

# Directional and quantitative phosphorylation networks

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## Abstract

Directionality in protein signalling networks is due to modulated protein–protein interactions and is fundamental for proper signal progression and response to external and internal cues. This property is in part enabled by linear motifs embedding post-translational modification sites. These serve as recognition sites, guiding phosphorylation by kinases and subsequent binding of modular domains (e.g. SH2 and BRCT). Characterization of such modification-modulated interactions on a proteome-wide scale requires extensive computational and experimental analysis. Here, we review the latest advances in methods for unravelling phosphorylation-mediated cellular interaction networks. In particular, we will discuss how the combination of new quantitative mass-spectrometric technologies and computational algorithms together are enhancing mapping of these largely uncharted dynamic networks. By combining quantitative measurements of phosphorylation events with computational approaches, we argue that systems level models will help to decipher complex diseases through the ability to predict cellular systems trajectories.

**Keywords:** systems biology; mass spectrometry; network modelling; phosphorylation; quantitation; prediction

## INTRODUCTION

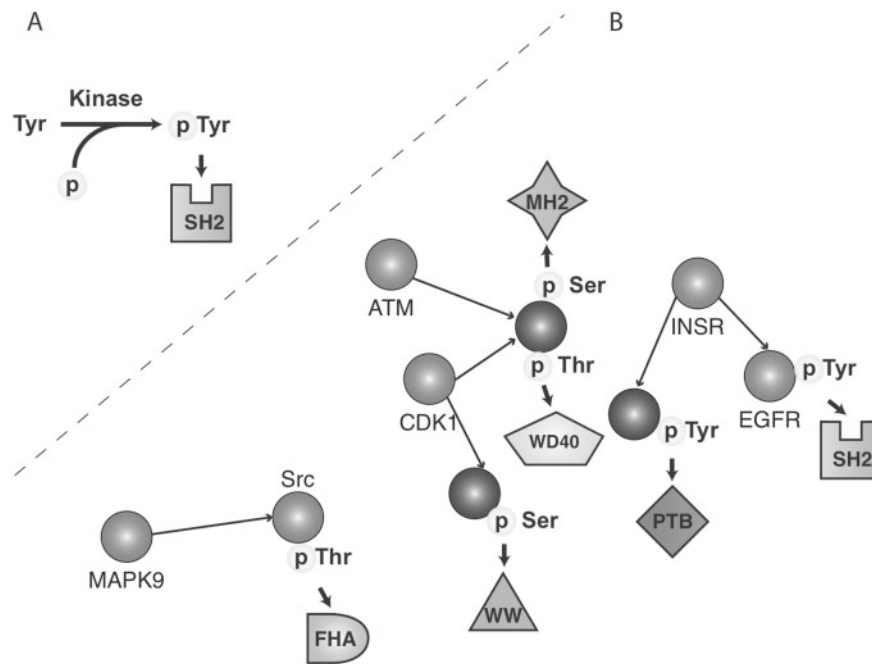
Any given cell in a physiological environment receives numerous simultaneous input cues that must be processed and integrated to determine changes in cellular behaviours such as migration, proliferation, apoptosis and differentiation. Reversible protein modifications are one of the underlying mechanisms that govern such cellular information processing. In particular, protein phosphorylation has proven to be a primary driving force behind cellular signal propagation. Through its ability to control protein–protein interactions, protein–phospholipid interactions, structural complex nucleation, allosteric structural reorganization, enzyme activity, degradation and translocation, phosphorylation impacts every aspect of cellular biology. All these events are fundamental for cellular organization, disease and response to input cues, and can be defined as phosphorylation networks (Figure 1). Recent technical

developments in mass spectrometry (MS) have permitted the identification and quantitation of thousands of *in vivo* protein phosphorylation sites. In combination with methods such as single-cell monitoring by phospho-flow, kinase-activity assays and conventional immunoblotting, this has broadened our ability to monitor the states of cellular phosphorylation networks under various stimuli. However, integration of these data is essential for their interpretation and utility in modelling of cellular phenotypes. Therefore, computational tools have been developed to combine these heterogeneous data sets and construct predictive models. These approaches have provided novel insights in the complex cellular biology of signalling systems. Here, we review recent technological advances in our ability to systematically gather information of cell signalling networks and how subsequent computational modelling is key to their interpretation.

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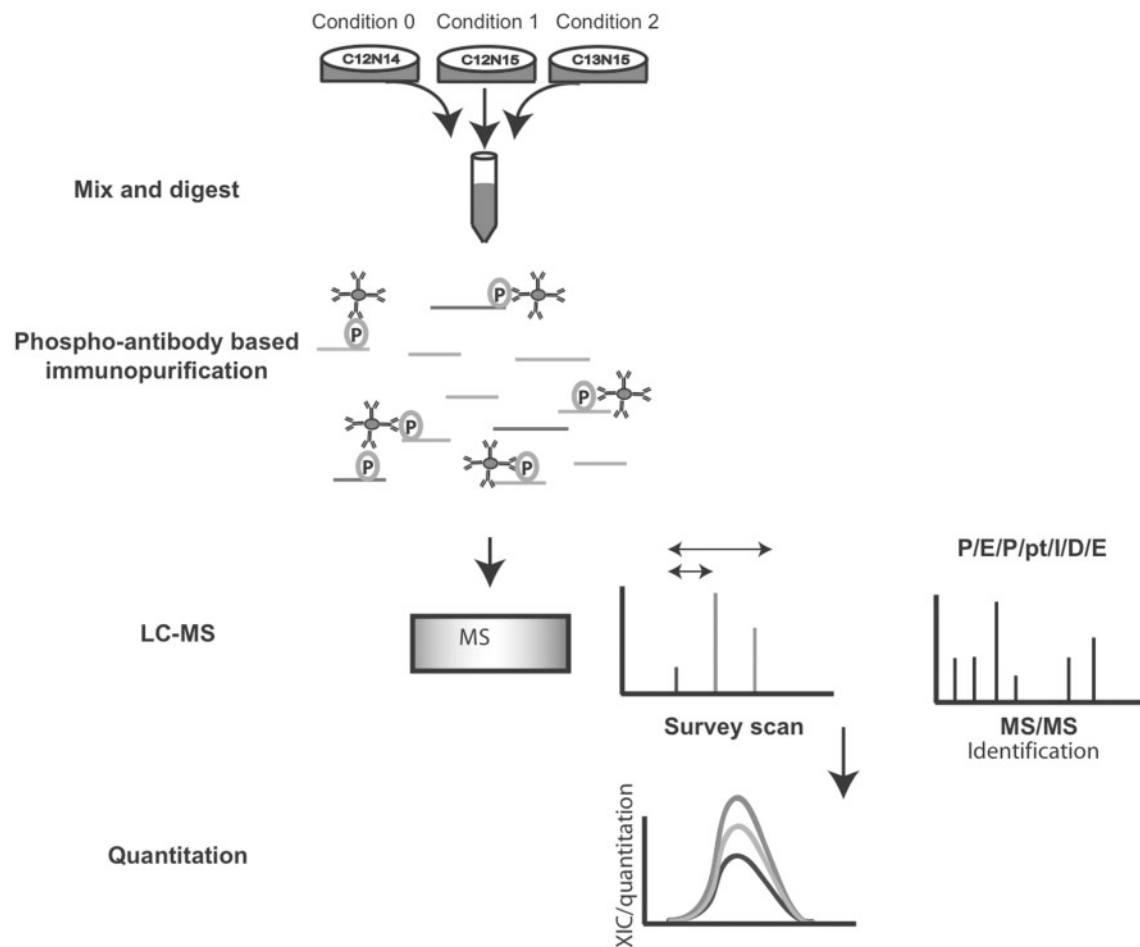
**Figure 1:** Directional interaction networks. Protein interactions that depend on protein phosphorylation (or other types of dynamic modifications) generate directional information flow within the cellular signalling networks. Here, this is illustrated by combining kinases (writing devices) with phospho-binding domains such as SH2 and BRCT (reader devices). In order to pulse signals through the system, phosphatases are required to remove phosphorylations to dampen the signals. This feature is absolutely crucial for any information processing system including the cell [58]. **(A)** A kinase can drive a signal through phosphorylation of a tyrosine on a substrate protein, which then subsequently is recognized by a SH2 containing protein. The effect of this small system is a net information flow from the kinase to the SH2 containing protein. It should be noted that we do not make any distinction as to whether the information transfer is ‘activating’ or ‘deactivating’ or ‘neutral’, as that is entirely context dependent. **(B)** A toy modular interaction network built from kinases and phospho-binding modules. The reader can anticipate how complex and very fast logic gates can be constructed by combining phosphorylation-driven interactions with dynamics depending on the external cellular input cues.

We will highlight trends we think will become essential to gain insight into the diseases arising from defects in cellular signalling networks such as cancer. Finally, we argue that these data-driven models are important for the understanding of cellular function under a variety of conditions such as differentiation, migration and apoptosis.

### Identification and quantitation of cellular phosphorylation sites

Identification of protein phosphorylation remains a major challenge. On average, phospho-proteins are phosphorylated on three sites [1], each of which may be regulated and have independent effects on cellular behaviour [2, 3]. In order to fully understand cellular decision-making processes, each phosphorylation site must be measured as an independent entity. However, despite recent technological improvements in MS, which have increased our

ability to identify thousands of phosphorylation sites from living cells, it is still a challenging undertaking. This is mostly due to the low stoichiometric nature of phosphorylations, thus prior enrichment of phosphorylated peptides is essential for their identification in MS-based approaches [4, 5]. Phospho-tyrosine-binding domains (i.e. SH2 and PTB) and generic anti phospho-tyrosine antibodies have been used to enrich tyrosine phosphorylated proteins [6–8] and peptides [9, 10]. However, the same has only proven feasible in a few cases for anti-phospho-serine/threonine antibodies [11, 12]. Instead, techniques such as IMAC,  $\text{TiO}_2$ , SCX and other approaches have been applied to enrich phosphorylated peptides in a more generic manner [5, 13–17]. In a recent study, it was shown that although these methods infer good reproducibility, they resulted in extensive variation in their ability to enrich for identical phosphorylated



**Figure 2:** Mass-spectrometry. An example of workflow of phospho-proteomics using relative quantitative MS (here exemplified by Stable Isotope Labelling with Amino acids in Cell culture, SILAC [20]). Cells are grown in special media containing stable isotopomeric versions of amino acids (for example Arginine and Lysine). Following perturbation, cells are lysed and mixed in a 1:1:1 ratio. For isolation of tyrosine phosphorylated peptides [9, 10] proteins are first digested, purified and then incubated with a generic anti-phospho  $\zeta$  Serine/Threonine/Tyrosine antibody. Isolated peptides are identified by liquid chromatography mass spectrometry (LC-MS). Following acquisition of data and identification of peptides, SILAC quantitation can be achieved by extracting the ion current (XIC) for individual peptides. Identical peptides will co-elute and are separated by a predicted mass difference (depending on the isotopomeric version of amino acid employed), thus differences in abundance can be directly determined due to differences in XIC.

peptides [18], suggesting that further development of enrichment strategies is still required.

Several large-scale MS projects have identified numerous phosphorylated peptides, emphasizing the frequent use of phosphorylation as a post-translational modification [2, 15, 19]. The combination of relative or absolute quantitation [20] with these approaches has provided an invaluable tool to investigate how phosphorylations are selectively modulated following perturbation of a signalling system [2, 10, 19] (Figure 2). Several of these approaches have studied the temporal regulation of phosphorylation events and have provided insight into the directionality of information

flow and the extensive cross talk between signalling networks. Epidermal growth factor receptor (EGFR) signalling is probably one of the best-studied receptor tyrosine kinase (RTK) systems by MS. Recently, numerous phosphorylation sites were identified and quantitated by  $\text{TiO}_2$  purification following epidermal growth factor (EGF) stimulation in HeLa cells [2]. An important observation in this study was that regulation of both tyrosine and serine/threonine phosphorylation was widespread following stimulation by EGF. However, the paper demonstrated that the regulation of tyrosine phosphorylation was more dramatic and precedes serine/threonine phosphorylation, suggesting that tyrosine

phosphorylation signalling events feeds into serine/threonine phosphorylation networks. In addition, since the temporal-profile of phosphorylations were measured on a systems-level, insight was gained into how potential sites could act as initiating signals, whereas others were predicted to act in negative feed-back control. Typically, these approaches have used 1 or 5 min of EGF stimulation as their earliest time-points, where receptor auto-activation and tyrosine phosphorylation was already at a maximal level [2, 10, 19]. Thus, to interrogate very early signalling events below the 1 min mark, a new technology was developed; instead of manually adding ligand (EGF) to cells grown on tissue-culture plates, a process which can be hard to execute for very fast signalling events, suspension cells were mixed with ligand through a pump system. After the cells were mixed with ligand, they would be combined with  $-20^{\circ}\text{C}$  Ethanol, thereby quenching the signalling virtually instantaneously. By adjusting the length of tubing where cells were mixed with the ligand before the signal was quenched, signalling events from 1 min down to a few seconds were examined [21].

Detection of tyrosine phosphorylation events has also been performed through the use of generic anti-phospho-tyrosine antibodies. With this approach, White and colleagues have addressed several important aspects of EGFR signalling networks, where recently they identified interplay between EGFRvIII and the c-MET receptor in Glioblastoma Multiforme. Through this system-wide approach, the authors demonstrated that the phosphorylation on c-MET activation loop correlated with the expression level of EGFRvIII, which subsequently was demonstrated to affect the cells responsiveness to EGFR inhibitor treatment (see below) [22].

Specific enrichment of tyrosine phosphorylated peptides has also been used to identify RTK-selective networks in EGFR or HER2 expressing cells stimulated with either EGF or heregulin (HRG). In addition to probing which networks that are preferentially employed by EGFR homodimers or EGFR-HER2/HER2-HER3 heterodimers, these studies have also been used to identify predictive phospho-markers for proliferation versus migration in a network specific manner [10, 19, 23]. Apart from leading to novel insight into the signalling networks and aiding in our understanding of EGFR biology, these studies have also provided

potential biomarkers, which can be used to assay the signalling state of tumours with increased EGFR or HER2 levels.

### **MRM: monitoring individual phosphorylation sites**

Even though MS enables us to identify and quantitate phosphorylation sites, selective interrogation of a specific network is difficult due to inconsistent selection of precursor ions for fragmentation and quantitation. The issue of random sampling is mostly because data acquisition is performed in an information dependent analysis (IDA) mode, where peptides are selected for fragmentation in an automated manner, which results in poor reproducibility between samples. To circumvent this hurdle, White and colleagues [24] recently implemented a MS strategy based on multiple reaction monitoring (MRM). MRM is well suited to specifically assay for and quantitate any given peptide [3, 20, 25, 26]. The principle of MRM is to program a triple quadrupole mass-spectrometer to select and analyse a particular peptide. As a population of peptides enters the mass-spectrometer, peptides are resolved according to their mass to charge ratio in the first quadrupole (Q1) and only the peptide of interest is selected and fragmented (in Q2). Following, fragments are resolved according to their mass to charge ratio in the third quadrupole (Q3). Finally, the detector will only elicit a response, when a specific fragment ion, related to the selected peptide, is passed through the third quadrupole (Q3). Thus, in contrast to peptide identification based on a full scan of all fragment ions, MRM is based on the detection of specific fragment ion(s) from pre-selected peptide(s). Because the mass-spectrometer is scanning for a selected set of peptide fragment ions instead of acquiring full MS/MS scans the duty-cycle become very high.

Following initial characterization of the EGFR signalling network, a list of selected nodes (phosphopeptides) were chosen and the signalling networks were specifically examined with increased coverage. Therefore, due to this highly selective approach the authors obtained an 88% increase in network coverage compared to IDA based approaches [24].

### **S531 not S532—organization of phosphorylation sites**

One significant problem with MS-based identification of phosphorylation sites is the ambiguity in

assigning the amino-acid residue that is phosphorylated. To better understand phosphorylation networks, new technology is needed to resolve this issue. Thus far, high throughput (HTP) MS identification of phospho-peptides utilizes a probability matching score, which is defined as the probability of a phosphorylation being correctly assigned to a particular amino-acid in the peptide [2, 27]. However, a very promising development is the application of soft ionization techniques for peptide fragmentation such as electron transfer dissociation (ETD) and electron capture dissociation (ECD) [28, 29]. These techniques prevent the loss of labile modifications such as phospho-serine and -threonine during fragmentation, and thus allow unambiguous identification of the phosphorylated residue within a peptide [30].

Application of these technologies will hopefully be able to streamline the current databases such as Phospho.ELM and PhosphoSite by re-analysing potential ambiguous phosphorylation sites. It will be important that the community places efforts into re-analysing some of these large datasets as new technology comes about, which is expected to dramatically reduce the errors in phosphorylation sites assignment.

Organizing thousands of phosphorylation sites and their temporal regulation to obtain novel biological insight can be very time-consuming and information may be missed due to the richness of data. Several approaches have been applied to cluster phosphorylation sites, such as Heat-Maps, Self-Organizing Maps (SOMs), hierarchical clustering and Fuzzy C-Means. These algorithms have all been used, with varying degree of success, to categorize phosphorylation sites into groups with sites regulated in a similar manner. By applying this approach and by assuming that sites regulated in a similar fashion would have similar function, insight can potentially be obtained when phosphorylation sites with known function co-cluster with novel sites. Through these approaches, phosphorylations that initiate signalling events displayed a different temporal regulation than ones that are proposed to be involved in negative feedback loop [2].

## FROM SITES TO NETWORKS

Above, we defined phosphorylation networks as cellular protein interaction networks spanned and modulated by dynamic protein phosphorylation.

Kinases phosphorylate substrates in a typical transient manner (i.e. high  $k_{off}$ ), thus in general, kinases do not form stable complexes with their substrates, but rather target the substrate, phosphorylate it and leave subsequently. This makes cellular (*in vivo*) kinase-substrate relationships difficult to capture by affinity-based methods (MS, pull-downs, etc.). Moreover, kinase specificity is highly context dependent, rendering it virtually impossible to derive meaningful cellular phosphorylation networks from *in vitro* data. The fact that a kinase can be observed to phosphorylate a substrate *in vitro* merely indicates that the catalytic subunit is recognizing and phosphorylating one or more compatible linear motifs in the substrate. However, if this information is derived in a *sequence-specific* manner, it can be highly useful for deriving linear motifs sequence models for kinases and phospho-binding modules [31–33]. These models can in turn be combined with contextual information (see below).

Currently there is a large gap between our understanding of *in vivo* phosphorylation sites and the kinases that modulate these. In the Phospho.ELM database of *in vivo* data, there are currently 11 197 annotated phosphorylation sites, of which only 21% have been linked to at least one *in vivo* kinase. Thus, our understanding of cellular signalling networks is increasingly fragmentary.

To challenge this problem and attempt to assign *in vivo* kinases to identified phospho-proteins, the NetworKIN algorithm was developed [34]. Introducing the novel concept of combining probabilistic modelling of network context with the linear motifs recognized by the catalytic domain, the NetworKIN algorithm can assign a specific kinase to an observed *in vivo* phosphorylation site with a 2.5-fold higher accuracy than previous methods such as Scansite and Netphos. However, even given powerful predictions, it is still challenging to experimentally validate kinase substrate relationships. As proof of principle, the authors showed Rad50 and p53BP1 are phosphorylated by ATM and CDK1. Importantly, they showed that by combining MRM with NetworKIN, it might be feasible to perform higher throughput validation of predicted kinase-substrate networks. This idea was exemplified by showing that the BCLAF1 protein was likely a direct target of GSK3 since inclusion of a GSK3 inhibitor resulted in a decrease of the predicted target phosphorylation (S531).

The NetworKIN work underlined the utility of network data to resolve biological not possible without. As NetworKIN is enhanced with kinome-wide coverage [31], addition of docking motifs, phospho-binding modules and phosphatases it will facilitate the move from *prediction to descriptive modelling* of mammalian signalling networks.

### Chemical network biology

Engineering of kinases to either accommodate an ATP analogue (analogue sensitive; AS) or to be specifically inhibited (inhibitor-sensitive) have proven a powerful way to interrogate specific kinase-substrate interactions. For example, modifying the kinase domain to accommodate an ATP analogue, such as the bio-orthogonal ATP $\gamma$ S, allows for selective detection and purification of substrates containing the thio-phosphate. Thus substrates containing the analogue-phosphate can be concluded to be *direct* targets of the AS kinase [35–37]. In a similar manner, kinases have also been modified to selectively bind a chemical inhibitor, but in contrast to the ATP analogues used, the inhibitor readily passes the cell membrane and can selectively and rapidly inhibit the engineered kinase. Inhibitor-sensitive kinases have been a good alternative to temperature-sensitive alleles in yeast, and knock-in of inhibitor-sensitive alleles have been generated in mice as well [38, 39]. However, one potential shortcoming is that the AS kinase may have distorted substrate recognition *in vivo* or the inhibitor used may have off-target effects.

## DETERMINING SIGNALLING NETWORK STATES

### Phospho-specific flow cytometry

In addition to conventional immunoblotting by phosphorylation-specific antibodies, only a few methods have the throughput and sensitivity to systematically analyse the state of multiple signalling nodes. One of these, phospho-specific flow cytometry, has provided insight into dysfunctional signalling networks on a single cell level [40, 41]. This technology is based on the detection of a phosphorylation event by a specific antibody within a single cell. Cells are labelled by antibodies only recognizing phosphorylated proteins (for example p-ERK or p-PKB) and cell markers (fx CD20 or CD33). Following, they are sorted and

measured by FACS, thereby providing quantitative measurement of cell specific signalling events. Thus, single-cell-signalling network signatures are obtained for specific cell populations, thereby allowing a heterogeneous sample to be analysed. For patient tumour samples, this is a particularly promising way to examine the state of the signalling network that subsequently can be used to predict patient outcome.

### Protein microarrays

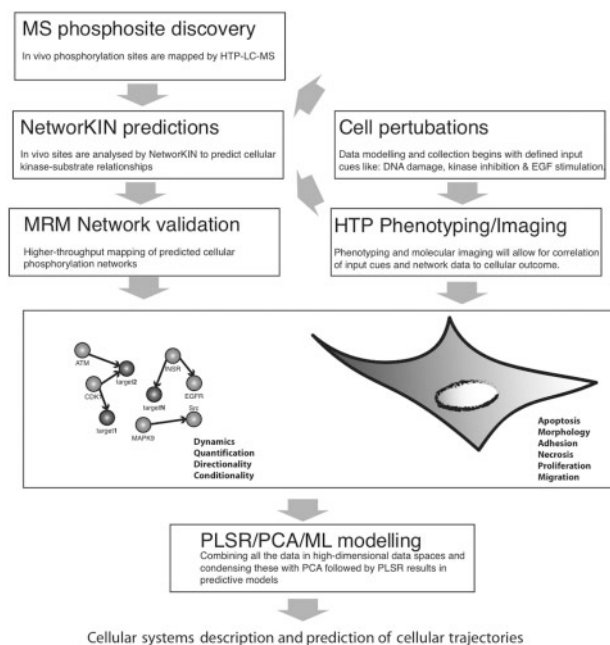
Modification-specific antibody based microarrays are a good alternative to phospho-flow for detection of phosphorylation events using tissue-culture samples or patient samples [42–46]. One clear advantage to this approach is the potential for higher sample throughput and that tissue sections has been analysed, providing a broad applicability. The utility of this tool for identifying distortions in signalling networks was recently highlighted: by employing antibody arrays it was determined that development of resistance to EGFR inhibition was dependent on increased c-MET activation (see below) [44, 45].

### HTP kinase assay

In addition to measurements of signalling networks by different phospho-profiling methods, read-outs of kinase activity by high-throughput kinase assays have provided important information for systems modelling [46, 47]. To improve throughput and specificity, various forms of kinase assays have been developed either through kinase-specific immuno-purification, or through development of specific kinase chemosensors [48, 49]. The clear advantage of these activation-based assays is that they provide a direct measurement of enzyme activity, rather than an assumed activity based on alterations in phosphorylation state.

### Real time imaging

Development of various microscopic techniques has allowed for real-time measurement of signalling events and how their specific sub-cellular localization is regulated. Using FRET and FLIM to investigate ErbB1 activation provided a quantitative measurement of lateral signal propagation and the combination of FRET with enzyme-substrate (ES) interactions has provided insight into the function of PTP1B in signal-termination



**Figure 3:** Data analysis. The figure illustrates a few potential routes that can be used for proteomics powered systems biology. Deciding on a cellular perturbation (input cue) is key in order to obtain a focused and smaller data set of phosphorylation sites, which display a dynamical response to the stimulation. These sites can then be used for kinase-substrate network modelling with the NetworkKIN algorithm. In particular, the predictions can be validated through MRM, which will enable the generation of dynamic, quantified, directional and conditional kinase-substrate interaction networks. In parallel, it will be important to measure the cellular outcomes of the input cues for instance by morphology or cell death. Taken together these and other data can be combined by numerical modelling methods such as PLSR, PCA and machine learning to derive models of the cellular cue, signal response spaces. In particular it is the aim to obtain models that can predict the trajectory the cell will take in time or response space.

of RTKs [50, 51]. Real-time imaging of cellular morphology combined with RNAi disruption of signalling components has provided another manner in which cellular signalling processing can be directly linked to phenotypic measurements using machine-learning (ML) approaches [52] (Figure 3). From a systems biological perspective, single-cell measurements, such as imaging and phospho-flow, are crucial as they allow insight into noise and fidelity of fundamental biological processes such as transcription [53] and phosphorylation networks.

## CUE, SIGNAL AND RESPONSE—FROM NETWORKS TO DISEASE MODELS

Constructing network models from methods like LUMIER [54], Y2H [55] and MS (as described above) is essential for systems level understanding of the cellular machinery. However, these networks are even more powerful when integrated with dynamic data, which makes it possible to describe the information flow within the system. In a series of papers from MIT, Lauffenburger, Yaffe and White labs have been combining cue, signal and response measurements to construct models of cellular decision processes [22, 46, 56, 57]. The activity-state of nodes within a signalling network was interrogated (‘signal’) in a systematic manner using phospho-flow, kinase assays, antibody-arrays and MS and correlated to cellular phenotypes (‘response’) such as apoptosis, proliferation and migration. The signals used to perturb the system (‘input cues’) were varied and combined to obtain measurements of cellular responses to conflicting (‘orthogonal’) signals.

The resulting large data set was subsequently subjected to data-driven modelling [in contrast to ordinary differential equation (ODE) modelling], in order to reduce the complexity of measurements and make it more amenable to human interpretation. In particular, the combination of partial least square (PLS) regression and principal component analysis (PCA), for numerical modelling and data condensation, respectively, of large phospho-data sets has proven a powerful way of deriving knowledge about critical phosphorylation sites and establishing predictive models for the cellular systems trajectories.

For example, Kumar *et al.* [23] and Wolf-Yadlin *et al.* [19] applied PLSR to phospho-proteomic data and phenotypic measurements (migration and proliferation) from cells expressing EGFR alone or in combination with HER2, in order to produce a coefficient vector indicating the importance of the identified phosphorylation site with respect to either migration or proliferation. Constructing a model to recapitulate the measured data allowed the authors to identify 9 phosphorylation events that alone could be used to predict a cellular decision (proliferation versus migration). PLSR has also been applied to generate systems models for cytokine or sepsis induced apoptosis and to predict a common effector hypothesis for signal integration [46–48]. These models have proven very powerful in predicting

cellular decision processes, for example the kinase MK2 was identified to play a role in pro-survival based on its activation profile in the PLSR model [46, 58].

To study how the signalling networks in AML cancer cells were correlated with clinical outcome, unsupervised clustering of phospho-flow data has been used to group samples from patients according to their differential network profiles. Importantly, patient response to chemotherapy was highly correlated with the initial clustering based on the signalling network state and thus indicating that the behaviour of the individual network can be used to determine and predict patient responsiveness and choice of treatment [41].

In other cases, a clear hypothesis can be defined by directly interrogating the signalling network alone. For example, recent identification of c-MET amplification as a means, whereby ErbB3/EGFRvIII expressing tumours develop resistance to EGFR inhibitor treatment was based on a systemic assessment of the signalling network. Using phospho-specific antibody arrays or phospho-proteomics, U87 cells expressing EGFRvIII or a lung carcinoma cell line resistant to Gefitinib (Iressa) was all identified to exhibit increased c-MET activation. Combinatorial treatment with both an EGFR inhibitor and a c-MET inhibitor had synergistic effects on growth inhibition, survival and anchorage-independent growth [22, 44, 45]. This work clearly shows the power of network approaches in deriving new drug strategies.

## REFLECTIONS

There is a tendency in the literature to consider the proteome a static entity that is just awaiting sequencing. We would argue that there is not one proteome but a continuum of proteomes and which interchange during cellular signal processing. The challenge facing today's systems biologists is to monitor the proteome continuum through time and along relevant systems trajectories to identify the basis vectors [57] that drives these changes. With this knowledge in hand, the next step is to translate it into models of cellular systems, and their responses to input cues and disease states. With an increased number of dynamic networks characterized, this information can be put together to create specific positive and negative identifiers or network signatures relevant for specific disease states. We speculate

that this will facilitate future drug development that focuses on these network signatures rather than individual genes in a novel network medicine.

### Key Points

- Phosphorylation modulated protein interaction networks are essential for cellular information processing.
- Quantitative mass-spectrometry combined with computational network modelling will become key tools in understanding cancers and cellular processes leading to disease.
- Cellular signaling networks mediate response to environmental cues and compute outcomes.
- It is important to analyse multi-variant input systems (such as cells) accordingly, thus applying orthogonal cues to the cells.

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