation (Lu et al., 2013), and O-glycosylation (Sun et al., 2016), which together will offer clues about potential points of intersection between different signaling modalities.

Though functional protein microarrays represent an attractive, high-throughput platform for the rapid identification of a large number of direct enzyme–substrate relationships, there are several caveats that must be considered when using this approach. For instance, when using functional protein microarrays, a stringent cutoff is typically used to reduce the rate of false positives. As a consequence, substrate identification may be biased toward

those proteins that contain multiple sites of modification. This is because these substrates will exhibit higher signal-to-noise ratios than other bona fide substrates that may only be modified at a single site and, therefore, may exhibit a signal intensity that falls below the cutoff. Likewise, proteins that are immobilized in greater quantities on the microarrays may exhibit higher signal-to-noise ratios or may be preferentially modified by the enzyme of interest. On the other hand, recognition of some substrates may be impaired due to misfolding of the substrates during purification or by immobilization of substrates in orientations that are inaccessible to the modifying enzyme.

Importantly, because direct enzyme–substrate relationships are generally determined using purified protein components in the mobile phase, interactions that are dependent on auxiliary factors, such as scaffold proteins, cellular cofactors, or other posttranslational modifications, may be missed using functional protein microarrays and other bottom-up approaches that use purified components. This has led to the development of several complementary approaches wherein more complex solutions, such as preformed protein complexes (Hu et al., 2015) or even whole-cell lysates (Goodwin et al., 2016; Woodard et al., 2013), are used in place of purified proteins in the mobile phase. This can be combined with computational approaches to identify novel KSRs that are dependent on auxiliary interactions. For instance, Hu et al. recently employed a bioinformatics strategy to identify novel scaffold proteins involved in the coordination of kinase signaling pathways (Hu et al., 2015). To this end, the authors mined protein–protein interaction databases to identify those proteins that interacted with each of the components within a given linear KSR pathway. These studies, which involved over 1100 known kinase–substrate pairs, identified 212 putative scaffold proteins, of which 194 had not been previously reported. They then used functional protein microarrays composed of >17,000 unique human proteins to validate their predictions. To this end, two putative scaffolds, activating transcription factor 2 (ATF2) and peptidylprolyl *cis/trans* isomerase, NIMA-interacting-1 (PIN1), that are predicted to be involved in casein kinase II (CKII) and c-Jun N-terminal kinase (Jnk) signaling, respectively, were tested. Consistent with their hypothesis, select substrates were only phosphorylated by the kinase of interest in the presence of the corresponding scaffold protein, suggesting that the scaffold protein was indeed facilitating phosphorylation of the substrate by the kinase of interest. These studies will be particularly valuable for understanding the spatial organization of phosphorylation networks and, as will be discussed in [Section 3](#), can be used in

conjunction with genetically targetable FRET-based biosensors to study the regulation of specific pools of a given cellular kinase in situ.

### **2.2.2 Analytical Protein Microarrays**

The next class of protein microarrays, known as analytical protein microarrays, is composed of a collection of purified “capture” proteins that bind to distinct analytes (e.g., a phosphorylated residue on a cellular protein) that are present in the mobile phase (Fig. 2B, middle). Though antibodies are by far the most common type of capture proteins utilized on analytical protein microarrays, other biomolecules that bind their targets through specific, high affinity interactions, such as nanobodies and even RNA aptamers, have also been used successfully (Chen, Nakamoto, Niwa, & Corn, 2012; Even-Desrumeaux, Baty, & Chames, 2010). Meanwhile, the mobile phase typically consists of a complex protein solution, such as cell or tissue lysates, under nondenaturing conditions.

In a typical analytical protein microarray experiment, a collection of proteins derived from a population of  $\sim 10^5$  cells are either prelabeled with a fluorescent dye before being incubated with the microarray or added directly to the arrays without prior labeling (Hughes et al., 2014; Zhang & Pelech, 2012). Following incubation, the array is washed and, if the lysate was not labeled beforehand, a second fluorescently labeled antibody that recognizes a protein of interest is added to the arrays. Multiplexing of this secondary detection step can provide information about the phosphorylation status of many different proteins using a single array. Importantly, while such a sandwich assay format potentially increases the time required to complete the experiment, the use of a second antibody for detection can substantially increase the sensitivity and specificity of the assay while reducing artifacts stemming from cross-reactivity of immobilized capture antibodies, poor labeling of low abundance proteins in the lysate, and/or disruption of the epitope through conjugation of the small-molecule fluorescent dye (Sutandy et al., 2013). By examining several lysates in parallel (e.g., at different times after treatment or from different patient samples), information about changes in the phosphorylation levels of many proteins in a given system can be obtained in a reasonable timeframe.

As for all antibody-based techniques used for the analysis of phosphorylation networks, one of the primary obstacles associated with the development of analytical protein microarrays is the availability of high-quality, phospho-specific capture antibodies that do not cross-react with other protein species. To help overcome this limitation, Koerber et al. recently

described an innovative, structure-guided approach for the development of phospho-specific antibodies (Koerber, Thomsen, Hannigan, Degrado, & Wells, 2013). Using this approach, which begins with a library of single-chain variable fragments (scFv's) containing an engineered anion-binding pocket (or "nest") and then uses phage display to enrich for scFv's that recognize a specific immobilized phosphopeptide, the authors were able to identify >50 antibodies that exhibit high specificity for the phosphorylated forms of their respective targets. This generalizable approach has the potential to dramatically expand the toolkit of reliable phospho-specific antibodies, which will not only facilitate the development of analytical protein microarrays but also promises to increase the utility of other approaches based on immunological detection of phosphorylated residues (Stoevesandt & Taussig, 2013).

In addition to antibody specificity, other factors can also impact the interpretation of data obtained from experiments designed to study global phosphorylation profiles using analytical protein microarrays. For instance, the relatively low fractional stoichiometries of many phosphorylated proteins, coupled with differences in avidity among capture antibodies, can contribute to false negatives using this approach—particularly when a given protein is expressed at relatively low levels in the cells under study. On the other hand, because lysates must be applied to the array under nondenaturing conditions, protein-protein interactions may lead to the formation of protein complexes that could cause false positives. Therefore, to guard against such artifacts, it is important that any hits identified during microarray experiments be validated using traditional immunological methods, such as Western blotting. Nonetheless, due to their ability to probe the phosphorylation status of a large number of cellular proteins simultaneously, analytical protein microarrays can provide important information about global changes in phosphorylation networks under a variety of conditions.

### **2.2.3 Reverse Phase Protein Microarrays**

Like analytical protein microarrays, RPPAs represent powerful tools for tracking changes in cellular phosphorylation profiles in cell lysates. However, instead of antibodies, the solid phase of RPPAs contains lysates derived from cell or tissue extracts (Fig. 2B, bottom). Accordingly, each microarray contains a collection of lysates obtained from various sources (e.g., from different clinical samples or from cells treated with a drug for various times and/or at different doses). Once immobilized, changes in the expression levels and/or phosphorylation status of select targets in the lysates are assessed

by probing the arrays with an antibody that is specific for the epitope of interest. Since many microarrays can be probed in parallel, a large number of targets can be tested simultaneously. Such a multiplexing strategy has the benefit of reducing the effects of interassay variability, thereby increasing confidence in the relationships observed between different species in a network.

Since each spot on the array contains only a few nanograms of lysate, many arrays can be prepared using only a small amount of starting material (Pierobon, Wulfschuhle, Liotta, & Petricoin, 2015). This feature is particularly attractive when studying phosphorylation profiles in lysates derived from precious samples, such as patient samples from clinical studies. For instance, Shull et al. recently used RPPA's composed of B-cell lysates from 24 different donors to gain a better understanding of the molecular profiles associated with chronic lymphocytic leukemia (CLL) (Shull et al., 2015). To accomplish this, the authors used 167 different antibodies to compare the levels of various cellular proteins and phosphoproteins in lysates obtained from either CLL patients or healthy controls. Interestingly, nonsupervised hierarchical clustering revealed a clear molecular signature among all CLL samples, regardless of the genetic subtype of CLL being tested. This may suggest that, at the network level, a common molecular profile exists across all CLL subtypes. In support of this notion, supervised hierarchical analysis, which focused on differences in probe intensities between CLL patients and healthy controls, revealed increased expression of several kinases involved in cancer progression in CLL lysates, including B-Raf, Akt, mTOR, Lck, Btk and Syk, as well as several other proteins, such as SF2/ASF and eIF4G, which play important roles in RNA processing and translation initiation, respectively. Moreover, increased phosphorylation of several cellular proteins, including PDK1, 4E-BP1, p70S6K, BAD, and PRAS40, was also observed. Interestingly, interrogation of the molecular functions associated with those proteins that were differentially expressed/phosphorylated between the two groups revealed an overrepresentation of proteins involved in signaling events controlling translation initiation. Together, these observations suggest that increased protein translation may be a key event involved in the initiation/progression of CLL.

### 2.3 Ensemble Approaches Based on Microwestern Arrays

As alluded to earlier, one of the most important considerations for most antibody-based approaches is antibody specificity. In the case of RPPAs,

antibody specificity is particularly important because each spot on the array contains a complex mixture of thousands of different cellular proteins, each of which could lead to a false-positive response if the antibody being used for detection has not been carefully validated. In this respect, RPPAs are akin to dot blots which, unlike Western blots, do not permit a second level of discrimination based on the electrophoretic mobility of the protein(s) recognized by the antibody. Therefore, to increase the accuracy of the RPPA approach, Ciaccio and colleagues recently developed so-called microwestern arrays that combine many of the attractive features of RPPA's with the ability to separate proteins in the lysates based on their apparent molecular weights (Fig. 2C) (Ciaccio et al., 2010). Not only can the increased resolution afforded by microwestern arrays reduce the incidence of false positives caused by antibody cross-reactivity, but they can also potentially expand the repertoire of antibodies that are available for analysis. For instance, previous studies demonstrated that only 4 of the 34 (<12%) antibodies evaluated behaved identically using both RPPA and traditional Western blotting protocols (Sevecka & MacBeath, 2006). In contrast, when a set of eight antibodies—four of which had produced consistent results using both RPPA and Western blotting and another four that had exhibited substantial reduction in their dynamic ranges in the RPPA due to cross-reactivity—were compared side by side using microwestern arrays and traditional “macrowestern” blotting, all of the antibodies tested produced similar quantitative results using both methods (Ciaccio et al., 2010). Though this approach is relatively new, it has already been used to gain important insights into a number of phosphorylation-dependent signaling processes, including those downstream of the epidermal growth factor receptor (EGFR) following EGF stimulation (Ciaccio et al., 2010) and the cytokine receptor during the host–pathogen response in macrophages (Huang et al., 2015).

## 2.4 Approaches That Provide Single-Cell Resolution

Together, the ensemble approaches described earlier provide important information about global changes in cellular phosphorylation profiles. However, because they report responses that are averaged across many cells within the population, ensemble methods lack the ability to distinguish cell-to-cell heterogeneities that can provide important clues about the regulatory mechanisms underlying a particular cellular response. For instance, cell-to-cell differences in the phase and/or the amplitude of a given response

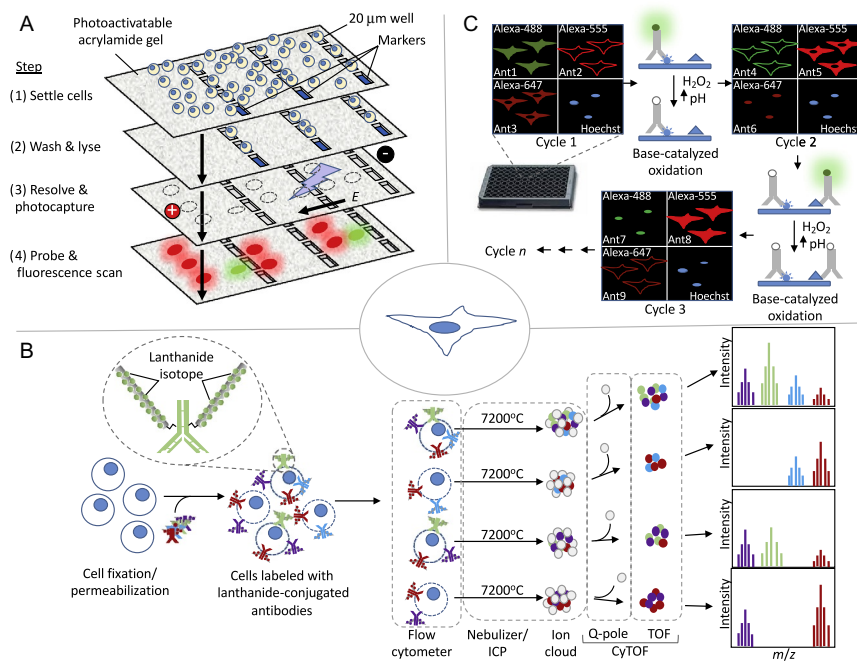
are likely to mask oscillatory and “switch-like” behaviors that encode critical information related to cellular function. In fact, emerging evidence suggests that several signaling systems are characterized by bistable (or multistable) responses (Albeck et al., 2008; Barr, Heldt, Zhang, Bakal, & Novak, 2016; Fosbrink, Aye-Han, Cheong, Levchenko, & Zhang, 2010; Montero et al., 2015; Spencer, Gaudet, Albeck, Burke, & Sorger, 2009). As a consequence, responses that appear to be graded when measured using ensemble approaches may, in fact, reflect differences in the *percentage* of cells that are in the “on” state rather than the degree to which a particular pathway is activated (Bagowski & Ferrell, 2001; Landry et al., 2015; Ni et al., 2011). Therefore, to better understand, how information is processed within individual phosphorylation networks, researchers have developed a series of analytical tools designed to study phosphorylation dynamics at single-cell resolution.

#### **2.4.1 Single-Cell Westerns**

As outlined in Section 2.3, microwesterns provide increased resolution of cellular species compared to RPPA's. However, because  $\sim 10^3$  cells are required per lysate, critical information about single cells is obscured using this approach. Therefore, to extend the microwestern array platform to single-cell applications, Hughes et al. recently developed single-cell western arrays (Hughes et al., 2014). These arrays, which consist of thousands of 20  $\mu\text{m}$  microwells patterned on a photoactivatable polyacrylamide gel attached to a solid glass support, have the potential to offer exciting new insights into systems-level changes in global phosphorylation profiles with single-cell resolution (Fig. 3A). For instance, as a proof of concept, the authors examined the phosphorylation status of extracellular-regulated kinase 1/2 (Erk1/2) and its upstream activator, MEK1/2, following fibroblast growth factor-2 (FGF-2)-mediated stimulation of neuronal stem cells. Interestingly, although these studies demonstrated an overall increase in the ratios of pMEK1/2 to total MEK1/2, and pErk1/2 to total Erk1/2, a large degree of heterogeneity was observed between the responses from cell to cell. This is consistent with the notion that each cell has a unique cellular profile based on its individual signaling history that may be masked by ensemble methods that pool material from thousands of cells.

#### **2.4.2 Mass Cytometry**

Flow cytometry, which is perhaps the most widely used method to study signaling dynamics within single cells, has provided important information



**Fig. 3** Single-cell methods to study global changes in phosphorylation status of cellular proteins. (A) Single-cell Western blotting. Individual cells are first applied to a photoactivatable acrylamide gel immobilized on a glass support. Once individual cells have settled into 20  $\mu\text{m}$  wells patterned on the surface, excess cells are removed and the remaining cells are lysed in a RIPA buffer. An electric field ( $E$ ) is then applied to the gel in order to resolve proteins present in the lysates. After electrophoresis, the proteins are fixed in the gel by irradiation (photocapture) before being probed with fluorescently labeled antibodies. (B) Mass cytometry. Following fixation and permeabilization, cells are incubated with up to 40 distinct antibodies, each labeled with a different isotope of a lanthanide metal ion (red, green, blue, or purple circles). The cells are then sorted using a flow cytometer before being nebulized and atomized/ionized using an inductively coupled plasma (ICP) torch containing superheated argon plasma. Atomization generates ion clouds that are introduced into a CyTOF mass cytometer, composed of a quadrupole (Q-pole) and a time-of-flight (TOF) mass analyzer, for elemental analysis. Signal intensities are proportional to the concentration of individual target molecules in each cell. (C) Cyclic immunofluorescence. During the first cycle, cells are fixed/permeabilized and treated with a set of antibodies labeled with spectrally distinct antibodies (e.g., Alexa-488 (green), Alexa-555 (red), and Alexa-647 (far-red)), as well as Hoechst stain (blue). Following imaging, the fluorophores are inactivated via base-catalyzed oxidation. The cycle is repeated using a second set of fluorescently labeled antibodies along with Hoechst stain as an internal control. This process is repeated through multiple rounds to build a multichannel image.

about signaling dynamics at the single-cell level. However, spectral overlap between fluorescently labeled antibodies effectively limits the number of cellular components that can be monitored simultaneously using this approach to less than 20 species (with upper limits of  $\sim 12$  species for most systems) (Tape, 2016). This, coupled with time-consuming sample preparation and measurement steps, substantially reduces the throughput of flow cytometric methods. Together, these considerations limit the utility of flow cytometry for systems-level analysis of cellular signaling networks. Therefore, to expand the number of distinct species that can be measured per cell, researchers have recently developed mass cytometry approaches that combine the single-cell capabilities of flow cytometry with the sensitivity of mass spectrometry (Fig. 3B) (Bandura et al., 2009; Baranov, Quinn, Bandura, & Tanner, 2002). For instance, to overcome the issue of spectral overlap, antibodies used in mass cytometry applications are conjugated to lanthanide metal ions rather than fluorescent dyes. To this end, each antibody is conjugated to a distinct lanthanide isotope via an acrylic acid polymer functionalized with multiple copies of a trivalent metal chelator, such as diethylenetriaminepentaacetic acid (Bjornson, Nolan, & Fantl, 2013; Di Palma & Bodenmiller, 2015). Cells are then permeabilized and labeled with the lanthanide-tagged antibodies before being sorted using a flow cytometer and nebulized into single-cell droplets. Once nebulized, each cell droplet is atomized/ionized in a stream of superheated argon plasma to generate single-cell ion clouds that are shuttled into a tandem quadrupole-time-of-flight mass cytometer (CyTOF) for elemental analysis. Since the lanthanide metal isotopes used for analysis do not exist at appreciable levels in most cell types, this approach benefits from very low background signal. This, coupled with the fact that between 100 and 150 metal ions can be conjugated to each antibody, results in very high signal-to-noise ratios, allowing a limit of detection of only a few hundred copies of a given phosphosite per cell (Di Palma & Bodenmiller, 2015). This feature is particularly important for measuring substoichiometric changes in the phosphorylation state of low abundance cellular proteins. Currently,  $\sim 40$  distinct lanthanide isotopes are available for mass cytometry applications, which permit the simultaneous analysis of subnetworks or key nodes within signaling networks (Bjornson et al., 2013; Di Palma & Bodenmiller, 2015; Gingras & Wong, 2016). Importantly, because a subset of the antibodies can be used to recognize distinct cell types within a complex heterocellular environment, mass cytometry can provide unique insights into the regulation of phosphorylation networks in complex cellular microenvironments more similar to those

found *in vivo* (Tape, 2016). For instance, Fernandez and Maecker recently developed a panel of  $\sim 30$  antibodies designed to track changes in the phosphorylation status of eight major nodes involved in cytokine, antigen, and toll-like receptor signaling across all major white blood cell lineages, including monocytes, and several B and T cell lineages (Fernandez & Maecker, 2015). However, because cellular phosphorylation networks can be perturbed during dissociation of cells from complex tissue matrices, mass cytometry and other flow-based single-cell strategies are better suited for cells that are naturally nonadherent, such as the hematopoietic lineages alluded to earlier (Tape, 2016). Moreover, like all of the methods discussed to this point, since mass cytometry requires cell lysis prior to detection, it offers little information about the spatial distribution of phosphorylation-dependent signaling events inside cells.

### 2.4.3 High-Content Imaging Approaches

Traditionally, antibody-based imaging approaches, such as IF, have been used to examine the spatial distribution of cellular signaling enzymes, such as protein kinases and phosphatases, and their downstream substrates. These studies have provided evidence of spatially defined signaling modules that are thought to be important for coordinated information flow inside the cell.<sup>a</sup> However, classical IF suffers from several limitations that reduce its utility for the systems-level analysis of dynamic cellular signaling networks. For instance, the throughput of classical IF is somewhat limited compared to many high-throughput technologies. Moreover, like flow cytometry, the number of distinct molecular species that can be reliably imaged in a single cell using standard IF-based protocols is limited by spectral overlap between fluorophores. To overcome these limitations, traditional IF protocols have been adapted in several innovative ways. For instance, microfluidic technologies, such as that used by the ImStain system developed by the Levchenko lab, can improve the throughput of IF experiments substantially (Cheong, Wang, & Levchenko, 2009). Likewise, to increase the number of distinct molecular species that can be monitored in a single experiment, Giesen et al. recently developed imaging mass cytometry (Giesen et al., 2014). This approach, which combines the multiplicity and precision of mass cytometry with a sophisticated laser ablation system that aerosolizes antibody-stained

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<sup>a</sup> In this context, a signaling module represents a collection of signaling molecules (e.g., receptors, signaling enzymes, small molecule second messengers, and downstream effectors) that form an integrated, functional circuit within the larger network.

cellular regions in a spatially defined manner and feeds them into the CyTOF system, permits the analysis of up to  $\sim 40$  distinct species in formalin-fixed tissue sections at a spatial resolution of  $\sim 1 \mu\text{m}$  (Di Palma & Bodenmiller, 2015). However, because aerosolization and subsequent atomization effectively destroys the cell present at each coordinate, analysis of additional species beyond the number of currently available lanthanide isotopes is not feasible.

As an alternative single-cell imaging strategy, Lin et al. recently developed cyclic immunofluorescence (CycIF) (Lin et al., 2015). CycIF is a generally applicable IF imaging method that involves repeated rounds of IF imaging using 3–5 fluorescence channels (plus an additional channel to visualize DNA) per round (Fig. 3C). Each round of imaging is followed by fluorophore inactivation using a combination of hydrogen peroxide, high pH, and irradiation with white light. Importantly, because the inactivation step is relatively straightforward, CycIF is compatible with 96- and 384-well formats, substantially increasing the throughput of CycIF experiments. Using this approach, the authors were able to track changes in the abundance of six cellular species, including different phosphorylation states of the S6 ribosomal protein (p-S6<sup>S240/244</sup> and p-S6<sup>S235/S236</sup>), following treatment of BRAF<sup>V600E</sup> melanoma cells with intermediate doses of the RAF inhibitor, vemurafenib. The relative levels of each species led to the identification of two distinct subpopulations in vemurafenib-treated BRAF<sup>V600E</sup> cells, one corresponding to proliferating cells in which the Erk/Rsk pathway is highly active and the other representing a quiescent, apoptosis-resistant population characterized by high TORC1 activity. In this study, while no loss of immunogenicity was observed after five cycles of staining and fluorophore inactivation, it remains to be seen whether visualization of the tens to hundreds of species required for systems-level analysis of signaling networks will be hampered by destruction of epitopes and/or modification of the cellular architecture during subsequent rounds of peroxide treatment.



### 3. STRATEGIES TO TRACK KINASE AND PHOSPHATASE ACTIVITY PROFILES IN LIVING CELLS

Together, the strategies outlined earlier provide a wealth of information about which cellular proteins are modified in response to various environmental changes. However, in order to gain a mechanistic understanding of how cellular phosphorylation networks are regulated under physiological conditions, this information must be supplemented with information about

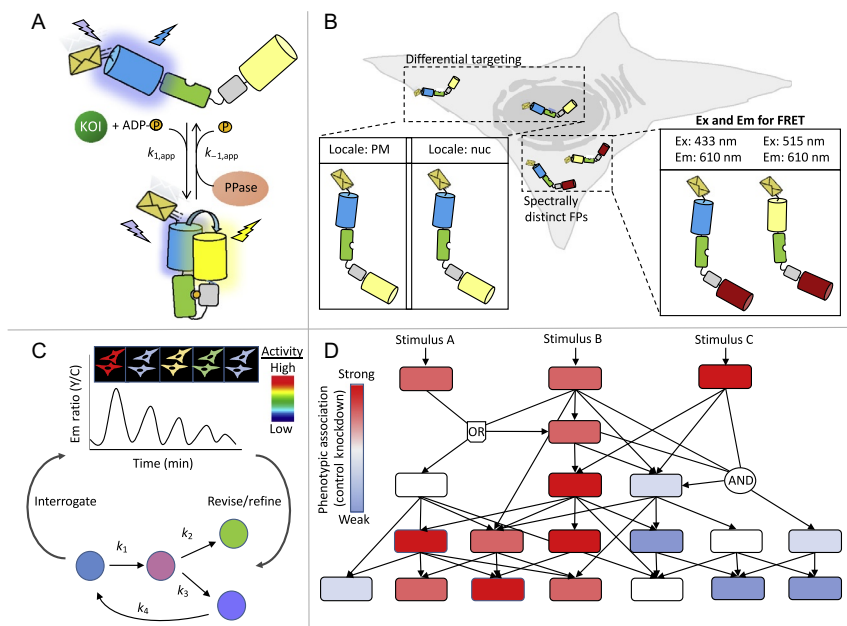
the spatial and temporal regulation of the kinases and phosphatases governing the phosphorylation state of these proteins. However, the methods described earlier generally do not provide direct information about the regulation of the signaling enzymes, themselves. Moreover, in most cases, these approaches lack the spatial and/or temporal resolution necessary to capture key aspects of phosphorylation-dependent signaling at the single-cell level. For example, due to the high cost and/or technical demands associated with many of the aforementioned methods, it is often not practical to take a large number of measurements over relatively short time intervals (e.g., every 30 s or 1 min). However, many important signaling processes, such as the oscillatory behaviors exhibited by protein kinase C (PKC), cAMP-dependent protein kinase (PKA),  $\text{Ca}^{2+}$ /calmodulin-dependent kinase II (CaMKII), and the  $\text{Ca}^{2+}$ /calmodulin-dependent protein phosphatase, calcineurin (CaN), occur on timescales of seconds to minutes (Markoulaki, Matson, & Ducibella, 2004; Mehta et al., 2014; Ni et al., 2011; Violin, Zhang, Tsien, & Newton, 2003). As a consequence, these behaviors may appear skewed or could be missed altogether during experiments that lack sufficient temporal resolution. Likewise, when using ensemble-based approaches like MS/MS and microwestern arrays, cell-to-cell heterogeneities are likely to mask dynamic behaviors that provide key information pertaining to a given cellular response. Finally, and perhaps most importantly, because each of the methods described earlier requires either cell lysis or cell fixation/permeabilization prior to analysis, they are not able to monitor real-time changes in single, living cells. Thus, they offer only a “snapshot” of the dynamic changes in kinase and phosphatase activities that characterize most phosphorylation-dependent signal networks.

### 3.1 Genetically Targetable Fluorescent Biosensors

The considerations outlined earlier are nontrivial, since critical information is often encoded in the dynamic properties (e.g., frequency and duration) and spatial patterning (e.g., compartmentalization) of signaling activities within the cell (Cheong & Levchenko, 2010; Ganesan & Zhang, 2012; Mehta et al., 2014). This, coupled with the fact that stochastic differences in expression patterns and signaling “history” exist between individual cells, suggests that significant cell-to-cell heterogeneities may exist within phosphorylation networks that can only be captured by tracking kinase and phosphatase activities at multiple times within individual, living cells. This requires molecular tools that are able to monitor dynamic changes in the

activity profiles of these enzymes with high spatiotemporal resolution. For instance, we and others have developed a series of genetically targetable fluorescent biosensors to track the activities of select signaling molecules within the endogenous cellular environment of single, living cells (Allen et al., 2008; Mehta & Zhang, 2011; Newman, Fosbrink, & Zhang, 2011). These biosensors, which can be directed to specific subcellular regions through the incorporation of a targeting motif (e.g., a nuclear localization sequence) or a component of a signaling complex (e.g., a scaffold protein) (Kunkel & Newton, 2014; Zhang, Ma, Taylor, & Tsien, 2001), are able to monitor real-time changes in the activity profiles of specific pools of a given signaling enzyme or second messenger under a variety of cellular conditions (Gao & Zhang, 2010; Kunkel & Newton, 2009). Importantly, as discussed in more detail later, the high spatiotemporal resolution afforded by these sensors provides kinetic data that can be integrated into computational models that provide insights into the behaviors of entire signaling networks (Greenwald, Polanowska-Grabowska, & Saucerman, 2014; Ni et al., 2011; Saucerman et al., 2006; Violin et al., 2008).

All fluorescent biosensors developed to date contain two basic elements: (1) a “sensor unit,” which undergoes a conformational change in response to a given cellular stimulus (e.g., phosphorylation or enzyme activation) and (2) a “reporter unit,” which converts the resulting conformational change into a change in the spectral properties of an attached fluorescent protein (FP) color variant(s) (Frommer, Davidson, & Campbell, 2009; Newman & Zhang, 2014) (Fig. 4A). While the reporter unit typically consists of either a circularly permuted variant of an FP (in the case of single fluorophore reporters) or a pair of FP color variants that are able to undergo FRET (in the case of FRET-based reporters), the sensor unit can take several forms depending on the cellular parameter being measured. For example, FRET-based biosensors that exploit an intrinsic conformational change in a cellular protein have been developed to track changes in the phosphatase activity of CaN (Mehta et al., 2014; Newman & Zhang, 2008), as well as the levels of several small molecule second messengers, such as  $\text{Ca}^{2+}$  (Geiger et al., 2012; Liu et al., 2011), cyclic AMP (cAMP) (DiPilato & Zhang, 2009; Klarenbeek, Goedhart, van Batenburg, Groenewald, & Jalink, 2015), cGMP (Honda et al., 2001; Niino, Hotta, & Oka, 2010; Nikolaev, Bunemann, Hein, Hannawacker, & Lohse, 2004; Russwurm et al., 2007), and nitric oxide (Pearce et al., 2000), involved in the regulation of cellular signaling enzymes. In other cases, the conformational change is generated via an engineered molecular switch. For instance, the sensor unit used by most FRET-based



**Fig. 4** Molecular and computational tools to gain a systems-level view of dynamic phosphorylation networks. (A) General design of a genetically targetable FRET-based kinase activity reporter. The biosensor consists of a "sensor unit" composed of a substrate domain (gray rectangle) linked to a phosphoamino acid binding domain (green) via a flexible linker. The sensor domain is sandwiched between a "reporter unit" consisting of two fluorescent protein (FP) color variants (cyan and yellow cylinders) that can undergo FRET. The sensor can be targeted to different subcellular regions via a targeting motif (envelope). Upon phosphorylation by the kinase of interest (KOI; green circle), the reporter undergoes a conformational change that brings the FP's into close proximity to one another, increasing FRET between them (blue arrow). Importantly, dephosphorylation by a phosphatase (PPase; orange oval) returns the biosensor to its open conformation. Apparent rate constants ( $k_{1,app}$  and  $k_{-1,app}$ ) can be determined based on the observed activity profiles. Lightning bolts represent photons of light at the excitation and emission maxima for the respective FP's (purple: CFP Ex: 433 nm; cyan: CFP Em: 477; yellow: YFP Em: 528 nm). (B) Coimaging strategies. FRET-based biosensors can be used to track the activities of two or more signaling enzymes simultaneously. For instance, two reporters that utilize the same FP FRET pair can be targeted to distinct subcellular regions (e.g., the plasma membrane (PM) and nucleus (nuc)). Alternatively, the activity profiles of two enzymes in the same compartment can be monitored simultaneously using reporters that utilize spectrally distinct FPs (e.g., CFP/RFP and YFP/RFP). Donor excitation and acceptor emission maxima for commonly used FP FRET pairs (e.g., Cerulean/mCherry and Venus/mCherry) are shown. Other coimaging strategies, such as FRET-fluorescence lifetime imaging (FRET-FLIM), are not shown. (C) Kinetic data obtained from live-cell imaging experiments can be used to construct computational models of individual signaling circuits based on ODEs/PDEs. The resulting models

kinase activity reporters is based on a modular design consisting of a “substrate domain” containing a sequence that is specifically phosphorylated by the kinase of interest (e.g., a consensus phosphorylation motif) and a “switching domain” encoding a phosphoamino acid-binding domain (PAABD) that binds the phosphorylated form of the substrate domain (Fig.4A). The substrate domain is attached to the PAABD by a flexible linker such that, upon phosphorylation by the kinase of interest, the biosensor undergoes an induced conformational change that alters the distance and/or orientation of flanking FP color variants that are able to undergo FRET (i.e., the “reporter unit”). While the reporter units of most FRET-based kinase and phosphatase reporters utilize a FP FRET pair consisting of variants of cyan FP (CFP) and yellow FP (YFP), other FP combinations, such as green/red FP (GFP/RFP), yellow/orange FP (YFP/OFP), YFP/RFP, and CFP/RFP, have also been used successfully (Ai, Baird, Shen, Davidson, & Campbell, 2014; Day & Davidson, 2009; Lam et al., 2012; Newman et al., 2011; Sample, Newman, & Zhang, 2009). The availability of spectrally distinct reporters facilitates coimaging experiments designed to measure changes in the activity profiles of multiple cellular signaling enzymes (e.g., two kinases or a kinase and a phosphatase) simultaneously in the same cell (Carlson & Campbell, 2009; Woehler, 2013) (Fig.4B). Such information is particularly valuable for understanding crosstalk between signaling pathways within phosphorylation networks. However, due to spectral overlap, currently it is only possible to track the activities of three to four distinct signaling enzymes simultaneously, depending on their subcellular localization (Woehler, 2013).

Using genetically targetable FRET-based activity reporters, researchers have uncovered important details about both the kinetics and the spatial distribution of endogenous kinase and phosphatase activities in a variety of cellular contexts (Allen et al., 2008; Cazabat et al., 2014; Fosbrink et al., 2010;

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can be used to generate hypotheses that can be interrogated using live-cell imaging approaches, the results of which can be used to further refine the model. Such an iterative approach can provide insights into complex signaling behaviors, such as oscillations. (D) Logic-based ordinary differential equation models are useful for studying larger phosphorylation networks. Analysis of these models (e.g., via sensitivity analysis) can reveal functional relationships within the network that may not be apparent from network topology alone (*warmer colored boxes*). These relationships can be evaluated using live-cell imaging experiments. Importantly, because each connection is represented by a differential equation, generalized parameters used for initial model development can be refined as experimental data become available, thereby improving the predictive power of the model.

Gao & Zhang, 2009; Hukasova, Silva Cascales, Kumar, & Lindqvist, 2012; Mehta et al., 2014; Ni et al., 2011; Tobias & Newton, 2016). For instance, recently it was proposed that, aside from simply bringing the atypical PKC family member, PKC $\zeta$ , into close proximity with its substrates, some scaffold proteins, such as partitioning-defective protein 6 (Par6) and p62, may actually regulate the activity of PKC $\zeta$  directly (Graybill, Wee, Atwood, & Prehoda, 2012; Tsai et al., 2015). This is believed to occur as a consequence of binding interactions between PKC $\zeta$  and a Phox and Bem1 (PB1) domain on the scaffold protein. According to this model, binding to the scaffold protein induces conformational changes in PKC $\zeta$  that displaces its auto-inhibitory domain, leading to its activation. To investigate this hypothesis further and to gain insights into the impact that Par6 and p62 have on the localized activity of PKC $\zeta$  during insulin signaling in living cells, Tobias and Newton generated fusion constructs composed of the FRET-based PKC activity reporter, CKAR, linked to the PB1 domains of Par6 and p62 (Tobias & Newton, 2016). Interestingly, these studies revealed that binding to the PB1 domain of Par6 (PB1<sup>Par6</sup>) activated PKC $\zeta$  approximately four times more efficiently than did binding to the corresponding PB1 domain of p62 (PB1<sup>p62</sup>). This, coupled with live-cell imaging experiments that demonstrated that insulin promotes interactions between PKC $\zeta$  and p62 (and, subsequently, with the insulin receptor substrate-1 (IRS-1)), suggests that the binding of PKC $\zeta$  to specific scaffolds can both relocalize and differentially tune its activity during the insulin response.

### 3.2 Integration of Live-Cell Imaging Data and Mechanistic Computational Models to Study Individual Signaling Pathways and Circuits

To better understand how spatiotemporal parameters impact endogenous signaling processes, information from live-cell imaging experiments such as those described earlier can be integrated into quantitative mathematical models of signaling pathways. These models, which are typically based on ordinary and partial differential equations (ODEs and PDEs, respectively), can reveal unexpected relationships and regulatory modules within the system that play important roles in controlling many aspects of cell physiology (Fig. 4C). Importantly, predictions made by computational models can lead to testable hypotheses that, in turn, can be evaluated experimentally using FRET-based reporters (Ganesan & Zhang, 2012; Landry et al., 2015; Ni et al., 2011; Sample et al., 2009; Tobias & Newton, 2016; Yaniv et al., 2015). For instance, to better understand crosstalk between Ca<sup>2+</sup>- and

cAMP/PKA-dependent signaling pathways during insulin secretion in pancreatic  $\beta$  cells, Ni et al. first used the FRET-based PKA reporter, A-kinase activity reporter 4 (AKAR4), to identify oscillatory changes in PKA activity in individual MIN6  $\beta$  cells treated with the  $K^+$  channel inhibitor, tetraethylammonium chloride (TEA) (Mehta et al., 2014; Ni et al., 2011). This oscillatory behavior, which was likely masked at the population level due to averaging artifacts, were shown to be synchronized with both  $Ca^{2+}$  and cAMP oscillations based on coimaging experiments with the  $Ca^{2+}$ -sensitive fluorescent indicator, Fura-2, and the FRET-based cAMP biosensor, indicator of cAMP using Epac (ICUE), respectively. To gain further insights into the molecular mechanisms underlying this  $Ca^{2+}$ -cAMP-PKA oscillatory circuit, the authors then constructed a mathematical model based on a detailed description of biochemical events involving these three molecular components. Not only was the model able to capture qualitative features of the experimental data but, importantly, it also made predictions about how the oscillatory circuit would respond to various pharmacological perturbations. These predictions were confirmed by subsequent live-cell imaging experiments, which together suggest that PKA may function as a frequency modulator during insulin secretion in  $\beta$  cells.

Moreover, based on model predictions, the authors were also able to identify other cellular factors that contributed to the observed oscillatory behaviors. The activity of one of these factors, CaN, was recently shown to be synchronized with  $Ca^{2+}$  oscillations in a manner similar to PKA in TEA-stimulated MIN6  $\beta$  cells (Mehta et al., 2014). Interestingly, these studies, which utilized targeted versions of the CaN activity reporter 2 (CaNAR2) to study CaN dynamics in different subcellular regions, identified distinct zones of CaN activity in response to TEA treatment. For example, while cytoplasmic and plasma membrane-targeted versions of CaNAR2 revealed integrative, step-like increases in CaN activity coincident with each  $Ca^{2+}$  spike, ER- and mitochondria-targeted versions of the reporter oscillated in register with the  $Ca^{2+}$  oscillations. Together, these studies highlight the unique perspectives that can be gained by integrating live-cell imaging data and computational models of cellular signaling networks. Indeed, such an integrated approach has provided key insights into the molecular mechanisms underlying (1) hysteresis and bistability of Aurora B kinase activity during mitosis in HeLa cells (Zaytsev et al., 2016); (2) acceleration of Ras-mediated activation of Raf by the scaffold protein, suppressor of clear homolog (Shoc2), in EGF-stimulated HeLa cells (Matsunaga-Udagawa et al., 2010); (3) cAMP/PKA-mediated regulation of spontaneous action

potential generation in sinoatrial node cardiac pacemaker cells (Yaniv et al., 2015); and (4) the existence of spatially restricted phosphorylation gradients downstream of the  $\beta$ -adrenergic receptor in cardiomyocytes (Saucerman et al., 2006), to name a few.

### 3.3 Perspectives: Strategies to Model Dynamics of Large, Highly Integrated Signaling Networks

To date, the construction and evaluation of computational models using FRET-based biosensors has been applied primarily to the study of individual signaling pathways within a given network, rather than to the broader network itself. This is due, in large part, to the fact that the detailed biochemical parameters necessary to build mechanistic models based on ODEs/PDEs are not currently available for many of the signaling enzymes in the network. Though FRET-based biosensors are well suited to provide this information, live-cell imaging protocols are not particularly amenable to high-throughput analysis (though some progress is being made in this area; e.g., see Maglica, Ozdemir, & McKinney, 2015). Moreover, despite their unique ability to track kinase and phosphatase activity in real-time and at single-cell resolution, FRET-based biosensors may not currently exist for key signaling enzymes within some networks. Finally, very little information about crosstalk between signaling pathways exists and, when it does, it is often cell-type specific. Together, these considerations make it difficult to build mechanistic models of integrated phosphorylation networks “from the ground up” using mechanistic data alone.

How, then, can we gain mechanistic insights into larger, more complex networks? One popular approach involves the development of logic-based computational models. Logic-based models, which use a series of logical operators (e.g., “AND”, “OR”, and “NOR”) to describe the relationships between the activities of protein intermediates, have been used successfully to model large, tightly integrated systems like phosphorylation networks. For instance, logic-based models based on Boolean logic, fuzzy logic, and extreme pathways analysis have been useful frameworks for modeling feasible solution spaces, feedback loops, and global network topologies in a variety of cellular systems (Landry et al., 2015; Terfve & Saez-Rodriguez, 2012). The versatility of these models stems from the fact they do not require rate parameters to be measured or estimated. However, though this improves their scalability, it can be a double-edged sword when studying highly dynamic systems such as phosphorylation networks. For example, because logic-based models assume that the influences of all protein states in the

system are binary and equal in magnitude, they do not generally provide information about dynamic spatiotemporal relationships that underlie the regulation of cellular phosphorylation networks (Landry et al., 2015). Moreover, aside from nuclear vs cytoplasmic localization, these models rarely incorporate spatial information that may be critical to achieving signaling specificity within the system.

To overcome these limitations, methods have recently been developed that combine elements of logic-based models with those of kinetic models to create so-called logic-based differential equation models (Henriques, Rocha, Saez-Rodriguez, & Banga, 2015; Kraeutler, Soltis, & Saucerman, 2010; Ryall et al., 2012; Zeigler, Richardson, Holmes, & Saucerman, 2016). These hybrid models retain the scalability of logic models while providing semimechanistic information about dynamic, systems-level behaviors within the network. For instance, interrogation of logic-based differential equation models (e.g., via sensitivity analysis) can provide a global view of quantitative functional relationships between each species in the network, often predicting key signaling enzymes whose overall influence on the system is greater than would be expected based on the network topology alone (Kraeutler et al., 2010; Ryall et al., 2012; Zeigler et al., 2016). The basic approach used by these methods is to construct a logic model and then estimate kinetic parameters within the system by converting binary relationships into continuous logic-based ODEs (Yaniv et al., 2015). The variables within these ODEs, which can take on any value between 0 and 1, are generally estimated based on limited experimental data or assigned default values determined empirically for the system under study. For instance, the Saucerman lab recently described a logic-based differential equation approach based on a normalized Hill modeling framework (Kraeutler et al., 2010). This approach, which has been used to model signaling networks in both cardiomyocytes and cardiac fibroblasts (Kraeutler et al., 2010; Ryall et al., 2012; Zeigler et al., 2016), uses normalized Hill functions (i.e., logic-based ODEs) to describe individual reactions in the network and “AND” and “OR” logical operators to describe crosstalk between pathways. Accordingly, each interaction in the network is assigned a reaction weight ( $W$ ), a half-maximal effective concentration ( $EC_{50}$ ), and a Hill coefficient ( $n$ ). Meanwhile, the activity of each species is controlled by a reaction time constant,  $\tau$ , and the maximal fractional activation of the species,  $I_{\text{fmax}}$  (Fig. 1). In most cases, values for these parameters are generalized due to the limited availability of quantitative biochemical data for many of the species in the network. For instance, in the cardiomyocyte model, all species were

assigned default parameters, where  $W$ ,  $\tau$ , and  $I_{\text{fmax}}$  were each set to 1, and  $\text{EC}_{50}$  and  $n$  were set to 0.5 and 1.4, respectively. Likewise, all initial activities ( $I_0$ ) were arbitrarily set at 25% of the maximum activity. Despite these simplifying global assumptions, the resulting model was able to approximate dynamic behaviors within the system and correctly predict qualitative input–output relationships for many of the reactions reported in the literature. Notably, experimental data either in support of or counter to model predictions could not be found for over 50% of the predicted relationships, suggesting that substantial gaps still exist in the literature (Ryall et al., 2012). This is consistent with the notion that detailed biochemical and kinetic data are not currently available for many of the components in the system.

Importantly, because the parameters used to construct logic-based differential equation models are described by ODEs, they can be updated and refined as experimental data become available. Thus, logic-based differential equation models represent an attractive general modeling framework to construct semimechanistic models of cellular phosphorylation networks that can be tested in live cells using genetically targetable FRET-based kinase and phosphatase activity reporters, and subsequently refined using experimentally derived kinetic parameters (Fig. 4D). Moreover, the targetability of FRET-based reporters allows distinct pools of the signaling enzymes under study to be monitored, adding a crucial spatial component to the network models. Together, this information can be used to make predictions and generate testable hypotheses about complex network behaviors, such as the dynamic control of crosstalk and signal integration within the system. For instance, using sensitivity analysis, it is possible to predict signaling pathways that are likely to interact in response to a given cellular stimulus or set of stimuli. These predictions can be evaluated directly in living cells using FRET-based activity reporters, either by employing coimaging strategies to monitor the activity profiles of individual components in each of the pathways or by activating one pathway and measuring changes in the activity of a representative kinase/phosphatase in the other pathway. Incongruities between model predictions and experimental results can help inform further model refinement (e.g., revisions to the value of  $n$  for a given reaction) and the generation of new experimental hypotheses. Such an integrated, iterative approach represents a generalizable method to gain a systems-level view of dynamic phosphorylation networks.



## 4. CONCLUSIONS

To better understand how cells convert information about their internal and external environments into an appropriate response, it will be necessary to understand how cellular phosphorylation networks function at the systems level. This requires both information about the global phosphoproteome under various cellular conditions and details about how the activities of signaling enzymes that control their phosphorylation state are regulated in cellular space and time. To this end, researchers have developed a series of complementary strategies that, together, are beginning to reveal exciting new details about the organization and regulation of cellular phosphorylation networks, both at the population level and within single cells. By integrating this information into computational models of cellular signaling networks, researchers are gaining new insights into the molecular mechanisms underlying the complex, systems-level behaviors that lead to a given functional cellular response. Such an integrated approach promises not only to provide a clearer picture of how highly dynamic, tightly integrated phosphorylation networks function under normal physiological conditions, but it also has the potential to deepen our understanding of disease progression and improve the efficacy of pharmacological interventions in accord with systems pharmacology paradigms.

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