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Methods and approaches to disease mechanisms using systems kinomics

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ABSTRACT

All cellular functions, ranging from regular cell maintenance and homeostasis, specialized functions specific to cellular types, or generating responses due to external stimulus, are mediated by proteins within the cell. Regulation of these proteins allows the cell to alter its behavior under different circumstances. A major mechanism of protein regulation is utilizing protein kinases and phosphatases; enzymes that catalyze the transfer of phosphates between substrates [1]. Proteins involved in phosphate signaling are well studied and include kinases and phosphatases that catalyze opposing reactions regulating both structure and function of the cell. Kinomics is the study of kinases, phosphatases and their targets, and has been used to study the functional changes in numerous diseases and infectious diseases with aims to delineate the cellular functions affected. Identifying the phosphate signaling pathways changed by certain diseases or infections can lead to novel therapeutic targets. However, a daunting 518 putative protein kinase genes have been identified [2], indicating that this protein family is very large and complex. Identifying which enzymes are specific to a particular disease can be a laborious task. In this review, we will provide information on large-scale systems biology methodologies that allow global screening of the kinome to more efficiently identify which kinase pathways are pertinent for further study.

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1. Introduction

There is enormous variability in the complexity of living organisms. Small simple viruses may contain fewer than a dozen genes on a genome consisting of a few kilo-bases that encode up to a dozen proteins. Significantly more complex eukaryotic organisms possess genomes in the mega-base range that, with alternative splicing and various possible post-translational modifications, may encode upwards of millions of protein permutations. For many decades, much research effort went into either understanding the simpler organisms, or trying to delineate a few molecules within more complex systems. With advances in whole genome sequencing, bioinformatics and instrumentation, it has been possible for more than a decade to assess, both quantitatively and simultaneously, changes in the levels of total mRNA expression and in levels of thousands of proteins. Despite these advances, cellular regulation is more often determined by protein post translation modifications than by absolute quantity. This review will focus on one of the largest and best-studied subsets of proteins, which are proteins involved in kinase signaling. This field of “kinomics” encompasses kinases, kinase targets and antagonistic phosphatases [1].

The development of genomics and proteomics tools has made it possible to create large amounts of information about many processes that occur throughout a cell or tissue in response to a stimulus. The first such technologies - microarrays and quantitative proteomics - were revolutionary in their ability to simultaneously measure thousands of genes and proteins within a single experiment. This ability to globally assess the state of a cell or tissue has since expanded and evolved into numerous other techniques that have been adapted to allow more high-throughput analyses. In an effort to probe even deeper into the cellular proteome, tools have been developed to detect and isolate specific subsets of proteins that might not otherwise be detected. Examples of these protein subsets include those with post-translational modifications (e.g. phosphorylation, ubiquitination, lipidation) and localizations in response to different stimuli. Similarly, different classes of enzymes (e.g. kinases, proteases, hydrolases) can be probed for their activity levels in response to various conditions.

Kinase signaling is a powerful and central cellular mechanism that mediates signal transduction events and is involved in a wide range of nearly all cellular processes including, but not limited to, the control of cell cycle progression, transcriptional regulation, cell transformation, proliferation, differentiation, and apoptosis. Given its central role in cellular function, aberrant regulation of kinase signaling can profoundly affect homeostasis and has been found to be involved in many disease states including insulin resistance [3,4], autoimmunity [5,6], viral infection [7,8], and oncogenesis [9,10]. Hence, assessing the kinome can provide insight into complex pathological processes across a wide array of diseases and has also been a well-studied target for therapeutics. It is therefore not surprisingly that many approved pharmaceuticals target kinases in an effort to restore homeostatic cell signaling events, and that efforts have been made to explore repurposing these drugs for other diseases [11–14]. Notably, kinase signaling may also be exploited clinically as a diagnostic tool and will be discussed below.

In this current review, we provide an overview of some of the popular high-throughput methodologies, analysis tools and databases that are commonly used in kinase signaling studies and how they may be used to understand particular disease processes in virology, cancer and clinical diagnostics. Some important areas of current research include the purification and characterization of protein kinases (both natural and recombinant), the elucidation of biological functions and ligands of kinases and the development of specific kinase inhibitors.

2. High throughput systems methodologies for studying the kinome

2.1. Nucleic acid-based approaches

2.1.1. siRNA

siRNAs, or small interfering RNAs, are regulators of expression and function of genes [15]. Double stranded precursors are cleaved by dicer proteins into short fragments. The siRNA consists of a guide strand that is assembled into a RISC-loading complex which binds to dsRNA, cutting it into a single stranded functional siRNA. This RISC complex will recognize a complementary mRNA strand and cleaves this strand at a single site, releasing the fragments, which are ready to cleave more mRNA. The resulting decrease in mRNA in the cell leads to a decrease in expression of the gene. By using this endogenous host mechanism, siRNA fragments can be transfected into the cell for targeted gene knockdown.

RNAi panel screening is a high throughput method using siRNAs to determine the effects of multiple genes in a specific experimental system. siRNA panels can be purchased against target genes of choice in each well of multiwall plates from different companies. Some panels are specific for particular cellular functional pathways, such as Qiagen's SureSilencing® siRNA arrays (http://www.sabiosciences.com/pathwaymagazine/pathways9/suresilencing-sirna-arrays.php), which are

predesigned to target main cellular genes in a biological or functional pathway.

By using siRNA panels specific to targeting kinases or phosphorylases, expression patterns or cell pathways affected by these enzymes can be determined. For example, Azorsa et al. used this method to determine kinases involved in tau protein hyperphosphorylation, which is known to occur during Alzheimer's disease [16]. In this study, siRNA transfection was used for 572 kinases, then increases or decreases in tau phosphorylation levels in a neuroglioma cell line were observed. Alternatively, using kinase or phosphorylase siRNA screens in cell lines to determine cell viability can also be performed. Hu and colleagues used a genome-wide siRNA library screen of 206 phosphatases and 691 kinases to observe those detrimental to the viability of a Rhabdomyosarcoma cell line, which is the most common type of sarcoma in children [17]. Using siRNA screening of a panel of inhibitors that target kinases, Kim et al. were able to identify a host factor kinase that is involved in hepatitis C virus entry [18]. Jansen and colleagues used a kinome-wide siRNA screen to identify kinases that have a role in acquired resistance to CDK4/6 small-molecule inhibitors which are associated with breast cancer [19]. This study was able to determine which kinase was a key modifier of sensitivity to the inhibitor, and was also able to restore sensitivity by targeting this factor. This example clearly shows how powerful this tool can be to narrow the focus from a large number of possible targets to one or two key kinases. In a contrasting direction of focus, another study by Giroux et al. was able to probe the kinome with an siRNA panel to try and induce apoptosis in a pancreatic adenocarcinoma cell line, directly identifying therapeutic targets [20].

2.1.2. Polymerase chain reaction

Polymerase chain reaction (PCR) is a highly sensitive technique that rapidly amplifies specific segments of DNA in a sample [21]. Amplification of DNA fragments into billions of copies allows detection and identification of gene sequences in a sample using specific primers that are short DNA fragments with defined sequences complementary to the target DNA sequence. Quantitative measurements of gene expression can be obtained using a modified technique called real-time PCR, and is used to determine how much DNA is present in a sample while it is being synthesized. Two methods are commonly used; either fluorescent dyes that non-specifically integrate into double stranded DNA, or sequence specific fluorescent DNA probes. In these experiments, specialized thermal cyclers equipped with fluorescence detection modules are used. The fluorescence signals are measured as amplification occurs and the signals are proportional to the total amount of target DNA, allowing the determination of the initial number of copies of template DNA with accuracy and high sensitivity. Gene expression measurements can be determined by coupling qPCR with reverse transcription (RT-qPCR), where RNA is reverse transcribed into cDNA, then the cDNA is used as the template for qPCR. This can be used to target specific phosphatases or kinases of interest. For example, Fu and colleagues targeted inositol polyphosphatase-like 1 by qPCR and showed a significantly higher level of this phosphatase in hepatocellular carcinoma tissue in comparison to non-cancerous tissues [22].

The expression of gene copy number for numerous kinases and phosphatases in a sample can be determined using qPCR assays that are tailored to detect these enzymes. Arrays predesigned to profile gene expression in a sample for a specific pathway can be purchased from a variety of companies, such as the MAP kinase signaling pathway, or a specific family of enzymes such as tyrosine kinases. In one example, Wu et al. used RT-qPCR to identify a phosphatase (among other genes) to characterize the mechanism of stretch-induced cell rearrangement [23].

2.1.3. DNA microarrays

DNA chips (microarrays) are assays that examine the expression levels of a large number of genes simultaneously. Microscopic slots are fitted onto a microchip slide with DNA molecules (nucleic acid sequences) attached to the surface in each slot [24]. The DNA attached to the slides are probes and the relative concentration of DNA in a mixture is detected by hybridization events that are measured in multiple ways. The two most common detection methods are by the incorporation of fluorescently-labeled nucleotides during synthesis, or the incorporation of biotin-labeled nucleotides which are subsequently stained with fluorescently-labeled streptavidin. mRNA molecules are taken from a sample and a reference, the samples are converted into complementary DNA, then are labeled with separate fluorescent probes. After mixing, they are added to the chip for hybridization and scanned to measure the relative expression of the two colored samples. Expression of the kinome can be observed with this method by using probe sequences of kinases and phosphatases. Using a cDNA microarray, Lee and colleagues were able to identify a cysteine-rich receptor-like kinase that is responsive to a fungal pathogen *Alternaria brassiciola*, and went on to determine the function of this kinase in plant immunity [25].

2.2. Protein & peptide arrays

2.2.1. Protein microarrays

Protein microarrays, set up similarly to DNA microarrays, can be used to identify kinase activity by spotting proteins onto microarray slides, then adding kinases with radiolabeled ATP. The amount of kinase activity is detected and quantified by autoradiography [26].

2.2.2. Peptide arrays

Peptide arrays have become a productive, high-throughput method for studying cellular or tissue kinomes [27]. This technique involves spotting glass arrays or 96 well plates with peptide sequences that are kinase-targets. The array is then exposed to a lysate containing active kinases, which results in peptide phosphorylation and which generates a visual signal of substrate-enzyme phosphorylation such as HRP or autoradiography [26]. By comparing the relative signals of experimental cell or tissue samples, one can identify changes in signal transduction pathways and phosphorylation-regulated events. A detailed description of this technique can be found in Daigle et al. [28].

A particular advantage of peptide arrays is their versatility. For example, the peptides spotted onto the array can be designed for a variety of different consensus sequences, such as those specific to different classes of kinase activity such as tyrosine kinases, serine kinases or threonine kinases. A second very useful aspect of peptide arrays is that they can be adapted for any particular species for which the genome sequence is known [29–31]. This is particularly useful for species which do not have many, or any, other tools available, such as microarrays, specific phospho-antibodies or commercial kits that measure kinase activity. For example, the use of this technology in agricultural species has been immensely valuable (reviewed by Daigle 2014 [28]) and has been applied to studies with cows [32] and poultry [33]. Protein arrays have been equally successful in smaller models for which reagents are difficult to find, such as hamsters [34], guinea pigs [35] and yeast [36]. The main disadvantage of this method is the upfront cost of the detection scanner and a substantial amount of needed expertise in setting up custom arrays; thus, these are typically provided by a core facility.

2.2.3. Phospho-ELISAs

Phospho-ELISAs are another technique that can be used to

detect phopho-peptides and are readily available from a variety of biochemical companies. Just like regular ELISAs, phospho-ELISAs are quick, sensitive, and relatively high throughput. In addition, ELISAs can analyze small volumes of either cell or tissue lysates as well as purified enzymes. Phospho-ELISAs can be found both for specific kinases, such as ERK1/2 or EGFR, as well as for broader kinase classes such as phospho-tyrosine [37] or phospho-serine/threonine activity. A common use of this technique is to determine or confirm whether specific kinase signaling pathways are activated under different conditions. For example, Janssens and colleagues showed that the nitration of chemokine CXCL12 decreased ERK1/2 phosphorylation in monocytes, which may result in the downregulation of inflammatory responses [38].

2.3. Cellular assays

2.3.1. Flow cytometry

Flow cytometry can be easily used to evaluate phosphorylation events where fresh or fixed cells are incubated with phospho-specific antibodies and then conjugated to a fluorescent tag for detection. This method has the distinct advantage of providing detailed and quantitative information about single cells in addition to the entire population of cells. For example, one can calculate the number of cells that become phosphorylated, as well as measure the relative amount of phosphorylation per cell. Newer flow cytometers can also analyze samples in a 96-well plate format and run in an automated fashion [39] (For example: BDSciences http://www.vp-scientific.com/bd_biosciences_protocol.htm). A second advantage of flow cytometry is the ability to multiplex multiple antibodies. For example, an increasing number of kits and protocols have optimized conditions that allow for 8- to 27-plex assays (see Krutzik et al. [40] for one such protocol). Multiple samples may also be barcoded and combined into a single tube for high-throughput analysis [41]. One popular application of flow cytometry is to monitor changes in immune cell populations and activation states in response to cancer treatments [42]. Increasingly, single cell flow cytometry is also a useful tool for analyzing a multitude of cell signaling pathways in one experiment, for example by examining patterns of B-cell activation [43].

2.3.2. Microscopy

Adherent cells can also be rapidly screened for phosphorylation by using automated microscopy in 96-well plates [44]. Similar to flow cytometry, cells may be fixed and stained with a phospho-specific antibody that is then conjugated to a fluorescent tag for detection. Alternatively, other assays have been designed to monitor wound healing in the presence of different chemical compounds [45] or for proteins that carry a fluorescent reporter that is modified under given experimental conditions after which the cells are fixed and analyzed [46]. McDonough et al. were able to quantify lipase phosphorylation and colocalization using automated digital microscopy coupled with high content analysis [47], while Bhadriraju and colleagues used automated fluorescence microscopy to measure relative myosin light chain phosphorylation in individual adherent cells with simultaneous correlations to cellular morphology [48]. Trask et al. used high content screening and fluorescent microscopy in a kinase-focused library to identify novel kinase inhibitors of p38-mediated MK2-EGFP translocation [49].

2.4. Phospho-proteomics

Identifying the phosphorylation target of a kinase is important to understand its function within phosphorylation networks. Phosphorylation can trigger a signaling cascade, which leads to activation of transcription factors and changes in protein expression [50]. Proteins can also contain multiple phosphorylation sites, each of which may alter the function of the protein differently [51]. Furthermore, the amount of phosphorylated protein can also affect downstream signaling. It is estimated that upwards of two-thirds of all proteins in the human proteome are phosphorylated, although the majority of their functions are currently unknown. Given the clear importance of phosphorylation as a post translational modification, much research has been performed to develop the methods necessary to identify phosphorylation sites and also quantify the extent of phosphorylation.

Because of the sheer number of potential phosphorylation sites, high-throughput, tandem mass spectrometry based techniques have become the main method for identification of protein phosphorylation sites. These techniques allow one to collect phosphorylated peptides from any protein sample of interest and identify thousands of phosphorylation sites within a reasonable timeframe. Phosphorylated proteins can be isolated using affinity chromatography, digested with trypsin to obtain peptides and then sequenced with liquid chromatography tandem mass spectrometry (LC-MS/MS). Alternatively, proteins can first be digested with trypsin and affinity chromatography is used to isolate phosphorylated peptides.

A comprehensive review of all techniques to isolate and identify phosphorylated peptides is beyond the scope of this review, but for a more in-depth discussion of the methods behind phosphorylation analyses we refer the reader to a review by Leitner et al. [52]. What follows is a discussion on two of the main techniques – metal affinity and liquid chromatography based methods to isolate phosphorylated peptides.

2.4.1. Metal oxide affinity chromatography (MOAC)

Metal oxide affinity chromatography (MOAC) uses beads composed of metal oxides such as TiO_2 or ZrO_2 to bind phosphorylated peptides and separate them from their non-modified counterparts. Under acidic conditions, phosphorylated peptides will adsorb to the surface of metal oxide beads. After washing, phosphorylated peptides are eluted at high pH where they can be purified and subsequently sequenced by mass spectrometry. This method can be performed either in batch format by adding beads directly to the peptide solution, by using a TiO_2 column in an HPLC format [53], or in a microcolumn format where metal oxide beads are packed into a pipette tip containing C8 material [54,55]. The C8 acts as a frit that holds metal oxide beads in place and does not bind non-modified or phosphorylated peptides to a significant extent.

As is the case for all metal affinity based methods, nonspecific binding of negatively charged acidic peptides with high aspartate or glutamate content was identified in early studies as a significant problem in metal affinity chromatography. This problem was somewhat alleviated through the addition of hydroxy acids such as glycolic or lactic acid to the dissolution and wash buffers. The addition of hydroxy acids prevents interaction between the acidic peptides and the positively charged surface but leaves the interaction between phosphorylated peptide and metal oxide surface intact. This technique increases the efficiency of phosphorylated peptide binding and allows for the isolation of phosphorylated peptides from as low as 100 μg of cell lysate [56].

The type of metal used may affect the binding affinity of specific phosphorylated sequences. Sugiyama et al. evaluated the ability of TiO_2 and ZrO_2 beads for phosphopeptide enrichment in the presence of different aliphatic hydroxy acids [57]. They found that lactic acid and B-hydroxypropanoic acid (HPA) provided the best results in terms of preventing non-specific binding of acidic peptides. Of the 1645 phosphopeptides they identified, 636 were identified by both methods. This suggests that a combination of both methods may be the best approach for more comprehensive

phosphopeptide analysis. In a later study, the same research group used a combination of six different methods to analyze the phosphoproteome of *Arabidopsis*. They used a combination of TiO_2, ZrO_2 and iron-immobilized metal affinity chromatography (Fe-IMAC) coupled to ammonia or phosphate buffers for phosphopeptide elution. In total, they identified 2171 phosphorylation sites on 1346 proteins, including 94 phosphorylated tyrosine residues.

2.4.2. Immobilized metal affinity chromatography (IMAC)

Immobilized metal affinity chromatography (IMAC) enriches phosphopeptides through interaction between a metal ion immobilized by a chelating agent coupled to the surface of sepharose or agarose beads. In one of the first examples, Anderssen and Porath used Fe^{3+} ions held in place by bidentate iminodiacetic acid to separate phosphoproteins from ovalbumin [58]. The phosphate groups organize the free coordination sites on the metal ion under acidic conditions allowing them to be separated from non-phosphorylated proteins. Currently, it is common to use nitrilotriacetic acid (NTA) supports coordinated to a variety of metal ions including Fe, Ga [59], and Zn [60].

Like MOAC, non-specific binding of acidic peptides is a significant issue with IMAC. One method to prevent non-specific binding with IMAC is to chemically modify acidic residues via methyl esterification. Ficarro et al. used this method to analyze the phosphoproteome of *Saccharomyces cervisiae* and found increased efficiency of phosphopeptide binding [61]. They reported detection of 5 fmol of phosphoprotein within a standard mixture of proteins. They mention that another advantage of using methylesterification is that heavy and light labels can be used in the labelling reagent to perform phosphosite quantification. The disadvantage to using methylesterification is increased variation during the sample labelling steps. It is also possible to prevent non-specific binding by changing buffer conditions. Tsai et al. [62] tested a number of different acids and varied pH using a Fe-NTA resin to find optimal binding conditions for phosphopeptides. They found that a solution of 6% acetic acid adjusted to pH 3.5 with NaOH provided optimal conditions for phosphopeptide binding. In a single step procedure, they identified 386 phosphoproteins from 550 μg of non-small-cell lung cancer cell lysate with 96% specificity.

2.4.3. Chromatography based methods

The other option to enrich phosphopeptides is to exploit their hydrophilic and ionic nature and separate them from non-modified peptides with liquid chromatography-based methods. Ion exchange materials such as strong anion exchange (SAX) and strong cation exchange (SCX) interact with ionic molecules where they can be eluted by modifying the salt content and/or pH of the mobile phase. The anionic nature of phosphate allows the use of SAX to separate phosphopeptides from non-modified peptides. Han et al. compared the efficiency of Fe3+-IMAC to SAX chromatography to identify phosphorylation sites in human liver tissue [63]. A direct comparison of the two methods resulted in 47 phosphopeptides identified by SAX and 24 identified by IMAC at 0.96% FDR. Twelve of the phosphopeptides were identified by both methods, again showing how using multiple methods can increase the number of phosphopeptide identifications.

Hydrophilic interaction chromatography (HILIC) uses hydrophilic column material coupled with hydrophobic mobile phases to separate hydrophilic from hydrophobic compounds. Phosphopeptides are typically more hydrophilic than non-phosphorylated peptides, making them an ideal candidate for separation by HILIC. McNulty and Annan used HILIC with a combination of IMAC enrichment to analyze the phosphoproteome of HeLa cell digests [64]. The phosphopeptides from HeLa cell digests were first enriched by HILIC chromatography followed by enrichment by Fe-IMAC. After each fraction was enriched by IMAC they were able to increase the efficiency of phosphopeptide detection to 96%. From 300 μg of HeLa cell protein they were able to identify 1000 unique phosphorylation sites, including 700 novel sites not previously discovered. The disadvantages to using these chromatography-based methods is the extra de-salting and or purification steps after fraction collection and decreased throughput from having to analyze multiple fractions from a single replicate.

2.5. Measuring kinase activity

2.5.1. ATPase probes

When establishing kinase networks, it is important to identify both the kinase and the kinase target. Bottom-up proteomics cannot only identify the site of phosphorylation but can also identify potential kinases responsible for the phosphorylation event. Due to the large dynamic range of the proteome, it is usually necessary to separate kinases from protein mixtures prior to LC-MS/MS analysis in order to identify an acceptable number of kinases. The ActivX system is a commercially available system that utilizes active site probes that react specifically with the ATP-binding site of kinases and other ATP-binding enzymes [65]. The probe consists of an ATP moiety attached to a desthiobiotin molecule through an acyl linker. Upon binding to the ATP-binding site, the acyl group reacts with a conserved lysine residue in most ATP-binding sites leaving behind the desthiobiotin tag. Kinases and other ATPases are enriched through binding to avidin or streptavidin agarose and then eluted for LC-MS/MS analysis. Kinases can be isolated in their intact state after reaction with the ATPase probe and subsequently trypsin-digested on-bead, or desthiobiotin-modified peptides can be collected after trypsin digestion [66]. The former method will identify multiple peptides per kinase and provides stronger evidence for identification while the latter can be used to quantify the extent of active site labelling, thus quantifying that particular kinase. Ideally, both approaches should be used for a more comprehensive identification and quantification of kinases in the system of interest. The main disadvantage of this technique is that, although there is still significant enrichment of kinases, the probe will also bind non-kinases that have ATP- or nucleotide-binding capabilities.

The ActivX system can be used to comprehensively identify kinases at the global level. However, its most interesting application is the potential to identify inhibitors for aberrant kinases in a disease state. Phosphorylation signaling is involved in cell division and growth, among many other processes. The focus on kinases as potential drug targets led to the development of the kinase inhibitor Gleevec® as a treatment for chronic myelogenous leukemia, now used in clinical practice. Most kinase assays to measure activity and find potential inhibitors are performed with the purified kinase. The issue with this approach is that kinases exist in a state of constant interaction with other proteins in the cellular network that may modify their activity. This is at least part of the reason for poor translation of many kinase inhibitors from *in vitro* assays into being used as treatment for disease. Patricelli et al. [67] used the ActivX system to profile kinases and identify potential kinase inhibitors in HL60 and PC3 cancer cell lines. Using a combination of ADP- and ATP-binding site probes, they identified approximately 220 kinases in both cell types. To quantify these proteins, they specifically targeted the active site peptides of approximately 100 of these kinases. They measured the effect of several different inhibitors on the signal intensity of these peptides to find IC_{50} values and binding affinities. In most cases, the affinity of kinase inhibitors discovered through the ActivX system closely matched the affinities found using the recombinant, purified enzymes. However, they did find several cases where the affinity of inhibitors measured

by the ActivX system differed from the *in vitro* assays. They conclude that, although the reasons for these differences are unknown, it shows the potential of this system to identify kinase inhibitors that may behave differently in cell-based systems compared to their activities in the purified state [67].

2.5.2. Luciferase based detection of enzyme activity

Luciferase assays are based on an analytical technique that harnesses the bioluminescence phenomenon of light emission by live organisms [68]. The reaction is generally caused by the oxidation of luciferin with oxygen, catalyzed by luciferase, resulting in an oxyluciferin molecule that is in an excited state. The oxyluciferin molecule emits visible light, then returns to its original state. There are over 30 different bioluminescence systems, 9 of which have their chemical structures determined. The most well-defined is the D-luciferin bioluminescence system. The first step in this chemical reaction is the reversible conversion of D-luciferin to luciferyl adenylate using ATP. It is because of this reaction that this luciferase has been used to design a high throughput kinase activity detection method. In these assays, the luminescence signal is inversely proportional to the amount of kinase activity; the assays quantify the amount of ATP that remains in a sample after a kinase reaction. In short, kinase-dependent ATP depletion is measured. High throughput kits can be purchased through several companies, such as Promega's 'Kinase-Glo® Luminescent Kinase Assay'.

2.5.3. Kinase activity kits

A variety of companies offer a good selection of enzyme activity kits that measure specific kinase activities (*ex.* Abcam) as well as universal kinase activity (*ex.* R&D). These kits come with a purified, recombinant enzyme as a positive control and can be compared to experimental cellular or tissue lysates. Although the kits are not high-throughput, they have high specificity, relatively easy workflows, fast turnaround times and employ simple detection methods.

3. Application and integration of systems kinomics to diseases

As powerful as the above-described tools are when used separately, many studies employ a combination of two or more methods to determine biological effects in different diseases. Most of these methods benefit from the utilization of other expression assays such as coupling qPCR with immunohistochemistry (to look at tissue expression in *in vitro* models or clinical samples), or using siRNA knockdown with Western blotting (to detect protein expression of target pathways with the knockdown of phosphatase genes). A common strategy is to use expression methodologies such as siRNA or RT-qPCR to first identify the kinases and phosphatases that are present, then perform further investigations using kinase activity assays to determine the activities of those identified enzymes. In many circumstances, the high-throughput analyses are stepping stones to generate hypotheses that require more focused experiments, and kinase/phosphatase studies are no exception. Each method has advantages and disadvantages (Table 1) which need to be considered when designing an experiment. Since kinomics is such an important aspect of cellular processes, it can be applied to virtually any disease. However, as a novel technology, the scope of current studies is still small. Most studies to date have focused on infectious diseases (virology) and cancer, and these will be discussed below. We also include example studies that have applied kinomics to the evaluation and diagnosis of clinical samples.

3.1. Virology

A growing number of kinomic peptide arrays are being performed on virally infected cells, including Cytomegalovirus [69–71], Ebola virus [72], Herpesvirus [73] and Hepatitis C virus [18,74,75]. Some studies have published whole kinome screens with the identification of specific kinases that are activated during viral infection, while others have begun to evaluate the antiviral impact of inhibiting identified kinases or related signaling pathways *in vitro* [76–79]. Kindrachuk and colleagues examined specific kinases activated by *in vivo* Ebola virus infection [72]. Although many variables affect the kinases identified including the type of virus, species, and timepoint, several kinases have been identified in multiple screens. For example, Hutterer et al. showed Aurora A activity was upregulated during cytomegalovirus infection [69] and Gabrielli and colleagues showed upregulation of the same activity during papillomavirus infection [80]. Similarly, systemic screens of kinase inhibitors have proven useful in discovering novel drug targets as was recently performed against hepatitis D virus [81] in which GSK-3β and Cdk inhibitors were identified. Kinases involved in the host antiviral response against Sendai virus have also been elegantly characterized by using an interferon-gamma promoter-driven luciferase assay [82] which identified FYN and SRPK1 as novel contributors to the host immune response. Finally, the ability to probe a wide variety of species has proven particularly useful within virology and includes the development of arrays for hamsters [34], guinea pigs [35], cow [32], monkey [79], pig, and poultry [33].

3.2. Cancer

Kinomics has also been applied in a variety of cancer studies. For example, a major focus has been the identification of kinases that promote survival of cancerous cells, and, by extension, large screens to determine viability of cancerous cells treated with various kinase inhibitors [83–86]. Similarly, screenings of migration inhibitors have also led to the discovery of potential therapeutic kinase targets [45]. These large screens of uncharacterized compounds are well complemented by other methods such as activity-based protein profiling (ABPP) [87–90], peptide arrays or siRNA arrays that can identify the specific proteins and pathways targeted by the inhibitor.

A second area of kinomics research in cancer consists of phenotyping tumors [87] and cancerous tissues that may lead to improved diagnostics and course of therapies as well as the development of novel therapeutics. Single cell flow cytometry has been extremely valuable for these studies as tissues and tumors can be heterogeneous cell populations and are not always well represented or understood without a representation of their variability.

3.3. Clinical samples and diagnostics

In addition to its value in basic science, kinomics research has also led to improved or novel diagnostic tests. For example, many kinases that were initially identified in cancer studies are currently in various stages of clinical trials and have shown great promise in treatment of cancer (reviewed by Ku et al. [91]). In addition, the field of infectious diseases has been impacted by the discovery of kinases that are unique to infectious organisms; this can allow for rapid, sensitive and specific diagnosis testing as was recently described by Wayengera et al. for Mycobacterium [92]. Some kinases are well-known biomarkers of disease and many studies have actively screened serum, tissue and urine samples to identify new markers. Some new potential markers include STK1c in response to lung surgery [93], plasma creatine kinase B for injury in

Table 1
Advantages & disadvantages of high-throughput methods in kinomics.

Method	Advantages	Disadvantages
siRNA/siRNA array	Simple and rapid. Many different siRNA delivery systems available. Screens can be tailored to specific pathways.	Off target effects. Incomplete knockdown. Costly for large screens. Optimization required for specific conditions. Transient inhibition – short term. Unmodified siRNAs easily degraded by RNases.
Real-time qPCR	High sensitivity (trace sample needed). Quantitative. Targeted approach – tailored to specific question.	DNA polymerase prone to errors. Non-specific binding of primers to other similar DNA sequences. Sequence information of target is required.
Microarrays	Affordable. Robust. Well established data analysis/bioinformatics tools available. Whole transcriptome differentially expressed gene analysis. Protein microarrays can be used for kinase activity.	Low dynamic range. Need reference sequence. Limited probes. Saturation effect can alter relative quantification. Indirect measure of relative concentration.
Peptide arrays	Relatively cheap. Sensitive. High throughput. Small sample volume. Adaptable to any species.	Sophisticated detection and analysis. Requires expensive scanner.
Phospho-ELISA	Small sample volume. Colorimetric detection. Quick, simple workflow. Relatively cheap. High specificity. Absolute quantitation.	Dependent on availability of a good phospho-antibody. Can be difficult for some species.
Phospho-Flow Cytometry	Quantitative per cell. Characterizes heterogeneous populations. Can be multiplexed with other markers.	Dependent on availability of a good phospho-antibody. Can be difficult for some species.
Microscopy	Once set up it can be automated. Live cell applicability. Determines cellular localization of target protein/enzyme.	Dependent on availability of a good phospho-antibody. Can be difficult for some species. Optimization of antibodies needed for each target. Not highly sensitive for quantification.
MOAC	Can be adapted to HPLC systems for automated sample processing. Stable against a wide range of pH and buffer conditions.	Non-specific binding of acidic peptides reduces efficiency of phosphopeptide binding. Requires pre-fractionation of samples with SCX or HILIC to acquire binding efficiencies of 80+%.
IMAC	Appears to be better for identification of multiply phosphorylated peptides. Can isolate phosphoproteins from purified protein samples.	Non-specific binding of acidic peptides is an issue similar to MOAC. Less tolerant to buffer conditions than MOAC.
Chromatography based methods	Semi-automated enrichment and collection of phosphopeptides. Increased phosphopeptide identifications through fractionation.	Requires expensive equipment and HPLC expertise. Fractions have to be purified decreasing throughput.
ABPP	Can be combined with other technologies. Probe-bound enzymes can be isolated from complex samples. Not species specific.	Selection of probes currently limited. Expensive reagents.
Luciferase assay	Relatively non-toxic, non-reactive. Does not require external illumination. No sample heating or light source drift. Extremely sensitive. Low concentrations of reagents. High throughput ability.	Short stability time for some reagents (possible signal decrease during assay readout). Susceptible to luciferase inhibitors.
Enzyme activity kits	Sensitive. High specificity. Quick, simple workflow. Colorimetric detection. Small sample volume.	Not high throughput.

professional boxers [94], creatinine kinase activity in the development of high blood pressure [95] and various kinases in different types of cancer (reviewed by Quan et al. [96]).

3.4. Phosphorylation databases and integration tools

3.4.1. Detecting phosphorylation sites

It is estimated that there are potentially 500,000 phosphorylation sites in the human proteome [97], the majority of which have unknown cognate kinases. These estimates are based on the multitude of phosphoproteomic experiments conducted identifying thousands of phosphorylation sites. Organizing all of this information is a challenging task, taking into account all of the kinases and phosphatases involved in regulating phosphorylation reactions. There are several databases available for many organisms that organize the information we have on phosphorylation sites including Phospho.ELM [98], PhosphoGRID [99], SysPTM ([100]), UniProt ([101]), PhosphoPep ([102]), PhosphositePlus ([103]).

Phosphorylation site database (PHOSIDA) ([104]) is a database developed in the Matthias Mann lab that contains information on

thousands of phosphorylation events in both prokaryotic and eukaryotic species generated through mass spectrometry experiments. They give an example of using their database to study the protein structure found in phosphorylation sites using solvent accessibility calculations. They found that phosphosites are generally found in loop or hinge regions and are typically highly accessible to the surrounding environment. These results largely confirm other studies showing that phosphosites require accessibility due to the need to interact with other proteins in the kinase/phosphatase network.

3.4.2. Data integration

The large number of phosphorylation site databases available has led to attempts to integrate this data into databases that contain all of this information in one source. dbPAF ([105]) is one example that combined information from several databases into a single source of information on animal and fungal phosphosites. After integrating data from the literature and from public databases they found a total of 54,148 phosphoproteins with 483,001 phosphorylation sites. They used this database to detect conserved sequence motifs of phosphorylation sites, predicted kinases, and analyzed conservation of phosphosite sequences across several species. KinMap is a second very useful and user-friendly resource that can be found online and allows interactive navigation through biochemical, structural, and disease association data ([106]).

4. Conclusion

There are numerous powerful high-throughput systems biology tools available for the study of kinases, kinase targets, the presence of phosphorylated proteins, and antagonistic phosphatases. Since kinases are key regulators of cellular processes and are integral to many different signaling pathways, kinomics is an important topic with respect to determining the mechanisms of how a disease can cause damage to cells. However, there are currently 518 putative kinases in the cell [2] and it is difficult to focus on relevant pathways using single molecule style experiments. Using these high-throughput methods, the most pertinent kinases in the study system can be identified before more focused experiments are performed.

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