**Cell Signaling Regulation by Protein Phosphorylation:
A Multivariate, Heterogeneous, and Context-dependent Process**

Evan K. Day^{1,^}, Nisha G. Sosale^{1,^}, and Matthew J. Lazzara^{1,2,*}

¹Department of Chemical and Biomolecular Engineering

²Department of Bioengineering

University of Pennsylvania, Philadelphia, PA

[^]contributed equally, ^{*} corresponding author

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Contact information for correspondence:

Matthew J. Lazzara

Department of Chemical and Biomolecular Engineering

University of Pennsylvania

311C Towne Building, 220 S. 33rd Street

Philadelphia, PA 19104-6393

215-746-2264, mlazzara@seas.upenn.edu

Abstract

Proper spatiotemporal regulation of protein phosphorylation in cells and tissues is required for normal development and homeostasis, but aberrant protein phosphorylation regulation leads to various diseases. The study of signaling regulation by protein phosphorylation is complicated in part by the sheer scope of the kinome and phosphoproteome, dependence of signaling protein functionality on cellular localization, and the complex multivariate relationships that exist between protein phosphorylation dynamics and the cellular phenotypes they control. Additional complexities arise from the ability of microenvironmental factors to influence phosphorylation-dependent signaling and from the tendency for some signaling processes to occur heterogeneously among cells. These considerations should be taken into account when measuring cell signaling regulation by protein phosphorylation.

Keywords: phosphorylation, kinase, activity, localization, complex, heterogeneity, microenvironment, multiplexed

Cell signaling regulation by protein phosphorylation

Cell signaling is the biochemical process by which cells are cued to respond to perturbations in their environment. In one example, ligand binding to the extracellular domain of a receptor initiates a network of intracellular biochemical reactions that affect cell phenotypes by modifying gene expression, metabolism, or cytoskeletal arrangements. Signaling regulates most normal cell processes, but improper signaling results in numerous diseases.

Many biomolecules participate in signaling, including proteins, amino acids, lipids, and second messengers (e.g., cyclic AMP, inositol triphosphate). The focus here is signaling regulation by protein phosphorylation, which plays several mechanistic roles. Phosphorylation generally receives greater attention than other post-translational modifications, in part because kinases are often over-expressed or mutated in disease, especially cancer. As a result, many inhibitors and antibodies have been developed to antagonize the activity of kinases.

Protein phosphorylation by kinases: Mechanistic aspects and signaling regulatory roles

Protein phosphorylation is a reversible process wherein phosphate from ATP (or other nucleoside phosphates) is esterified to amino acids by protein kinases. Serine and threonine phosphorylation are the most common phosphorylation events, with tyrosine accounting for <1% of the total esterified phosphate. Other amino acids, including histidine and lysine, can be phosphorylated, but roles of these events have not been deeply investigated.

In humans, 518 protein kinases control phosphorylation. 90 are tyrosine kinases, and 56 are transmembrane receptors (receptor tyrosine kinases, RTK). The remaining 372 are serine/threonine kinases, some of which are receptors, and dual specificity protein kinases (tyrosine and serine/threonine). Protein kinases have conserved structural motifs including an activation loop, catalytic domain, and ATP binding domain. Many kinase structures have been solved, which has aided inhibitor design.

Protein phosphorylation regulates signaling in multiple ways (Fig. 1A). Phosphorylation of residues in kinase activation loops promotes an active kinase conformation (e.g., Thr²⁰²/Tyr²⁰⁴ in the extracellular regulated kinase, ERK). Conversely, some phosphorylation events negatively regulate kinase activity (e.g., SRC Tyr⁵²⁷ phosphorylation, which promotes inhibitory intramolecular tethering). Thus, some phosphorylation events are probed as proxies for host protein kinase activity. Protein phosphorylation also creates binding sites for proteins containing cognate motifs. For phosphotyrosines, these include SRC homology 2 (SH2) and phosphotyrosine binding (PTB) domains. Domains for phosphorylated serine or threonine also exist. Phosphorylation-dependent protein complex formation has regulatory roles including protein localization, activation of constituent proteins, and protein trafficking.

Protein phosphatases

Phosphorylation-dependent signaling is tightly regulated by protein phosphatases, which hydrolyze phosphate esters (Fig. 1A, top panel). 147 protein phosphatases are encoded in the human genome. Of the 100 protein tyrosine phosphatases (PTPs), 37 are phosphotyrosine-specific, including 21 receptor-like PTPs. 65 PTPs have dual specificity for phosphorylated serine or threonine and tyrosine. While phosphatase biochemical functions have been well studied, substrates of specific phosphatases remain largely unknown. Phosphatases are often referred to as non-specific, but some recent findings may argue against this notion [1].

Phosphatases are critical for normal cell signaling, and alterations to phosphatase expression or localization and phosphatase mutation arise in numerous diseases and can alter response to therapy [2,3]. Interestingly, elevated phosphatase activity can sometimes promote, rather than antagonize, pathogenic signaling. SH2-domain containing phosphatase 2 (SHP2), which is required for complete ERK activation downstream of most RTKs, provides a well-known example [4,5]. Although phosphatase catalytic domains have been difficult to drug with specificity, inhibition through allosteric mechanisms may be a viable approach [6].

An underappreciated aspect of phosphatase regulation is that dephosphorylation rates can be fast compared to other signal transduction rates, including those for receptor trafficking and signaling persistence. This issue has been studied for epidermal growth factor (EGF) receptor (EGFR) signaling using experimental and computational modeling [7,8]. These studies have revealed that EGFR tyrosine dephosphorylation occurs efficiently at the plasma membrane [8] and have been extended to predict the efficacy of different EGFR-targeted therapeutics [9] and the impact of different receptor dimerization schemes on receptor phosphorylation [10]. Whether or not all phosphorylation events have similarly rapid turnover remains unclear, but a broad survey may provide new understanding of signaling regulation across pathways.

Factors influencing phosphorylation-dependent signaling and signaling measurements

Control of phenotypes by signaling networks: Need for multiplexed measurements. Cells may integrate information from multiple signaling pathways to make decisions (Fig. 1B). In colorectal carcinoma cells, cytokine-mediated apoptosis is regulated by a network of signaling processes, with some initiated through autocrine feedback [11]. In glioma cells, the ERK and STAT3 pathways each contribute to the distinct phenotypes of proliferation and therapeutic resistance, but to different degrees [4]. Kinase-mediated signaling may also be dramatically rewired in cancer cells at the network level through transcriptional responses to therapeutics, and this plasticity is a common driver of therapeutic resistance [12]. The capacity for signaling rewiring can also be leveraged for therapeutic benefit, as in the sequential delivery of EGFR kinase inhibitors and DNA damaging agents to prime tumor cells for apoptotic response [13]. Data-driven computational models have been indispensable for providing quantitative understanding of network control of phenotypes [11,14] and provide motivation for quantitative approaches for multiplexed signaling measurements.

Cellular heterogeneity in signaling processes. Signaling regulation in cell populations may exhibit biologically relevant cell-to-cell variability (Fig. 1B). In flies and other organisms,

normal development requires signaling by mitogen-activated protein kinases (MAPK) to proceed with spatial regulation, leading to key transcription factor expression in some cells [15]. In tumors, spatial and temporal heterogeneity of phosphorylation-dependent signaling can influence various phenotypes [16], including resistance of cell subpopulations to therapy [17]. While heterogeneity can have a genetic basis, it may also arise from stochastic fluctuations in gene expression [18] or microenvironmental cues at the tumor/stroma interface [19]. Computational tools are helpful for predicting the consequences of heterogeneity, as in the use of multi-objective computational optimization to predict useful therapeutic combinations for targeting cell subpopulations in tumors [20].

Context dependence: Influence of the microenvironment. The microenvironment also plays important roles in signaling (Fig. 1B). For example, stiff extracellular matrices may promote epithelial-mesenchymal transition (EMT) by activating or upregulating specific signaling pathways or transcription factors [21,22] or by tuning response to ligands such as transforming growth factor beta [23]. In pancreatic ductal adenocarcinoma, SMAD4-mutant tumors engage STAT3-dependent signaling driven partly through integrins, leading to tumor stiffening and fibrosis and further driving tumor progression [24]. A number of engineered microenvironment systems have been developed to help uncover the physical basis for such observations. For example, cells can be grown on two- or three-dimensional gels of varying stiffness, including polyacrylamide gels where stiffness can be tuned and surfaces can be decorated with collagen coatings whose shape and structure can be manipulated to recapitulate *in vivo* contexts [25]. 3D cell spheroids that mimic the shape of tissue structures are also useful *in vitro* models [26].

Crosstalk among different cell types can also drive signaling. For example, tumor stromal cells (e.g., pancreatic stellate cells) may secrete ligands that impact tumor progression or response to therapy [27]. Conversely, tumor cells can secrete ligands that promote recruitment of macrophages or other immune cells that may support tumor progression or chemoresistance [28]. Advances have been made to reconstitute this complexity in engineered

microenvironments [29,30]. Methods also exist to modulate crosstalk *in vivo*, for example via transcription factor knockdown in tumor macrophages [31]. Signaling crosstalk can also be mechanically modulated. For example, CD47-mediated inhibition of macrophage phagocytosis, which is regulated by the PTP SHP1, can be overcome by sufficient stiffness of phagocytosed particles [32]. This effect may synergize with the tendency for stiffer cancer cells to migrate less than softer counterparts [33] to promote retention and immune capture of stiff tumor cells.

Local concentrations of diatomic oxygen or reactive oxygen species can also affect phosphorylation-dependent signaling. In tumors, hypoxia initiates angiogenic signaling through vascular endothelial growth factor receptor and transcriptional adaptation mediated by hypoxia-inducible factors [34], which can substantially rewire signaling networks. Reactive oxygen species in tumors can also regulate signaling by oxidizing reactive cysteines in PTPs and reversibly inactivating the catalytic domain [35].

Measuring cell signaling processes involving protein phosphorylation

The ability to measure protein phosphorylation was greatly aided by the development of phosphorylation-specific antibodies in the 1980s. Such antibodies are now used in a wide array of techniques. Phosphotyrosine is generally easier to detect, and is more immunogenic, than other phosphorylated residues. This may explain why phosphotyrosine measurements are disproportionately represented in the literature compared to the prevalence of phosphotyrosine in the phosphoproteome. In all measurements, antibody specificity is critical, and kinase inhibitors or siRNA reagents can aid in antibody validation. Measurement techniques not involving site-specific antibodies are also available (e.g., activity assays, mass spectrometry). While these approaches are sometimes thought of as being more quantitative, this may not necessarily be the case. Regardless of the method used, careful sample preparation is required to preserve phosphorylation. Indeed, the rapid dephosphorylation that occurs upon cell lysis without proper handling (phosphatase inhibition and cooling) can have negative consequences

for phosphoprotein analysis. The following discussion is not comprehensive, but highlights advantages of certain conventional and newer measurement approaches, with interwoven discussion of the issues of context dependence, heterogeneity, and multivariate signaling.

Western blotting. Western blotting is the original multiplexed signaling measurement. The size-based electrophoretic separation of proteins, specificity of antibodies, and ability to strip and re-probe membranes allows for the survey of many analytes in a single blot. Western blots can be employed quantitatively if appropriate experimental and image analysis techniques are used after diagnostic testing [36]. Small-format blots can also increase the bandwidth of this approach, enabling 576 lanes to be run within the dimensions of a 96-well plate [37]. Thus, western blotting can be a powerful quantitative technique even for network-level analysis [13]. While conventional western blotting analyzes lysates of cell populations, recent advances enable western blotting with single-cell resolution [38]. Of course, a disadvantage of western blotting, and all approaches using cell lysates, is the inability to make measurements easily in 3D microenvironments. While tissues and gels can be enzymatically disaggregated to create lysates, substantial changes in protein phosphorylation may occur during preparative steps due to the rapid kinetics of dephosphorylation. Similar concerns apply for tissue samples [39].

Fluorescence microscopy. Fluorescence microscopy has long been used for measuring protein phosphorylation with single-cell resolution and subcellular localization detail. Confocal platforms allow for the direct analysis of cells embedded in complex 3D microenvironments. Variations on standard fluorescence microscopy, including those with single-molecule resolution, can be used for quantifying protein density in membranes, binding rates, and diffusivities. Fluorescence microscopy can be multiplexed to a modest degree on typical microscopes (~3-4 independent signals), but higher end instruments can separate 6-8 conventional fluorophores, or larger numbers of quantum dot reagents.

Ectopic expression of fluorescent fusion proteins is commonly used for live-cell microscopy to provide high temporal resolution of signaling processes (e.g., receptor trafficking).

A caveat is that standard expression methods can create over-expression artifacts. However, gene editing now enables the knockin of fusion proteins at endogenous loci. This approach was recently used to visualize differential endocytic trafficking of EGFR and H-Ras in response to EGF in HeLa cells and to understand the consequences for MAPK activation [40].

One of the most commonly implemented fluorescence microscopy techniques in signaling research is fluorescence resonance energy transfer (FRET) imaging. FRET can be used for measurements of protein-protein interactions [41], protein conformation [42], or kinase activity. A well-known example of a FRET-based activity reporter is the extracellular signal-regulated kinase activity reporter (EKAR) [43], which has been used to study oscillatory EGF-mediated ERK activation and relationships between ERK activity and nucleocytoplasmic shuttling [44,45]. While FRET is powerful, a number of caveats must be considered, including potential difficulties in obtaining robust signals due to competition of endogenous substrates with reporters, a general issue that can affect MAPK regulation of endogenous genes [46]. It is also possible to image phosphorylation-dependent signaling with single-molecule resolution, which has aided in quantifying phosphorylation-dependent binding rates [47].

Luminex. Phospho-specific antibodies can also be utilized in multiplexed bead arrays that can be sorted and measured using the Luminex platform. In this approach, antibodies are immobilized on beads that are identified by the amount of fluorescent dye contained within. After incubation with lysates, beads are mixed with fluorophore-conjugated secondary antibodies, enabling the simultaneous detection of more than 100 distinct analytes from the same lysate. The Luminex platform is widely used for cytokine analysis, but kits are also available for phosphoproteins and have been utilized for protein phosphorylation measurements at the network level [48]. With careful validation of antibodies and protein loadings, Luminex can provide quantitative measurements with a relatively high degree of multiplexing.

Mass spectrometry. Mass spectrometry can be used to measure phosphorylation, and other post-translational modifications, at the proteomic level. Enrichment for particular

phosphorylation sites prior to analysis provides especially deep network views of kinase-dependent signaling [49]. Analysis can be done with relative or absolute quantitation using isobaric tags, and methodological improvements continue to be made. A recent study described a new multiplexed method with absolute quantification and large dynamic range of protein phosphorylation and applied the method to quantify the effects of EGFR inhibition in glioblastoma patient-derived xenografts [50]. Mass spectrometry datasets provide a useful basis for data-driven computational models connecting multivariate signaling to phenotypes, as in a study that identified HER2-regulated signaling events that modulate mammary epithelial cell migration and proliferation [51]. Despite its ability to broadly interrogate phosphorylation mediated signaling, mass spectrometry is not without limitations. Tryptic protein digestion can fail to produce appropriately sized peptides for detection, leading to missed phosphorylation events. Many detected phosphorylation sites may be of unknown biochemical function. This may aid discovery, but can also reduce the efficiency of data-driven computational modeling.

Flow cytometry. In flow cytometry, single cells flow through an optical bench for light scattering and fluorescence measurements. While live-cell cytometry is limited to surface markers tagged by fluorophore-conjugated antibodies or cells expressing fluorescent proteins, fixed cells can be probed for surface and intracellular proteins. Whereas sophisticated flow cytometers can quantify up to 17 fluorophores in 1000s of cells in minutes, lower end instruments may detect 4-6 distinct colors. The lower limit may suffice for standard applications, but is typically insufficient for signaling analysis at the network level. Some cytometers are now equipped with in-line imaging for analysis of protein localization, cell morphology, and other characteristics. These instruments have lower throughput (~5- to 10-fold reduction compared to conventional cytometers), but the increased information content can be a worthwhile tradeoff.

Mass cytometry. Mass cytometry is a relatively new approach in which metal-tagged antibodies are used to label cells that are passed in single file through a microfluidic chamber and nebulized, creating single-cell peptide clouds that are subjected to mass spectrometry.

Mass cytometry has been used in numerous studies, such as the multiplexed analysis of 14 protein phosphorylation levels in response to 27 inhibitors from many samples simultaneously using mass-tag barcoding [52] to probe inhibitor activity at the signaling network level and identify off-target effects [53]. Because detection is based on metal tags, many more signals can be resolved than with fluorescence approaches and without compensation. The current multiplexing limit seems to be ~45 analytes, but 90 or more may eventually be possible. To interpret mass cytometry datasets, dimensionality reduction approaches including t-distributed stochastic neighbor embedding are helpful [54].

Antibody and protein arrays. Phosphorylation-specific antibodies can also be used in high-throughput arrays [55]. One strategy is to survey many analytes for a small number of conditions. In forward phase protein arrays, antibody libraries are arrayed on substrates to localize antigen binding to specific locations. In one example, 91 phosphorylation sites on 67 proteins were surveyed for six conditions to probe RTK co-activation in response to EGF [37]. Alternatively, investigators may be interested in changes in a small number of phosphoproteins across many conditions. For such cases, reverse-phase protein arrays can be implemented by arraying lysates on substrates and probing with a small number of antibodies. In one example, 56 breast cancer samples were analyzed for phosphorylation of AXL and MET [56].

Phosphoprotein measurements in resected tissues. Immunohistochemistry is often used to detect phosphorylated proteins in fixed or frozen tissues, often using peroxidase-conjugated secondary antibodies to overcome fluorescence limitations in relatively thick sections. More recently, it was demonstrated that phosphorylation-dependent protein complexes can also be detected in tissue samples using proximity ligation assays, as in a study demonstrating a correlation between EGFR:GRB2 complex abundance and lung cancer sensitivity to EGFR kinase inhibitors [57]. To parallelize such measurements, small tissue sections can be arrayed on glass. While tissue arrays have clear throughput advantages, data must be interpreted with

caution given that protein phosphorylation is sensitive to tissue handling and is highly variable within resected tissues [39].

Transcriptional profiling. Transcriptional profiling is also being increasingly utilized to provide inferences on relevant phosphorylation-dependent signaling processes. In one example, a survey of 132 distinct transcripts using commercially available RNA microarrays revealed that ERK reactivation after MET receptor inhibition in gastric cancer cells resulted from diminished expression of dual specificity phosphatases [58]. Deep sequencing of RNA (RNA-Seq) is also being increasingly utilized to gain insight into signaling regulation. While these measurements are expensive to make, they provide gene expression analysis across the entire transcriptome and are extremely useful for discovery projects [59]. Inferences on phosphorylation-dependent signaling mechanisms can be made from RNA sequencing data through pathway analysis using WebGestalt [60] or other available computational tools.

Concluding remarks

The control of cellular phenotypes by networks of protein phosphorylation events, context-dependence of signaling, and signaling heterogeneity among cells bring special considerations to bear when measuring phosphorylation-dependent signaling. While some questions may be reasonably addressed using conventional techniques that produce relative quantitative comparisons at the cell population level, other questions may require single-cell resolution or absolute quantitation. The choice of method should ultimately be dictated by balancing scientific objectives with considerations of cost, sample and instrumentation availability, and processing time. Investigators should also consider how data will ultimately be utilized, especially when large datasets will be produced. A wide range of computational approaches have been developed to aid in the interpretation of signaling measurements made at various scales or to generate predictions from them. For maximal scientific utility,

measurement and modeling approaches selected together as part of the study design process.

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Figure Legends

Figure 1. (A) Protein phosphorylation (primarily on serine, threonine, or tyrosine) can influence cell signaling in multiple ways including: regulation of biochemical activity of host proteins; reversible formation of protein complexes (e.g., through SH2 domain-phosphotyrosine binding interactions); and regulation of protein localization. (B) Complete analysis and understanding of phosphorylation-dependent signaling processes may need to account for: the abilities of multiple signaling pathways to coordinate to determine complex cellular phenotypes; the tendency for cell signaling processes to occur heterogeneously among cells in populations; and the ability of the local cell context (microenvironment) to influence signaling processes through numerous effects including matrix mechanical effects on adhesion proteins, hypoxia-initiated transcriptional regulation, and ligand-mediated intercellular crosstalk.

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(A)

Some regulatory roles of protein phosphorylation

activity regulation

complex formation

cellular localization

(B)

Factors influencing phosphorylation-dependent signaling

multivariate signaling

cell-to-cell variability

context dependence

Heterogeneity

Context dependence

Multivariate signaling