Analyst

Accepted Manuscript

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/analyst

Towards Single Cell LC-MS Phosphoproteomics

Ayşe Nur Polat[1, 2], Nurhan Özlü[1]

[1]Department of Molecular Biology and Genetics, Science Faculty, Koç University, Istanbul, Turkey

[2] Schools of Engineering and Science, Jacobs University, Bremen, Germany

To whom correspondence should be addressed:

Ayşe Nur Polat
Department of Molecular Biology and Genetics
Koç University
Istanbul, Turkey
Email: anpolat@ku.edu.tr

Nurhan Özlü, PhD
Department of Molecular Biology and Genetics
Koç University
Istanbul, Turkey
Email: nozlu@ku.edu.tr

Keywords: mass spectrometry, phosphoproteome, microfluidic chip, quantitative phosphoproteomics

Running Title: Phosphoproteomics

Summary/Abstract

Protein phosphorylation is a ubiquitous posttranslational modification, which is heavily involved in signal transduction. Mis-regulation in the protein phosphorylation is often associated with a decrease in the viability and complex diseases such as cancer [1, 2]. The dynamic and low abundant nature of phosphorylation makes studying phosphoproteome challenging [3]. In this review, we summarize state of the art proteomic techniques to study and quantify peptide phosphorylation in biological systems and discuss their limitations. Due to its short-lived nature, the phosphorylation event cannot be precisely traced in a heterogonous cell population, which highlights the importance of analyzing phosphorylation events in a single cell level. Mainly, we focus on the methodical and

instrumental developments in proteomics and nanotechnology, which will help to build more accurate and robust systems for the feasibility of phosphorylation analysis in a single cell level. We propose that an automated and miniaturized construction of analytical systems holds the key to the future of phosphoproteomics; therefore, we highlight the benchmark studies in this direction. Having advanced and automated microfluidic chip LC systems will allow us to analyze single cell phosphoproteomics and quantitatively compare it with others. Progress in the microfluidic chip LC systems and feasibility in the single cell phosphoproteomics will be beneficial for early diagnosis and detection of the treatment response for many crucial diseases.

Introduction:

Proteins are composed of small subunits, "amino acids", which are translated from a genetic code [4]. After translation, the diversity of proteins can be increased with the addition of modifications [5]. The human genome is resourced with the static information of genes whereas the proteome expands this to multiple different species by covalent modifications and non-covalent interactions [6, 7]. Traditionally, using the Edman degradation reaction, amino acids are sequenced via labelling amino acid terminal residue and cleaving from the peptide without disrupting the remaining peptide bonds [8]. Unfortunately, this method is not efficient enough to analyze the dynamic proteome and detect different modifications on the proteins. On the other hand, several hundreds of post-translation modifications are detected by methods using Mass Spectrometry (MS) [9] [10]. Amongst these identified modifications, s peptide phosphorylations are essential for the catalytic activity of kinases with regard to signal transduction [5]. A considerable amount of knowledge has been accumulated over the years on the phosphorylation state of serine, threonine and tyrosine side chains; additionally, the phosphorylation of histidine, arginine and possibly lysine have also been reported to a far less extent and their role in signal transduction is yet to be determined [11-14].

Phosphorylation through kinases lies at the heart of signalling pathways; thereby protein kinase and phosphatase activities are attractive research topics [15]. Mis-regulation of protein phosphorylation is often associated with a decrease in the viability and diseases such as cancer [1, 2].

Mass spectrometry-based phosphoproteomics technologies have been steadily developing for over a decade; however, studying phosphoproteomics is still quite challenging. Due to low abundance and the physicochemical properties of the phosphopeptides, sample preparation, fractionation and instrumentation techniques are the key steps that determine the success rate of phosphopeptide detection [3, 16, 17]. Phosphoproteomics suffers from pitfalls of instrumental techniques and biological systems. Examples include poor reproducibility of MS analysis, time dependence of phosphorylation events and sub-stoichiometry of phosphorylated peptides, cell to cell protein amount and phosphorylation

activity variation in a heterogonous population [18]. In this review, we summarize state of the art proteomic techniques to study protein phosphorylation in biological systems and discuss their limitations and challenges. Due to short life and low abundance, quantifying phosphorylation events in a heterogeneous cell population introduces ambiguity; it would be more accurate to analyze phosphorylation events on a single cell level. A major barrier to perform phosphoproteomic analysis on a single cell level is the lack of sensitive methods to process very low amounts of material in a single cell for LC/MS. One potential direction towards single cell phosphoproteome applications is integrating microfluidic systems to proteomics. Having advanced and automated microfluidic chip LC systems combined with effective phosphopeptide enrichment and detection methods would allow us to analyze single cell phosphoproteomics and quantitative comparison with others. Both the microfluidics and proteomics fields have made tremendous advancements in the last decade and more efforts are emerging to take advantage of both technologies. Figure 1 shows the timeline of landmark studies in proteomics and microfluidics and highlights their intersection in recent years.

One of the first intersections was the use of a Lab-on-a-Chip system for the separation and fractionation of samples of interest from complex biological systems [19-22]. Another advanced application is the integration of microfluidics into HPLC-chip systems for MS applications [23-26]. Detailed reviews have been published by Gao *et al.* and Feng *et al.* about advances on microfluidics and their combination with MS [27, 28]. Therefore technical advances on microfluidic devices will not be discussed further in this review.

The following sections explain each step of the phosphoproteomics analysis and discuss the progress made towards single cell proteomics by making use of microfluidic chip LC systems. We propose that the integration of these two technologies is a next step for phosphoproteome analysis and pushing such integrated technology will open up new avenues of research.

Sample preparation:

Sample preparation is a critical step for increasing the sensitivity of a phosphoproteome analysis as reviewed in [3].The type of the sample and the aim of the experiment are the key parameters to decide how to treat samples. In general, cells or tissue samples are lysed with a buffer containing different reagents for various purposes. For example, denaturing agents enhance cell and tissue solubility and protein unfolding [29]. All sample preparation should be performed on ice to minimize enzyme activities. A mixture of protease inhibitors should be used to prevent undesirable protein degradation. Negatively charged phospho groups are stable on proteins at acidic and physiological pH conditions; only through catalysis reaction can phosphatases remove phospho groups. Therefore, a cocktail of phosphatase inhibitors should be used to prevent dephosphorylation. A detailed list of phosphatase inhibitors used in a lysis buffer and their applications can be found in [18, 30]. Depletion of surfactants and protein inhibitors is a prerequisite for an efficient mass spectrometric analysis because their favourable ionizability and their relative abundance hamper the peptide spectrum [31].

Different types of proteolytic enzymes can be utilized for protein digestion to generate a specific peptide pool. Unique peptide distributions can be obtained with different sequence characteristics, length, solubility and charged with the appropriate choice of enzymes. Lys-C, Trypsin and Lys-N are the most common proteolytic enzymes; each of them cleaves the proteins at specific sides. Trypsin cleaves the C-terminal side of lysine and arginine residues; their activity is strictly dependent on the buffer and the reagents concentrations [32]. Trypsin with LysC is a popular enzyme combination for CID and HCD fragmentation based MS analysis [33]. Conversely, LysN, that cleaves at the N-terminal side of lysine, is a commonly used enzyme to generate simplified fragmentation spectra during ETD fragmentation [34-36]. Using a different combination of proteolytic enzymes would increase the coverage phosphoproteome [37].

Figure 2 shows a general workflow of a typical phosphoproteomic study, which has multiple steps with various tasks. Each step has a possibility of sample loss and contamination introduction. Single cell level studies testify to the power of microfluidics as Lab-on-a-Chip systems for sample preparation and digestion due to their liquid handling capacities and capability to manipulate single cells in one system. These systems can provide highly accurate measurements even for very low abundant species in a complex

mixture because of their sensitivity and compatibility with high-throughput detection and selection systems [38, 39].

Microfluidic systems can be integrated into phosphoproteomics at the very first stage of the workflow while dissecting a part of a tissue or by selecting the required cell population. Agresti *et al.* used an integrated drop-based microfluidic device to establish an ultrahigh-throughput screening platform[39].

Their platform consists of two devices. The first mixes yeast cells with fluorogenic substrates into low picoliter volume droplets. A second device redirects the droplets and sorts the cells subsequently according to their dielectrophoretic forces. The sorting of cells is based on their intensity of fluorogenic substrates binding to the enzymes on the cell surface. Since the cells remain encapsulated in the drops, the entire reaction vessel is assayed and sorted with this microfluidic system. Using a microfluidic design instead of a traditional Fluorescence-activated cell sorting (FACS) not only increased the rate of screening 1000 fold but also the versatility of cells [39].

Marcy and colleagues performed a promising study for the integration of microfluidic systems into a biochemical process. They used a fabricated microfluidic device to lyse the isolated cells and amplify their genetic material [38].Another noteworthy phosphorylation study done by Jen *et al.* lysed HeLa cells using a micro well device with 20-μm diameter for single-cell-based chemical lysis experiments. At the single-cell level, cells are fully lysed 12 seconds after the lysis buffer injection [40]. These studies propose that microfluidic devices are suitable for multiple single cell applications from cell lysis to monitoring biochemical activities. Integration of microfluidic devices with different capabilities can enable us to perform single cell phosphoproteomics analyses on chips. Therefore phosphoproteomics analysis can greatly benefit from microfluidic based chip systems for high throughput studies [41, 42].

Phosphopeptide analysis by LC-MS:

Phosphopeptide fragmentation:

Phosphopeptide sequencing by tandem MS can induce the loss of a labile phosphate group and makes assigning the phospho-site to the correct residue challenging. Determining the site-specific phosphorylation is complicated because CID typically results in the partial elimination of the phosphoric group (H_3PO_4, 98 Da or HPO_3, 80 Da, neutral loss) of phosphoserine and phosphothreonine [43],whereas phosphotyrosine does not allow loss of phospho-group because of the aromatic ring. But, occasionally, phosphotyrosine can undergo with an irregular loss of HPO_3 from phosphotyrosine and H_2O from another residue [33, 44]. The low abundance of the phosphopeptides is a well-known issue so that choosing the fragmentation method can be crucial for phosphoproteome analysis. Since the analysis of phosphorylation at the single cell level will be more demanding, the sensitivity and the coverage of the detection need to be improved. This can be done by combining different fragmentation methods [45-50].

Sample Fractionation:

Dynamic range and the peptide sequencing speed of the instrument are two main limiting factors of the complex peptide mixture analysis and prevent full proteome identification [51]. Phosphorylated peptides are under-represented in a complex sample and their detection by MS is further impaired by low ionization efficiency and signal suppression. Thus, enrichment and fractionation techniques are obligatory for phosphoproteomics studies [52].

In proteomics, the majority of the sample analysis is performed with reversed phase chromatography coupled with tandem mass spectrometry (nanoLC-MS). Although new instruments have high resolving power and detection speed, pre-fractionation of samples prior to MS analysis is a prerequisite for a comprehensive analysis [53]. Their use in phosphoproteomics is limited by their capability to resolve highly complex samples like whole cell lysate. Thus, a single cell analysis would require massive optimization of several parameters, from liquid flow rates, chip channel dimensions to waste line and mixing chamber distributions. Well-known phosphoproteomics protocols should be adapted for microfluidic device dimensions.

Different strategies can be utilized at a different stage of the workflow for this purpose. Employing an additional agent at the enrichment stage can increase the selectivity or reagents can be used for signal enhancement by improving sample solubility and spray. For example, Winter *et al*. showed that a citrate addition to the phosphopeptide sample could effectively improve the sensitivity of LC-MS analysis of phosphopeptides [54].

Larsen *et al.* also showed that binding of highly acidic peptides onto TiO_2 material can be circumvented by adding DMSO (Dimethyl sulfoxide), KF (Potassium fluoride) and HNa_2PO_4 (Sodium phosphate dibasic) reagents to the loading buffer. A study by Ficarro *et al.* demonstrated that using low flow rates at the nanoliter range can enhance the phosphopeptide detection [55]. A recent study from the Kuster group revealed the importance of spray to enhance phosphopeptide detection. They used DMSO to improve the solubility and evaporation efficiency [54, 56].

Several other LC methods based on different chemical properties of peptides have been developed for sample fractionation. Therefore, using the combination of different separation procedures is required to increase the peak capacity, and the overall LC resolving power. The most commonly used methods are ion exchange chromatography (SCX), strong anion exchange (SAX), weak anion exchange (WAX) hydrophilic interaction liquid chromatography (HILIC) and Electrostatic Repulsion-Hydrophilic Interaction Chromatography (ERLIC) [51, 57-59 55].

The frequent use of and the new improvements in microfluidics are contributing greatly to single cell proteomics at different levels of the process like single cell trapping, lysis, separation and analysis. It has become possible to use extended nano-space by down scaling the size of the microfluidic to nanofluidics [19 22]. Extended nano-space helps to manipulate biological systems efficiently according to their physicochemical properties which lead to elevated performances by rapid, time efficient and reagent consuming reactions. This results in reproducible and high throughput data for single cell phospho proteomics [60]. A recent study published by Huft J. and colleagues showed a successful integration of a microfluidic device to a solid phase LC. They automated a multifunctional

platform, which permits flexible generation and complex manipulation of low picoliter-scale droplets. Their study supports that this kind of design can handle enzymatic assays and DNA purification at a single cell level [61].

Improvements in technology will make microfluidic parts for the LC systems cheaper and more disposable which will increase the quality of the chromatographic separation. Also, working with such a miniaturized system will allow us to exploit the resolution of the chromatography accordingly.

Phosphopeptide enrichment:

Systematic and large scale analysis of the phosphorylation events in the cell is challenging because of the dynamic range and the complexity of the sample. Physicochemical properties of phosphopeptides are predictable so they can be fractionated and enriched using multiple methodologies. But each additional step introduced to the workflow also introduces a new possible error and variant for the analysis. To maximize the analytical sensitivity, the workflow should be as simple as possible, with relatively few sample preparation steps, so as to prevent loss of phosphopeptides. To increase coverage and sensitivity, samples should be handled cautiously prior to analysis. Similarly, a robust system is needed to minimize the variation across the replicates. Introduction of microfluidic chip systems and their automation for sample preparation and fractionation is an effective solution to prevent sample losses and to enhance reproducibility. Employing microfluidic devices minimizes the sample and reagent consumption; it also helps us manipulate the physicochemical properties of the reagent. Consequently, by using microfluidic chips, we can design a controllable, repeatable and rigid system for sample analysis. In the previous section we briefly explained the fractionation methods and in this section we will continue with enrichment techniques and applications. Although several selective targeted phosphoproteomics methods have been developed and are widely used, lab-to-lab enrichment efficiency and identification rates differ for the same protocols [62]. Obtaining full coverage of the phosphoproteome with various enrichment strategies with the combination of fractionation techniques is still a demanding task. A wide range of techniques has been developed to enrich the population of phosphopeptides. These enrichment methodologies

are based on different principles and are employed according to their orthogonality, with each having its own advantages and disadvantages [63]. The most frequently used techniques are HPLC-based fractionation, and antibody affinity chromatography (immuno-precipitation (IP), immobilized metal ion affinity chromatography (IMAC), metal oxide affinity chromatography (MOAC), and chemical derivatization based chromatography) [64-67] . Although MOAC, SIMAC and IMAC have quite similar principles we will explain each in a separate section to highlight the slight discrepancies among these methods.

IMAC:

The IMAC method is based on the affinity of positively charged metal micro particles (Fe^{3+},Al^{3+},Co^{2+},Ga^{3+},Ti^{4+}), forming a stationary phase that captures negatively charged phosphopeptides under acidic conditions and releases at alkaline pH [68, 69]. Non-specific binding of acidic peptides is a major obstacle to this technique [70]. One approach to circumvent this shortage is through O-methyl esterification which derivate carboxyl groups on acidic residues into less acidic functional groups. This approach increases the specificity for the selective phosphopeptide detection [71, 72]. IMAC has frequently been coupled with strong cation exchange chromatography (SCX), and hydrophilic interaction liquid chromatography (HILIC). These systems are used prior to the IMAC enrichment to reduce sample complexity and amplify selectivity towards phosphopeptides [73].

MOAC:

MOAC is an alternative method to IMAC which uses a similar binding chemistry. Acidic residues are neutralized by protonation with the acidification of the loading buffer. As phosphopeptides retain their charge at highly acidic pH, their binding affinity to the metal oxide functionalized ZrO_2 and TiO_2 beads are enhanced [64]. The phosphate group of phosphopeptide binds to the oxide groups of the TiO_2 beads in a bidentate mode [72]. A drawback to this method is the non-specific binding of the acidic non-phosphopeptides. Employing an additional agent at the enrichment stage can increase the selectivity and partly overcome this shortage. For example, using a low pH loading buffer containing

reagents such as 2, 5-dihydroxybenzoic acid (DHB), dimethyl sulfoxide (DMSO), phtalic acid and glycolic acid reduces the nonspecific binding of acidic peptides [74].

Winter *et al*. showed that phosphopeptide enrichment sensitivity could improve by using phosphate, EDTA or citrate as a chelating reagent [54]. A similar study by Larsen *et al.* also showed that binding of highly acidic peptides onto TiO_2 material can be circumvented by adding DHB to the loading buffer [75].

To our knowledge, phosphopeptide enrichment by using TiO_2 beads is a widely used method because of its offline and online applications and compatibility [76]. Thus, this method has been successfully coupled with reverse phase liquid chromatography. Furthermore, a novel microfluidic chip device based on a TiO_2 column integrated to RP-HPLC-MS is manufactured by Agilent. This HPLC-chip enables applications like fully automated phosphopeptide quantification [77-79].

SIMAC:

Several different phosphopeptide enrichment methods have been well-established and their weak and strong features rigorously evaluated [80]. To increase the identification rate of phosphopeptides, different enrichment methods are combined for Sequential elution from the IMAC (SIMAC) method [81]. This method combines strengths of both IMAC and TiO_2 and allows the enrichment of both mono and multi-phosphorylated peptides from complex samples. The rationale behind this methodology is to elute multi-phosphorylated and mono phosphorylated peptides differently based on their binding efficiency to IMAC beads. Mono phosphorylated peptides are eluted from the IMAC beads in an acidic condition. As a second step, TiO_2 chromatography is applied to these elutes and flow-throughs to remove most of the non-phosphorylated peptides from the pool of mono-phosphorylated peptides in a complex mixture. IMAC has a stronger selectivity for multi-phosphorylated peptides; for these peptides a parallel IMAC chromatography is performed. Phosphopeptides are eluted in a basic condition, which selectively elutes multi-phosphorylated peptides from the IMAC beads. This method has found its own application in large-scale phosphoproteomics experiments [64, 76 82, 83].

Immuno-precipitation (IP):

Phosphorylation of tyrosine has key functions for most of the signalling pathways. But in a mammalian cell, phosphorylation of tyrosine occurs at low frequency compared to serine and threonine [84]. Thus, enrichment of phosphotyrosine requires explicit methods like immuno-precipitation (IP). Phosphopeptide enrichment with IP is strictly limited with specificity, the lot-to-lot variability of the antibody and reproducibility of the enrichment protocol [85]. IP can be performed at protein or peptide levels by using phosphotyrosine antibodies [86, 87]. Several IP experiments should be done in parallel to acquire a comprehensive set of phosphotyrosine peptides [66, 88]. Thus, this method is not feasible for experiments with a limited sample amount. Performing IP experiments at the peptide level is more effective [89]. However using IP strategies at the peptide level requires a large amount of protein as a starting material [90]. Recent studies on microarrays and phosphotyrosine antibodies showed that one can enrich different phosphotyrosine populations by using different types of antibodies [91]. Therefore, antibody cocktails will likely be more popular for this sort of strategy.

Ti-IMAC:

Recently a new phosphopeptide enrichment protocol has been successfully used by the Heck group [92]. This method is based on Zr (IV) or Ti (IV) phosphate/phosphonate chemistry, chelating and immobilization of Ti^{4+} via coordination between Ti^{4+} and the P-O bond of the phosphate group [93,92]. Structural improvements on the Ti-IMAC materials made this method more orthogonal with other peptide fractionation techniques such as SCX and HILIC [73].

Promising results for the miniaturization of LC systems as microfluidics (HPLC chips) for sample enrichment and fractionation have been published. Several nano-LC-chip systems have been commercialized by different vendors such as TiO_2 enrichment, HILIC fractionation and protein digestion chips [77, 79, 94-97]. Ti-IMAC based nano-LC-chip systems will be beneficial for selective and sensitive phosphopeptide enrichments of small materials such as a single cell. The next challenge will be to establish a full phosphoproteomics workflow in one automated system (Figure 3).

Chemical Derivatization:

Various chemical derivatization protocols are based on a specific reaction under strict conditions. Thus, most of the developed phosphopeptide enrichment protocols have low efficiency and low reproducibility. A general application of a particular derivatization protocol for a wide range of applications is almost impossible. Beta elimination of the phosphate group from phosphoserine and phosphothreonine under basic conditions is possible but its efficiency is strictly dependent on the peptide sequence [98] [99]. In addition, this approach cannot be used for the phosphotyrosine peptides. Another similar approach is to replace the phosphate group with biotinylated tags. This approach is also not applicable to phosphotyrosine residues but can modify phosphoserine and phosphothreonine O-glycolysated forms and non-modified residues [100]. Because of the non-specific reaction and low reproducibility nature of the approach, it has yet to be improved to be compatible with other enrichment strategies.

Table 1a presents a review of potential applications of the current phosphoproteomics methods to microfluidics. Each method's compatibility and future adaptations to the microfluidic systems are rated as low, moderate or high with respect to their current trends and applications. Since several successful applications have already been published with IMAC and MOAC based enrichment methods, they are rated as highly compatible. Chemical derivatization is rated as moderate because the application is dependent on the microfluidic designs. A complicated design with well-defined fluid trafficking is required to prevent the side reactions caused by the residual chemicals. Designing such systems would take a long time and require a lot of expertise in the microfluidic field. IP based enrichment strategies is the least compatible with microfluidics. Implementation of these protocols into a microfluidic chip is demanding, since the reproducibility of the IP enrichment protocols are low, and highly specific antibodies are needed for high affinity bindings.

Polat et al. recently published another example of employing a microfluidic device to perform a phosphoproteomic enrichment method. A chip LC system was used which consists of four columns; a TiO_2 column is replaced in between two reverse phase (RP)

columns and followed by an analytical column. The first RP column was used for quantitative labelling and cleaning followed by a TiO_2 column where phosphopeptide enrichment was conducted. A second RP column was employed for a controlled released of the sample from the analytical column to be processed by the MS analysis. This automated system was shown to be sensitive (10 fold ratio differences measured successfully), efficient (high labelling and enrichment efficiency), highly reproducible, less time and sample consuming (1µg sample and whole analysis time was 8 hours instead of a whole day) and robust [77]. Thus with this approach, comparable results can easily be achieved in high throughput studies. Several studies have been conducted on phosphoproteins by either labelling or label free quantification strategies. Only a few were performed by using microfluidic/chip LC and single cell systems [77, 79, 96, 101]. Up to now, phosphoproteomics studies mainly used averaged extrapolated results from heterogeneous cell populations; however, as it is further discussed in the coming sections, individual cell analysis is more definitive and accurate compared to population analysis [102].

Quantitative Proteomics:

Quantification of phosphoproteome is essential for studying its dynamic nature and has greatly benefited from the development of advanced instruments, new software, and sample preparation methods. Different types of labelling can be introduced at various stages of the workflow [103, 104]. The step where quantification is introduced to the workflow and the quantification strategy should be considered carefully which vary largely based on the question being addressed.

Different strategies such as chemical labelling, metabolic labelling, label free and absolute quantification strategies have been employed in several studies [105]. In this section we will briefly explain the principles of the methods and the successful adaptations for quantitative the studies. Common quantification strategies can be broken down into two main categories: relative quantification and absolute quantification. The idea behind microfluidics is to minimize user interference and maximize the automation to prevent bias

and contaminations prior to quantification. In general, microfluidics will be most beneficial for quantitative analysis since it provided accurate and consistent sample-to-sample experimentation.

Metabolic Labelling:

Metabolic labelling is introduced during the cell growth and division by the substitution of the natural ^{14}N or ^{12}C sources with their heavier isotopes, such as ^{15}N or ^{13}C or by substituting one or more essential amino acids with their heavy-labelled counterparts to obtain full incorporation of the synthesized proteins [106]. The main advantage of metabolic labelling is the incorporation of the label in the living cell at the earliest stage of the work flow. Samples can be combined before the sample preparation steps and any error coming from the sample handling steps will be identical for each sample. The quantification accuracy will be equally affected for each sample. Metabolic labelling is commonly used in invertebrate model organisms such as yeast, *C. elegans* and *drosophila*; a new metabolic labelling method introduced by the Yates Lab extended this technique to an entire mammal. The SILAM (stable isotope labelling of mammals) labelling of rodents was performed with a diet of ^{15}N-enriched (>99%) blue-green algae *Arthrospira platensis,* as the sole protein and nitrogen source for the animal [107]. Rauniyar *et al.* demonstrated a quantitative application of SILAM to analyze protein expression levels in the rat brain at two different developmental stages. They found that ^{15}N labelled rat can be an optimal source of a tissue-specific internal standard to facilitate the quantitative proteomic [108]. As the incorporation reaction is quite specific apart from the preventable arginine proline conversion, no side reactions or side products have been observed in the sample [109]. The limiting factors for the use of this method are the time required and the cost of the experiments.

SILAC:

The most frequently used metabolic labelling method is the Stable Isotope Labelling by Amino acids in Cell culture (SILAC) [106]. Simply, the procedure is the in vivo incorporation of

the stable isotope containing versions of essential amino acids (especially arginine, lysine and leucine) during the cell growth and division. Arginine and lysine residues are particularly popular since trypsin cleaves peptides at the carboxyl terminal of these amines; when SILAC is combined with trypsin digestion, every peptide will be labelled at the carboxyl-terminus. After several cell doublings, the complete cellular proteome is labelled with the of isotope-containing amino acids [110]. The number of labelling channels for this method is limited with three but this can be extended to four or five labels at the expense of accurate quantification hindrance. A recent paper published by the Augustin group established 5-plex SILAC, which was employed to monitor the phosphotyrosine signalling perturbations induced by a drug treatment. SILAC is generally used for cell systems that are grown in a culture but has limited use for body fluids and tissues [111] [106, 110, 112]. New SILAC applications are also emerging, like SILAC labelled mice [113]. By in vivo labelling, the entire proteome prior to sample preparation prevents quantitative labelling based sample loss; therefore, we believe that in the future the SILAC method will be the method of choice for single cell relative quantification studies.

Chemical Labelling:

When metabolic labelling cannot be used due to sample related reasons, chemical labelling can be an alternative solution for the quantification. For chemical labelling purposes, any reactive group of peptides can be altered and targeted by labels, particularly the peptide/protein N-terminus and Ɛ-amino group of lysine [114]. One of the primary disadvantages of the chemical labelling method is side reactions and products; those may complicate the MS detection and spectra analysis. Peptide precursor ions (MS) and/or fragment ions (MS/MS) can be utilized for the quantification. Another accepted method for chemical labelling is stable-isotope dimethyl labelling [115, 116]. This method targets the primary amine groups (lysine and amino termini) of proteins/peptides. Three channels of labels can easily be generated by using a combination of isotopomers of formaldehyde and cyanoborohydride, including isotopes ^{2}H and ^{13}C atoms [103, 105]. Since deuterium has a different physical and chemical property from the hydrogen analogue, the deuterium effect reveals itself as retention time shifts during liquid chromatography (LC) separations.

Although ICAT itself is not an isobaric tagging strategy, developing this method was the first step for isobaric tagging-based quantification. The implementation of two isobaric tag quantification methods, (iTRAQ) and tandem mass tag (TMT) have increased the popularity and the usage of chemical labelling for proteomics [115-118].

Stable-isotope dimethyl labelling:

Dimethyl labelling is applied to the sample at the peptide level generally after a tryptic digest; each label differs from others by at least 4 Da [119] [120]. The low cost of the method and micrograms to milligrams of sample labelling range, its well established labelling protocols and applicability to any biological experiment make it a newly emerging quantitative method for many research groups [121]. A drawback to this method is the deuterium effect during LC separations, which can affect the quantification [122] [123]. But this affect can be minimized by using retention time alignment software. Dimethyl labelling can prevent unspecific binding of phosphopeptides into IMAC columns, with the esterification of the acidic groups such as C-termini of peptides and carboxylic acids in side chains of glutamic and aspartic acids. Consequently, this method can improve the enrichment of the phosphopeptides [77, 79, 85].

ICAT:

ICAT (Isotope-coded affinity tags) labelling is performed at the protein level. This chemical labelling strategy works based on its specificity toward sulfhydryl groups and consists of an isotopically coded linker and an (biotin) affinity tag for the purification of labelled proteins/peptides. Its applicability is only for cysteine-containing proteins which reduces the reliability of the quantification and limits the number of labelling channels; as a result, ICAT is less popular compared to other methods [118] [115, 124] .

Isobaric labelling (iTRAQ and TMT):

The isobaric tag for relative and absolute quantification (iTRAQ) and the tandem mass tag (TMT) have the main advantage of multiplexed analysis of four, six, eight, or 10 samples within one experiment setup. These isobaric labels consist of three different components: reporter, balance and reactive regions. N-hydroxy-succinimmide ester is employed for the reaction with primary amines of peptide N-termini chemistry. Through MS, isobaric labelled peptides cannot be distinguished so that MS spectra will be less complicated, but relative abundance can be determined when MS/MS reporter ion cleaves off during the fragmentation. This multiplexing strategy is presented in 4- or 8-plex formats for iTRAQ, and 6 or 10-plex for TMT. A good chromatographic separation to diminish co-elution of peptides and a ToF or HCD capable instrument allowing quantitation of low m/z fragmentation ions are required for better quantification by using an isobaric tag.

Recently, a detailed study comparing both identification and quantification of iTRAQ/TMT, SILAC, Dimethyl labelling was published by the Heck group [125]. Triple labelled samples were used for SILAC, dimethyl and iTRAQ/TMT and a separate 6-plex iTRAQ/TMT was employed to judge the performance of a complex design. Their results showed that SILAC and dimethyl labelling are both similar for quantification and identification. On the other hand, iTRAQ/TMT had a lower rate for MS2 based quantification due to the co-isolation problem. Their most precise quantitative results were obtained with MS2/MS3 based TMT experiments. Finally, the Heck group suggested using SILAC for affinity purification MS experiments and dimethyl labelling for primary cell cultures of tissue samples [123].

Label-free Quantification:

In the absence of the labels one can use a label free approach for the peptide/protein quantification. This method uses either a precursor intensity calculation, a spectral counting method (total number of the identified peptide-to spectrum matches, PSMs, per protein) or a sequence coverage of each protein [126 127]. An indefinite number of samples can be compared with label free methods but a label-free quantification experiment requires

multiple analyses of each sample and separate analysis for each condition. As a consequence, label-free experiments can be elaborate and time consuming.

Both intensity calculation and spectral counting based label-free approaches need a robust LC system and high precision MS for high reproducibility and to align the retention time and m/z of peptides in between analyses. Label free quantification using precursor intensity is based on extracted ion chromatogram (XIC) of the peptide of interest. Peptide/protein abundance can be predicted by accumulating differentiations of measured XIC variations [96]. An internal standard with a known concentration is needed for the relative quantification and normalization [128]. To our knowledge, the most frequently used approach is the spectral counting method because of its sensitivity (dynamic range) and high reproducibility. This method is generally used for the comparison of large datasets [129]. Results of a relative quantification with spectral counting are strictly dependent on the parameters used and data manipulation [130]. Each step of the workflow should be considered carefully: the number of data points, the length and abundance of the peptide, sample concentration, analogy of the sample analysis process, protein identification process, filtering parameters, and normalization of data.

A study by Megger et al. investigated three different hepatocellular carcinoma (HCC) cell lines HepG2, Hep3B, and SK-Hep-1, using both label free and TMT labelling. In this study, protein coverage obtained with label-free quantification outperformed the TMT labelling. But the protein identification rates were similar for both approaches. Although reproducibility was comparable for both TMT and label-free, TMT had higher quantification accuracy. They also demonstrated the necessity of using HCD fragmentation in combination with TMT labelling [131].

The message from peptide quantification methods comparison studies is universal; they all demonstrate that a quantification strategy should be chosen based on the type of the sample type and design of the experimental workflow.

Absolute Quantification (SRM/MRM):

SRM/MRM quantification is preferred by many groups because of its highly reproducible, sensitive and accurate nature for both relative and absolute quantification. Absolute protein quantification applications are performed by adding a known concentration of an internal standard (a peptide containing heavy amino acids or stable-isotope-containing tags) to the protein digest. Mass spectrometric signals of standard and endogenous peptide of the sample are compared for the quantification [132]. Different reference peptides/proteins and approaches such as AQUA and QconCAT, Protein standard absolute quantification (PSAQ), FlexiQuant (PSAQ), and absolute SILAC are used for the targeted SRM/MRM method [86, 133-136]. SRM/MRM is simply the isolation of specific fragments of the peptide and detection of its transitions by the mass spectrometer.

The transient and dynamic nature of phosphorylation, low abundance of phosphoproteins, and lack of phospho specific antibodies make the targeted absolute quantitative methods desirable. Even though this approach is applicable and favoured for the phosphoproteomics and in general proteomic experiment designs, determination of one or a few specific phosphoproteins could be quite challenging and time consuming [137]. Some of the limitations of the SRM/MRM method include: the necessity of discovery experiments for the selection of appropriate internal standards, optimizing assays for each peptide of interest, the high cost of internal standard synthesis and bias due to the late introduction of internal standards to the sample [138].

Although SRM/MRM requires prior knowledge of peptides/proteins, it is the most preferred method for the biomarker studies. Due to its sensitive absolute quantification, it is feasible for high-throughput clinical studies. New automation and chip applications will likely strengthen the power of SRM for discovering new biomarkers and extend its application to single cell studies [139]. In Figure 3 we showed a hypothetical design of a microfluidic chip for quantitative phosphoproteomics. This kind of design can circumvent the introduction of impurities and losses during sample handling. Therefore, it is beneficial to use a combination of this design together with sensitive analysis methods such as

SRM/MRM or label free quantification. All these approaches require minimal variation before and during sample analysis so that the robustness of microfluidic chip systems can significantly improve peptide quantification.

Table 1b presents a review of all quantitation methods discussed above for their adaptations to microfluidic systems for a single cell. Each method is rated based on its potential of integration to the microfluidics systems and prospective in single cell phosphoproteome quantification. High reproducibility and robustness are the main strengths of the microfluidics. For label free and absolute quantification methods no chemical labelling step is involved; only stable LC-MS systems and reproducible analysis with stringent protocols are required. Therefore, they are more compatible with microfluidics. Although numerous applications of chemical labelling using microfluidic HPLC chips have been published [77, 140], in general their compatibility is debatable[77, 78]. For instance, for TMT and iTRAQ, several different labelling reagents are needed and optimization is a pre-requisite. A comprehensive microfluidic design would be needed for such labelling strategies, and, as explained in the chemical labelling part, building such systems can be quite challenging. Therefore, chemical labelling is rated as moderate for compatibility. The metabolic labelling strategies on the other hand can be applicable. The previous sections showed examples of on chip cell growth. Implementing metabolic labelling into such designs could be less demanding compared to the other chemical labelling-based methods. Hence, metabolic labelling is rated as compatible for microfluidics.

Towards single cell phosphoproteomics:

The dynamic nature of protein phosphorylation and cross talk between the phosphorylation sites play an essential role in the specificity of signal transduction pathways and in fine-tuning the cellular response. Upon stimulus, protein phosphorylation may rapidly occur and reach its maximum level followed by a decrease in a short period of time [141]. However, it is not feasible to trace such a dynamic phosphorylation in a mixed cell population level due to the heterogeneity of cells. Figure 4 shows a hypothetical example

that illustrates the importance of single cell phosphoproteome in analyzing phosphorylation dynamics and shows plausible discrepancies in quantifying phosphorylation sites in a single cell versus a heterogeneous cell population. In the last decade, studies at the single cell level revealed new mechanisms in cellular signalling pathways. For example, the oscillatory response of p53 and Nf-κB signalling systems were only identified by quantitative analysis at a single cell level [142]. We suspect that many phosphorylation events in eukaryotic cells may have similar dynamics and would only be revealed by phosphoproteomics analysis at a single cell level. The single cell phospho-analysis approach becomes even more valuable in some fields such as stem cell, developmental biology and cancer where a small subpopulation of cells could have the biggest impact in decision-making of neighbouring cells. For example, reverse transcription quantitative PCR analysis in single cells allowed Diehn et al., to differentiate a subpopulation of cancer stem cells that are tumour radioresistant with lower reactive oxygen species (ROS) and increased expression of free radical scavenging systems [143].

Ma et al. showed a microfluidic design for a quantitative measurement of complex secreted proteins to assess multiple inflammatory cytokines from human macrophages [41]. In this study, they employed a single cell barcode system to assess the content of heterogeneity functionality in a single cell using a clinical microchip. Each channel of the chip was loaded with either single or a small number of cells. Experiments conducted with the microfluidic system showed heterogeneous functional diversity between two pools (healthy vs. patient). They claimed that this system is a high throughput low cost and portable system, which can be adapted for various fundamental and clinical applications. One can further speculate a derivative of such systems in which phosphorylation events between different pools can be quantified from single cells.

In parallel, developments in nanotechnology will serve the outcome of single cell analysis since it can enable scientists to manipulate and target at a molecular level in a single cell using nano systems, such as nano pocket delivery systems with colloidal chemistry, liposome, micelle encapsulation, dendrimers and carbon nano tubes [144]. More importantly, recent improvements in instrumental technology and material engineering have made

sample analysis possible at the attomole level with nanoliter amounts [25]. The advantage of these systems is the specific targeting and safely delivery of agents. In this way, drug releases can be controlled and patient safety will be increased by reducing side reactions and increasing compliance [145] [146].

Limitations and Challenges:

Although microfluidics applications are rapidly increasing, there are a few issues that need extra consideration in the mechanics and the fundamentals of devices. Most of the possible issues are the result of the wrong channel design (channel wideness, depth, junction points) and valves, which eventually affects flow rates, flow streams, shear stress and evaporation [147, 148]. Optimization of the system for the corresponding sample is challenging, requiring many parameters to be checked and adjusted [20, 149]. Most of the mechanical parts are affiliated with another; the switching speed of the valves affects the efficiency of the chemical mixing and flow continuation [150]. Another important aspect is the preservation of viable cells on the chip which is primarily based on the microfluidic chip material and coatings [151]. Elimination of the waste chemicals or side products is also crucial for cell viability and behaviour; therefore, a cell should be isolated enough to not be affected by another's toxic effect [150]. All these problems have been observed in several studies and various solutions have been utilized [21]. This research indicates that the microfluidic designs can also be challenging for inexperienced communities. Thus, stepwise integration of the Lab-on-a-Chip system into the complex quantitative phosphoproteomics workflows is utterly convenient for the transition process. For phosphoproteomics, one of the first applications of nano/micro fluidic-chip LC devices was used for the separation and sample preparation steps [152, 153].

Different columns systems are easily integrated into micro devices; thus various methods have been applied on one chip. Mass production limits the system-to-system variations and full integration allows automation of the whole process. Samples can be separated and analyzed simultaneously and repetitively without any loss [26] [154]. These systems are designed at micro sizes and require a micro-gram/liter sample. In fact, it is possible to

perform femtomole level quantification when microfluidic systems are combined with a sensitive detection system [79].

Integrated microfluidics has been used for almost twenty years but their adaptation to life sciences has increased dramatically in the last decade due to large-scale applications and mass production [155, 156]. Furthermore microfluidics provide huge advantages for single cell analysis such as scalability, enhancing the concentrations of small volumes and ability of handling and lysing single cells [157]. We could debate that the next step for this technology will be determining phosphorylation regulation at single cell level and a limited number of studies support our argument [158]. However, yet we are far away from global phosphoproteomic analysis in single cells. Improvements in the precision of single cell handling, sample preparation, instrumentation and enhanced sensitivity in phosphopeptide detection will greatly serve to this mission. From the future prospective, this technology will likely be applicable for the diagnosis and treatment of diseases with further advancements in the field of personalized medicine and medical care.

FIGURE LEGENDS

Figure 1. Milestones for proteomics and microfluidics. This figure indicates milestones for microfluidics and proteomics and their intersection times. Proteomics milestones are based on `Proteomics of industrial fungi: trends and insights for biotechnology` de Oliveira et al.[8, 28, 60, 65, 75, 86, 106, 118, 134, 135, 159-171] and microfluidics milestones are based on ` Timing is everything: using fluidics to understand the role of temporal dynamics in cellular systems ` Jovic et al. [8, 102, 171-181]

Figure 2. General phosphoproteomics workflow. This figure illustrates additional steps such as labelling, enrichment and fractionation, to proteomics workflow.

Figure 3. Hypothetical design of a microfluidic chip for quantitative phosphoproteomics. Hypothetical microfluidic chip design is the combination of all essential steps for quantitative phosphoproteomics. Each individual step is performed in the cited studies and their successful implications are discussed in the text. Employing such a microfluidic chip system for a single cell phosphoproteomics experiment will automate the procedure, which will lead to high throughput results.

Figure 4. A schematic illustration of a hypothetical cell surface protein's phosphorylation dynamics upon a stimulus in a single cell versus a mixed cell population. An exemplary graph is shown in the bottom. In a mixed cell population, the quantifying abundance of phosphorylation sites would be inaccurate due to the unsynchronized response of different cells upon a stimulus.

Table1. A review of the potential applications of the current phosphoproteomics methods to microfluidics.

a. Each enrichment method's pros and cons and their compatibility with future adaptations of the microfluidic systems are rated as low, moderate or high with respect to their current trends and applications.

b. Each quantification method's pros and cons and their compatibility to future adaptations of the microfluidic systems are rated as low, moderate or high with respect to their current trends and applications.

Acknowledgements

N. Ozlu is funded by TUBITAK (The Scientific and Technological Research Council of Turkey) 1001, EMBO (European Molecular Biology Organization) Installation Grant, European Union Marie Curie Career Integration Grant. A. N. Polat would like to thank Prof. Dr. Nikolai Kuhnert for his supervision and technical input. The authors would like to thank Dr. Victoria Taylor for careful reading of the manuscript.

REFERENCES

1. J. Reimand, O. Wagih and G. D. Bader, *Sci Rep*, 2013, **3**, 2651.
2. J. Zhang, P. L. Yang and N. S. Gray, *Nat Rev Cancer*, 2009, **9**, 28-39.
3. N. Ozlu, B. Akten, W. Timm, N. Haseley, H. Steen and J. A. Steen, *Wiley Interdiscip Rev Syst Biol Med*, 2010, **2**, 255-276.
4. A. A. Turanov, A. V. Lobanov, D. E. Fomenko, H. G. Morrison, M. L. Sogin, L. A. Klobutcher, D. L. Hatfield and V. N. Gladyshev, *Science*, 2009, **323**, 259-261.
5. M. Mann and O. N. Jensen, *Nat Biotechnol*, 2003, **21**, 255-261.
6. J. D. Watson, *Genome Research*, 2001, **11**, 1803-1804.
7. J. C. Venter, M. D. Adams, E. W. Myers, P. W. Li, R. J. Mural, G. G. Sutton, H. O. Smith, M. Yandell, C. A. Evans, R. A. Holt, J. D. Gocayne, P. Amanatides, R. M. Ballew, D. H. Huson, J. R. Wortman, Q. Zhang, C. D. Kodira, X. H. Zheng, L. Chen, M. Skupski, G. Subramanian, P. D. Thomas, J. Zhang, G. L. Gabor Miklos, C. Nelson, S. Broder, A. G. Clark, J. Nadeau, V. A. McKusick, N. Zinder, A. J. Levine, R. J. Roberts, M. Simon, C. Slayman, M. Hunkapiller, R. Bolanos, A. Delcher, I. Dew, D. Fasulo, M. Flanigan, L. Florea, A. Halpern, S. Hannenhalli, S. Kravitz, S. Levy, C. Mobarry, K. Reinert, K. Remington, J. Abu-Threideh, E. Beasley, K. Biddick, V. Bonazzi, R. Brandon, M. Cargill, I. Chandramouliswaran, R. Charlab, K. Chaturvedi, Z. Deng, V. Di Francesco, P. Dunn, K. Eilbeck, C. Evangelista, A. E. Gabrielian, W. Gan, W. Ge, F. Gong, Z. Gu, P. Guan, T. J. Heiman, M. E. Higgins, R. R. Ji, Z. Ke, K. A. Ketchum, Z. Lai, Y. Lei, Z. Li, J. Li, Y. Liang, X. Lin, F. Lu, G. V. Merkulov, N. Milshina, H. M. Moore, A. K. Naik, V. A. Narayan, B. Neelam, D. Nusskern, D. B. Rusch, S.

Salzberg, W. Shao, B. Shue, J. Sun, Z. Wang, A. Wang, X. Wang, J. Wang, M. Wei, R. Wides, C. Xiao, C. Yan, A. Yao, J. Ye, M. Zhan, W. Zhang, H. Zhang, Q. Zhao, L. Zheng, F. Zhong, W. Zhong, S. Zhu, S. Zhao, D. Gilbert, S. Baumhueter, G. Spier, C. Carter, A. Cravchik, T. Woodage, F. Ali, H. An, A. Awe, D. Baldwin, H. Baden, M. Barnstead, I. Barrow, K. Beeson, D. Busam, A. Carver, A. Center, M. L. Cheng, L. Curry, S. Danaher, L. Davenport, R. Desilets, S. Dietz, K. Dodson, L. Doup, S. Ferriera, N. Garg, A. Gluecksmann, B. Hart, J. Haynes, C. Haynes, C. Heiner, S. Hladun, D. Hostin, J. Houck, T. Howland, C. Ibegwam, J. Johnson, F. Kalush, L. Kline, S. Koduru, A. Love, F. Mann, D. May, S. McCawley, T. McIntosh, I. McMullen, M. Moy, L. Moy, B. Murphy, K. Nelson, C. Pfannkoch, E. Pratts, V. Puri, H. Qureshi, M. Reardon, R. Rodriguez, Y. H. Rogers, D. Romblad, B. Ruhfel, R. Scott, C. Sitter, M. Smallwood, E. Stewart, R. Strong, E. Suh, R. Thomas, N. N. Tint, S. Tse, C. Vech, G. Wang, J. Wetter, S. Williams, M. Williams, S. Windsor, E. Winn-Deen, K. Wolfe, J. Zaveri, K. Zaveri, J. F. Abril, R. Guigo, M. J. Campbell, K. V. Sjolander, B. Karlak, A. Kejariwal, H. Mi, B. Lazareva, T. Hatton, A. Narechania, K. Diemer, A. Muruganujan, N. Guo, S. Sato, V. Bafna, S. Istrail, R. Lippert, R. Schwartz, B. Walenz, S. Yooseph, D. Allen, A. Basu, J. Baxendale, L. Blick, M. Caminha, J. Carnes-Stine, P. Caulk, Y. H. Chiang, M. Coyne, C. Dahlke, A. Mays, M. Dombroski, M. Donnelly, D. Ely, S. Esparham, C. Fosler, H. Gire, S. Glanowski, K. Glasser, A. Glodek, M. Gorokhov, K. Graham, B. Gropman, M. Harris, J. Heil, S. Henderson, J. Hoover, D. Jennings, C. Jordan, J. Jordan, J. Kasha, L. Kagan, C. Kraft, A. Levitsky, M. Lewis, X. Liu, J. Lopez, D. Ma, W. Majoros, J. McDaniel, S. Murphy, M. Newman, T. Nguyen, N. Nguyen, M. Nodell, S. Pan, J. Peck, M. Peterson, W. Rowe, R. Sanders, J. Scott, M. Simpson, T. Smith, A. Sprague, T. Stockwell, R. Turner, E. Venter, M. Wang, M. Wen, D. Wu, M. Wu, A. Xia, A. Zandieh and X. Zhu, *Science*, 2001, **291**, 1304-1351.

8. P. Edman, *Archives of Biochemistry*, 1949, **22**, 475-476.
9. G. A. Khoury, R. C. Baliban and C. A. Floudas, *Sci. Rep.*, 2011, **1**.
10. R. Aebersold and M. Mann, *Nature*, 2003, **422**, 198-207.
11. H. R. Matthews, *Pharmacol Ther*, 1995, **67**, 323-350.
12. P. V. Attwood, M. J. Piggott, X. L. Zu and P. G. Besant, *Amino Acids*, 2007, **32**, 145-156.
13. X. L. Zu, P. G. Besant, A. Imhof and P. V. Attwood, *Amino Acids*, 2007, **32**, 347-357.
14. J. Fuhrmann, B. Mierzwa, Débora B. Trentini, S. Spiess, A. Lehner, E. Charpentier and T. Clausen, *Cell Reports*, 2013, **3**, 1832-1839.
15. J. D. Graves and E. G. Krebs, *Pharmacol Ther*, 1999, **82**, 111-121.
16. J. R. Yates, C. I. Ruse and A. Nakorchevsky, *Annu Rev Biomed Eng*, 2009, **11**, 49-79.
17. B. Domon and R. Aebersold, *Nat Biotechnol*, 2010, **28**, 710-721.
18. T. E. Thingholm, O. N. Jensen and M. R. Larsen, *Proteomics*, 2009, **9**, 1451-1468.
19. H. Andersson and A. van den Berg, *Curr Opin Biotechnol*, 2004, **15**, 44-49.
20. G. M. Walker, H. C. Zeringue and D. J. Beebe, *Lab Chip*, 2004, **4**, 91-97.
21. P. J. Hung, P. J. Lee, P. Sabounchi, N. Aghdam, R. Lin and L. P. Lee, *Lab Chip*, 2005, **5**, 44-48.
22. M. L. Kovarik and S. C. Jacobson, *Anal Chem*, 2009, **81**, 7133-7140.
23. D. Reichmuth, T. Shepodd and B. Kirby, *Analytical Chemistry*, 2005, **77**, 2997-3000.
24. N. Yin, K. Killeen, R. Brennen, D. Sobek, M. Werlich and T. van de Goor, *Analytical Chemistry*, 2005, **77**, 527-533.
25. J. Hardouin, R. Joubert-Caron and M. Caron, *J Sep Sci*, 2007, **30**, 1482-1487.
26. H. Yin and K. Killeen, *J Sep Sci*, 2007, **30**, 1427-1434.
27. D. Gao, H. Liu, Y. Jiang and J.-M. Lin, *Lab on a Chip*, 2013, **13**, 3309-3322.
28. X. Feng, B. F. Liu, J. Li and X. Liu, *Mass Spectrom Rev*, 2014.
29. M. Waas, S. Bhattacharya, S. Chuppa, X. Wu, D. R. Jensen, U. Omasits, B. Wollscheid, B. F. Volkman, K. R. Noon and R. L. Gundry, *Anal Chem*, 2014, **86**, 1551-1559.
30. N. Ozlu, M. Kirchner and J. J. Steen, *Methods Mol Biol*, 2012, **795**, 217-231.

31. J. R. Wisniewski, A. Zougman, N. Nagaraj and M. Mann, *Nature methods*, 2009, **6**, 359-362.
32. J. V. Olsen, J. C. Schwartz, J. Griep-Raming, M. L. Nielsen, E. Damoc, E. Denisov, O. Lange, P. Remes, D. Taylor, M. Splendore, E. R. Wouters, M. Senko, A. Makarov, M. Mann and S. Horning, *Mol Cell Proteomics*, 2009, **8**, 2759-2769.
33. P. J. Boersema, S. Mohammed and A. J. Heck, *J Mass Spectrom*, 2009, **44**, 861-878.
34. N. Taouatas, A. F. Altelaar, M. M. Drugan, A. O. Helbig, S. Mohammed and A. J. Heck, *Mol Cell Proteomics*, 2009, **8**, 190-200.
35. N. Taouatas, A. J. Heck and S. Mohammed, *J Proteome Res*, 2010, **9**, 4282-4288.
36. N. Taouatas, M. M. Drugan, A. J. Heck and S. Mohammed, *Nat Methods*, 2008, **5**, 405-407.
37. S. Gauci, A. O. Helbig, M. Slijper, J. Krijgsveld, A. J. Heck and S. Mohammed, *Anal Chem*, 2009, **81**, 4493-4501.
38. Y. Marcy, C. Ouverney, E. M. Bik, T. Losekann, N. Ivanova, H. G. Martin, E. Szeto, D. Platt, P. Hugenholtz, D. A. Relman and S. R. Quake, *Proc Natl Acad Sci U S A*, 2007, **104**, 11889-11894.
39. J. J. Agresti, E. Antipov, A. R. Abate, K. Ahn, A. C. Rowat, J. C. Baret, M. Marquez, A. M. Klibanov, A. D. Griffiths and D. A. Weitz, *Proc Natl Acad Sci U S A*, 2010, **107**, 4004-4009.
40. C. P. Jen, J. H. Hsiao and N. A. Maslov, *Sensors (Basel)*, 2012, **12**, 347-358.
41. C. Ma, R. Fan, H. Ahmad, Q. Shi, B. Comin-Anduix, T. Chodon, R. C. Koya, C. C. Liu, G. A. Kwong, C. G. Radu, A. Ribas and J. R. Heath, *Nat Med*, 2011, **17**, 738-743.
42. U. Vermesh, O. Vermesh, J. Wang, G. A. Kwong, C. Ma, K. Hwang and J. R. Heath, *Angew Chem Int Ed Engl*, 2011, **50**, 7378-7380.
43. R. S. Annan and S. A. Carr, *Anal Chem*, 1996, **68**, 3413-3421.
44. S. A. Beausoleil, M. Jedrychowski, D. Schwartz, J. E. Elias, J. Villén, J. Li, M. A. Cohn, L. C. Cantley and S. P. Gygi, *Proc Natl Acad Sci U S A*, 2004, **101**, 12130-12135.
45. P. Roepstorff and J. Fohlman, *Biomed Mass Spectrom*, 1984, **11**, 601.
46. B. Paizs and S. Suhai, *Mass Spectrom Rev*, 2005, **24**, 508-548.
47. A. Makarov, E. Denisov, A. Kholomeev, W. Balschun, O. Lange, K. Strupat and S. Horning, *Anal Chem*, 2006, **78**, 2113-2120.
48. J. Wu, P. Warren, Q. Shakey, E. Sousa, A. Hill, T. E. Ryan and T. He, *Proteomics*, 2010, **10**, 2224-2234.
49. K. Biemann, *Methods Enzymol*, 1990, **193**, 886-887.
50. H. Marx, S. Lemeer, J. E. Schliep, L. Matheron, S. Mohammed, J. Cox, M. Mann, A. J. Heck and B. Kuster, *Nat Biotechnol*, 2013, **31**, 557-564.
51. S. Di Palma, M. L. Hennrich, A. J. Heck and S. Mohammed, *J Proteomics*, 2012, **75**, 3791-3813.
52. S. Loroch, *Electrophoresis*, 2013, **34**, 1483–1492.
53. H. Zhou, S. Di Palma, C. Preisinger, M. Peng, A. N. Polat, A. J. Heck and S. Mohammed, *J Proteome Res*, 2013, **12**, 260-271.
54. D. Winter, J. Seidler, Y. Ziv, Y. Shiloh and W. D. Lehmann, *J Proteome Res*, 2009, **8**, 418-424.
55. S. B. Ficarro, Y. Zhang, M. J. Carrasco-Alfonso, B. Garg, G. Adelmant, J. T. Webber, C. J. Luckey and J. A. Marto, *Mol Cell Proteomics*, 2011, **10**, O111.011064.
56. H. Hahne, F. Pachl, B. Ruprecht, S. K. Maier, S. Klaeger, D. Helm, G. Medard, M. Wilm, S. Lemeer and B. Kuster, *Nat Methods*, 2013, **10**, 989-991.
57. M. Gilar, P. Olivova, A. E. Daly and J. C. Gebler, *J Sep Sci*, 2005, **28**, 1694-1703.
58. F. Zhou, T. W. Sikorski, S. B. Ficarro, J. T. Webber and J. A. Marto, *Anal Chem*, 2011, **83**, 6996-7005.
59. M. L. Hennrich, V. Groenewold, G. J. Kops, A. J. Heck and S. Mohammed, *Anal Chem*, 2011, **83**, 7137-7143.
60. R. Ishibashi, K. Mawatari and T. Kitamori, *J Chromatogr A*, 2012, **1238**, 152-155.
61. J. Huft, C. A. Haynes and C. L. Hansen, *Anal Chem*, 2013, **85**, 2999-3005.

62. F. Wang, C. Song, K. Cheng, X. Jiang, M. Ye and H. Zou, *Anal Chem*, 2011, **83**, 8078-8085.
63. A. M. Palumbo, S. A. Smith, C. L. Kalcic, M. Dantus, P. M. Stemmer and G. E. Reid, *Mass Spectrom Rev*, 2011, **30**, 600-625.
64. M. W. Pinkse, P. M. Uitto, M. J. Hilhorst, B. Ooms and A. J. Heck, *Anal Chem*, 2004, **76**, 3935-3943.
65. S. B. Ficarro, M. L. McCleland, P. T. Stukenberg, D. J. Burke, M. M. Ross, J. Shabanowitz, D. F. Hunt and F. M. White, *Nat Biotechnol*, 2002, **20**, 301-305.
66. J. Rush, A. Moritz, K. A. Lee, A. Guo, V. L. Goss, E. J. Spek, H. Zhang, X. M. Zha, R. D. Polakiewicz and M. J. Comb, *Nat Biotechnol*, 2005, **23**, 94-101.
67. R. C. Cheung, J. H. Wong and T. B. Ng, *Appl Microbiol Biotechnol*, 2012, **96**, 1411-1420.
68. T. S. Nühse, A. Stensballe, O. N. Jensen and S. C. Peck, *Mol Cell Proteomics*, 2003, **2**, 1234-1243.
69. J. Villén and S. P. Gygi, *Nat Protoc*, 2008, **3**, 1630-1638.
70. M. Kokubu, Y. Ishihama, T. Sato, T. Nagasu and Y. Oda, *Anal Chem*, 2005, **77**, 5144-5154.
71. C. F. Xu, Y. Lu, J. Ma, M. Mohammadi and T. A. Neubert, *Mol Cell Proteomics*, 2005, **4**, 809-818.
72. P. Connor and A. McQuillan, *Langmuir*, 1999, **15**, 2916-2921.
73. H. Zhou, S. Di Palma, C. Preisinger, M. Peng, A. N. Polat, A. J. R. Heck and S. Mohammed, *Journal of Proteome Research*, 2012, **12**, 260-271.
74. M. W. Pinkse, S. Mohammed, J. W. Gouw, B. van Breukelen, H. R. Vos and A. J. Heck, *J Proteome Res*, 2008, **7**, 687-697.
75. M. R. Larsen, T. E. Thingholm, O. N. Jensen, P. Roepstorff and T. J. Jørgensen, *Mol Cell Proteomics*, 2005, **4**, 873-886.
76. T. E. Thingholm, O. N. Jensen, P. J. Robinson and M. R. Larsen, *Mol Cell Proteomics*, 2008, **7**, 661-671.
77. A. N. Polat, K. Kraiczek, A. J. Heck, R. Raijmakers and S. Mohammed, *Anal Bioanal Chem*, 2012, **404**, 2507-2512.
78. R. Raijmakers, C. R. Berkers, A. de Jong, H. Ovaa, A. J. Heck and S. Mohammed, *Mol Cell Proteomics*, 2008, **7**, 1755-1762.
79. R. Raijmakers, A. J. Heck and S. Mohammed, *Mol Biosyst*, 2009, **5**, 992-1003.
80. L. Beltran and P. R. Cutillas, *Amino Acids*, 2012, **43**, 1009-1024.
81. K. Engholm-Keller, P. Birck, J. Størling, F. Pociot, T. Mandrup-Poulsen and M. R. Larsen, *J Proteomics*, 2012, **75**, 5749-5761.
82. J. Pan, B. Naumann-Busch, L. Wang, M. Specht, M. Scholz, K. Trompelt and M. Hippler, *J Proteome Res*, 2011, **10**, 3830-3839.
83. T. Verano-Braga, V. Schwammle, M. Sylvester, D. G. Passos-Silva, A. A. Peluso, G. M. Etelvino, R. A. Santos and P. Roepstorff, *J Proteome Res*, 2012, **11**, 3370-3381.
84. J. V. Olsen, B. Blagoev, F. Gnad, B. Macek, C. Kumar, P. Mortensen and M. Mann, *Cell*, 2006, **127**, 635-648.
85. P. J. Boersema, L. Y. Foong, V. M. Ding, S. Lemeer, B. van Breukelen, R. Philp, J. Boekhorst, B. Snel, J. den Hertog, A. B. Choo and A. J. Heck, *Mol Cell Proteomics*, 2010, **9**, 84-99.
86. S. A. Gerber, J. Rush, O. Stemman, M. W. Kirschner and S. P. Gygi, *Proc Natl Acad Sci U S A*, 2003, **100**, 6940-6945.
87. N. Ibarrola, D. E. Kalume, M. Gronborg, A. Iwahori and A. Pandey, *Anal Chem*, 2003, **75**, 6043-6049.
88. G. Zhang and T. A. Neubert, *Methods Mol Biol*, 2009, **527**, 79-92, xi.
89. B. Blagoev, S. E. Ong, I. Kratchmarova and M. Mann, *Nat Biotechnol*, 2004, **22**, 1139-1145.
90. A. D. Zoumaro-Djayoon, A. J. Heck and J. Muñoz, *Methods*, 2012, **56**, 268-274.
91. M. Tinti, A. P. Nardozza, E. Ferrari, F. Sacco, S. Corallino, L. Castagnoli and G. Cesareni, *N Biotechnol*, 2012, **29**, 571-577.

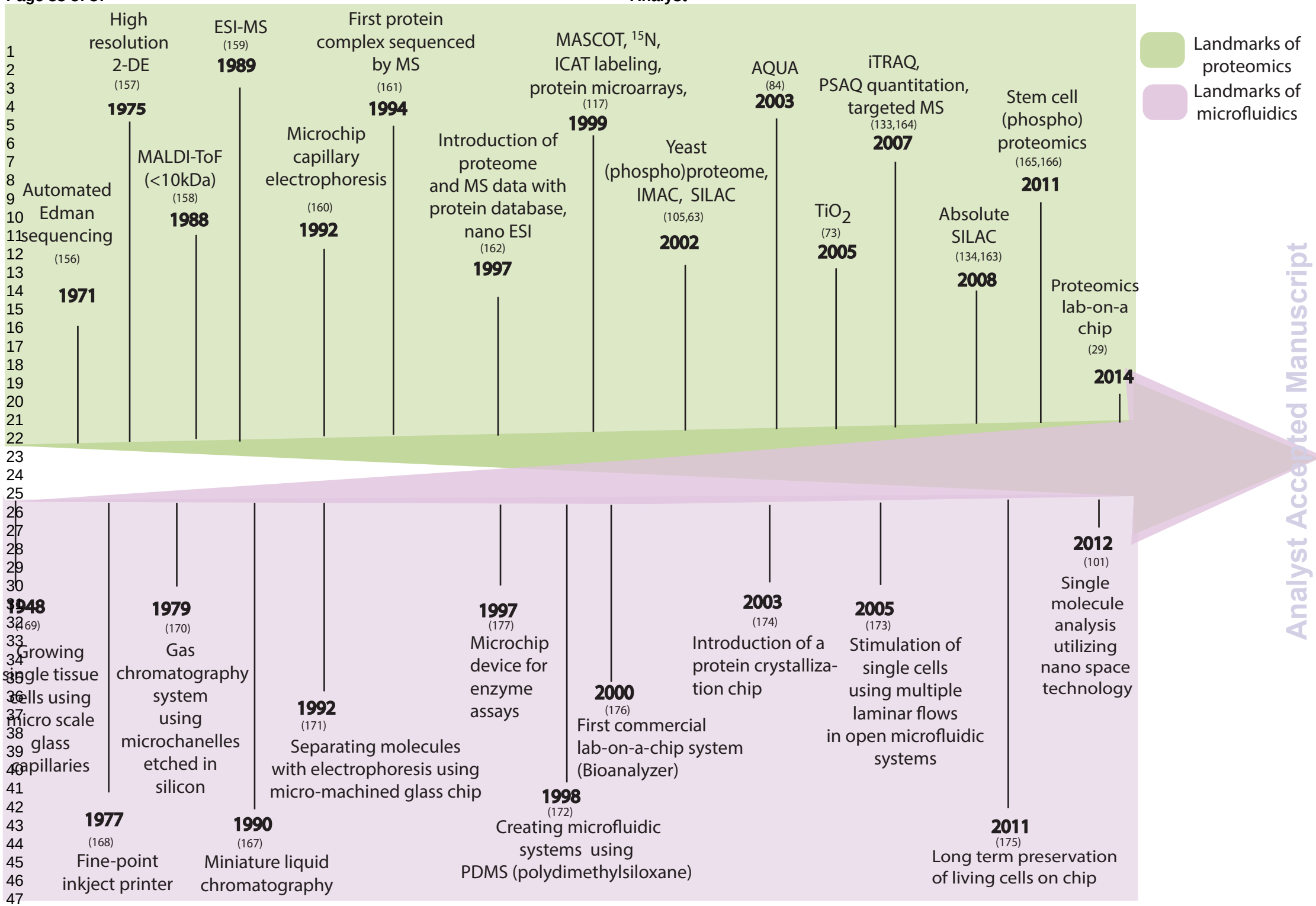
92. H. Zhou, M. Ye, J. Dong, E. Corradini, A. Cristobal, A. J. Heck, H. Zou and S. Mohammed, *Nat Protoc*, 2013, **8**, 461-480.
93. J. Ugelstad, P. C. Mórk, K. H. Kaggerud, T. Ellingsen and A. Berge, *Advances in Colloid and Interface Science*, 1980, **13**, 101-140.
94. R. Raijmakers, C. Berkers, A. de Jong, H. Ovaa, A. Heck and S. Mohammed, *Molecular & Cellular Proteomics*, 2008, **7**, 1755-1762.
95. R. Raijmakers, K. Kraiczek, A. de Jong, S. Mohammed and A. Heck, *Analytical Chemistry*, 2010, **82**, 824-832.
96. O. B. Bleijerveld, P. Wijten, S. Cappadona, E. A. McClellan, A. N. Polat, R. Raijmakers, J. W. Sels, L. Colle, S. Grasso, H. W. van den Toorn, B. van Breukelen, A. Stubbs, G. Pasterkamp, A. J. Heck, I. E. Hoefer and A. Scholten, *J Proteome Res*, 2012, **11**, 5235-5244.
97. A. Le Nel, J. Krenkova, K. Kleparnik, C. Smadja, M. Taverna, J. L. Viovy and F. Foret, *Electrophoresis*, 2008, **29**, 4944-4947.
98. L. Matheron, S. Clavier, O. Diebate, P. Karoyan, G. Bolbach, D. Guianvarc'h and E. Sachon, *J Am Soc Mass Spectrom*, 2012, **23**, 1981-1990.
99. D. T. McLachlin and B. T. Chait, *Anal Chem*, 2003, **75**, 6826-6836.
100. H. Nika, E. Nieves, D. H. Hawke and R. H. Angeletti, *J Biomol Tech*, 2013, **24**, 132-153.
101. K. R. Willison and D. R. Klug, *Curr Opin Biotechnol*, 2013, **24**, 745-751.
102. Y. Xu, K. Jang, T. Yamashita, Y. Tanaka, K. Mawatari and T. Kitamori, *Anal Bioanal Chem*, 2012, **402**, 99-107.
103. M. Bantscheff, M. Schirle, G. Sweetman, J. Rick and B. Kuster, *Anal Bioanal Chem*, 2007, **389**, 1017-1031.
104. J. Yates, C. Ruse and A. Nakorchevsky, *Annual Review of Biomedical Engineering*, 2009, **11**, 49-79.
105. M. Bantscheff, S. Lemeer, M. M. Savitski and B. Kuster, *Anal Bioanal Chem*, 2012, **404**, 939-965.
106. S. E. Ong, B. Blagoev, I. Kratchmarova, D. B. Kristensen, H. Steen, A. Pandey and M. Mann, *Mol Cell Proteomics*, 2002, **1**, 376-386.
107. D. B. McClatchy and J. R. Yates, 3rd, *CSH Protoc*, 2008, **2008**, pdb.prot4940.
108. N. Rauniyar, D. B. McClatchy and J. R. Yates, 3rd, *Methods*, 2013, **61**, 260-268.
109. D. Van Hoof, M. W. Pinkse, D. W. Oostwaard, C. L. Mummery, A. J. Heck and J. Krijgsveld, in *Nat Methods*, United States, 2007, vol. 4, pp. 677-678.
110. S. E. Ong, I. Kratchmarova and M. Mann, *J Proteome Res*, 2003, **2**, 173-181.
111. M. Mann, *Nat Rev Mol Cell Biol*, 2006, **7**, 952-958.
112. S. E. Ong, G. Mittler and M. Mann, *Nat Methods*, 2004, **1**, 119-126.
113. M. Kruger, M. Moser, S. Ussar, I. Thievessen, C. A. Luber, F. Forner, S. Schmidt, S. Zanivan, R. Fassler and M. Mann, *Cell*, 2008, **134**, 353-364.
114. S. E. Ong and M. Mann, *Nat Chem Biol*, 2005, **1**, 252-262.
115. P. L. Ross, Y. N. Huang, J. N. Marchese, B. Williamson, K. Parker, S. Hattan, N. Khainovski, S. Pillai, S. Dey, S. Daniels, S. Purkayastha, P. Juhasz, S. Martin, M. Bartlet-Jones, F. He, A. Jacobson and D. J. Pappin, *Mol Cell Proteomics*, 2004, **3**, 1154-1169.
116. A. Thompson, J. Schäfer, K. Kuhn, S. Kienle, J. Schwarz, G. Schmidt, T. Neumann, R. Johnstone, A. K. Mohammed and C. Hamon, *Anal Chem*, 2003, **75**, 1895-1904.
117. L. Dayon, A. Hainard, V. Licker, N. Turck, K. Kuhn, D. F. Hochstrasser, P. R. Burkhard and J. C. Sanchez, *Anal Chem*, 2008, **80**, 2921-2931.
118. S. P. Gygi, B. Rist, S. A. Gerber, F. Turecek, M. H. Gelb and R. Aebersold, *Nat Biotechnol*, 1999, **17**, 994-999.
119. J.-L. Hsu, S.-Y. Huang, N.-H. Chow and S.-H. Chen, *Analytical Chemistry*, 2003, **75**, 6843-6852.
120. J. L. Hsu, S. Y. Huang and S. H. Chen, *Electrophoresis*, 2006, **27**, 3652-3660.

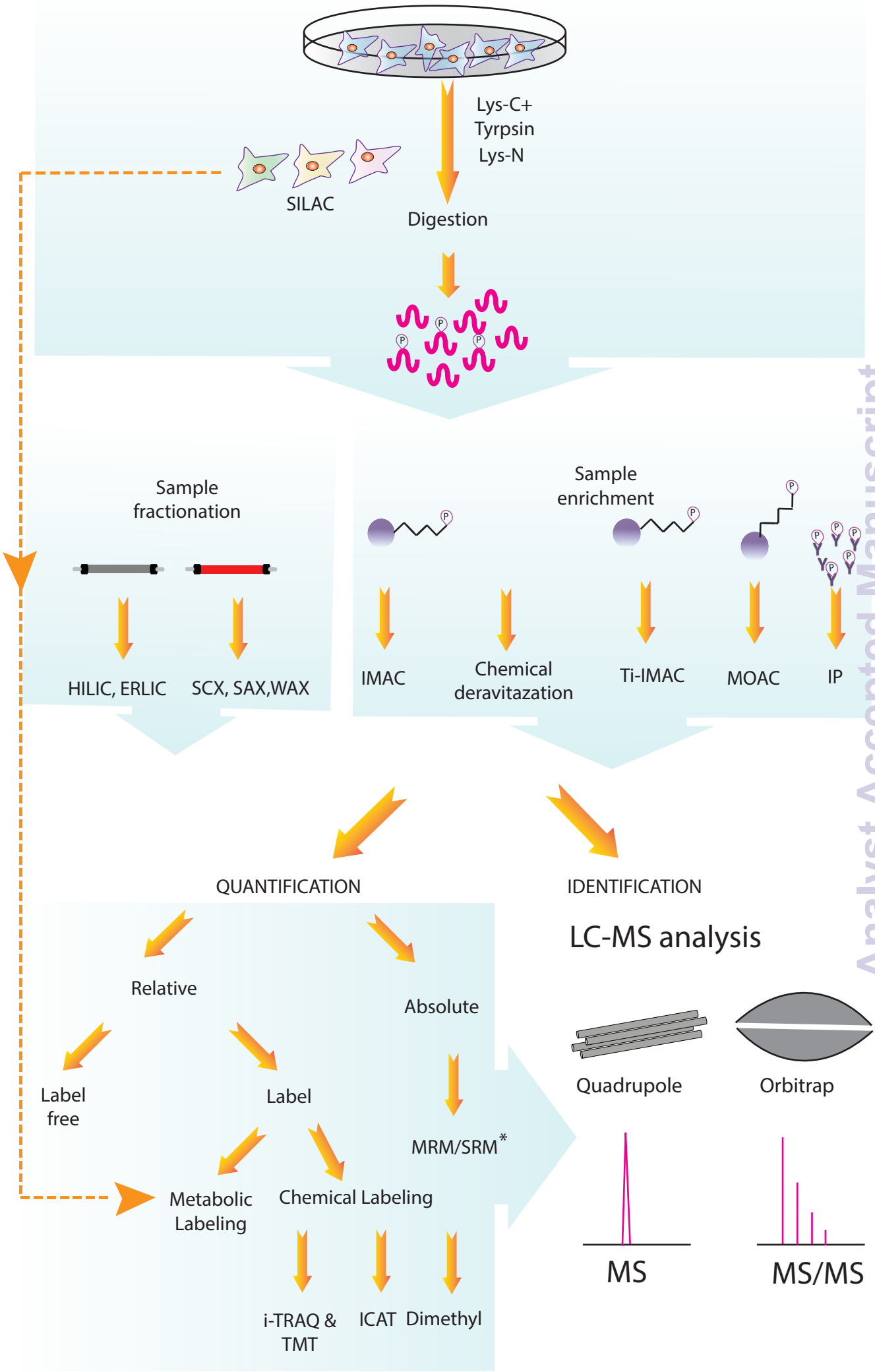
121. P. J. Boersema, R. Raijmakers, S. Lemeer, S. Mohammed and A. J. Heck, *Nat Protoc*, 2009, **4**, 484-494.
122. S. Di Palma, R. Raijmakers, A. J. Heck and S. Mohammed, *Anal Chem*, 2011, **83**, 8352-8356.
123. A. F. Altelaar, C. K. Frese, C. Preisinger, M. L. Hennrich, A. W. Schram, H. T. Timmers, A. J. Heck and S. Mohammed, *J Proteomics*, 2013, **88**, 14-26.
124. R. Zhang, C. S. Sioma, R. A. Thompson, L. Xiong and F. E. Regnier, *Anal Chem*, 2002, **74**, 3662-3669.
125. P. J. Boersema, R. Raijmakers, S. Lemeer, S. Mohammed and A. J. R. Heck, *Nature Protocols*, 2009, **4**, 484-494.
126. W. M. Old, K. Meyer-Arendt, L. Aveline-Wolf, K. G. Pierce, A. Mendoza, J. R. Sevinsky, K. A. Resing and N. G. Ahn, *Molecular & Cellular Proteomics*, 2005, **4**, 1487-1502.
127. J. M. Asara, H. R. Christofk, L. M. Freimark and L. C. Cantley, *Proteomics*, 2008, **8**, 994-999.
128. N. M. Griffin, J. Yu, F. Long, P. Oh, S. Shore, Y. Li, J. A. Koziol and J. E. Schnitzer, *Nat Biotechnol*, 2010, **28**, 83-89.
129. Y. Zhang, Z. Wen, M. P. Washburn and L. Florens, *Anal Chem*, 2009, **81**, 6317-6326.
130. H. Choi, D. Fermin and A. I. Nesvizhskii, *Molecular & Cellular Proteomics*, 2008, **7**, 2373-2385.
131. D. A. Megger, L. L. Pott, M. Ahrens, J. Padden, T. Bracht, K. Kuhlmann, M. Eisenacher, H. E. Meyer and B. Sitek, *Biochim Biophys Acta*, 2013.
132. D. S. Kirkpatrick, S. A. Gerber and S. P. Gygi, *Methods*, 2005, **35**, 265-273.
133. R. J. Beynon, M. K. Doherty, J. M. Pratt and S. J. Gaskell, *Nat Methods*, 2005, **2**, 587-589.
134. V. Brun, A. Dupuis, A. Adrait, M. Marcellin, D. Thomas, M. Court, F. Vandenesch and J. Garin, *Molecular & Cellular Proteomics*, 2007, **6**, 2139-2149.
135. S. Hanke, H. Besir, D. Oesterhelt and M. Mann, *J Proteome Res*, 2008, **7**, 1118-1130.
136. S. Singh, M. Springer, J. Steen, M. W. Kirschner and H. Steen, *J Proteome Res*, 2009, **8**, 2201-2210.
137. C.-Y. Chang, P. Picotti, R. Hüttenhain, V. Heinzelmann-Schwarz, M. Jovanovic, R. Aebersold and O. Vitek, *Molecular & Cellular Proteomics*, 2012, **11**.
138. R. Aebersold, A. L. Burlingame and R. A. Bradshaw, in *Mol Cell Proteomics*, United States, 2013, vol. 12, pp. 2381-2382.
139. M. Jovanovic, L. Reiter, A. Clark, M. Weiss, P. Picotti, H. Rehrauer, A. Frei, L. J. Neukomm, E. Kaufman, B. Wollscheid, M. J. Simard, E. A. Miska, R. Aebersold, A. P. Gerber and M. O. Hengartner, *Genome Research*, 2012, **22**, 1360-1371.
140. R. Raijmakers, A. Heck and S. Mohammed, *Molecular Biosystems*, 2009, **5**, 992-1003.
141. J. E. Purvis and G. Lahav, *Cell*, 2013, **152**, 945-956.
142. D. Bakstad, A. Adamson, D. G. Spiller and M. R. White, *Curr Opin Biotechnol*, 2012, **23**, 103-109.
143. M. Diehn, R. W. Cho, N. A. Lobo, T. Kalisky, M. J. Dorie, A. N. Kulp, D. Qian, J. S. Lam, L. E. Ailles, M. Wong, B. Joshua, M. J. Kaplan, I. Wapnir, F. M. Dirbas, G. Somlo, C. Garberoglio, B. Paz, J. Shen, S. K. Lau, S. R. Quake, J. M. Brown, I. L. Weissman and M. F. Clarke, *Nature*, 2009, **458**, 780-783.
144. X. Ma, L. H. Zhang, L. R. Wang, X. Xue, J. H. Sun, Y. Wu, G. Zou, X. Wu, P. C. Wang, W. G. Wamer, J. J. Yin, K. Zheng and X. J. Liang, *ACS Nano*, 2012, **6**, 10486-10496.
145. D. Majumdar, K. H. Jung, H. Zhang, S. Nannapaneni, X. Wang, A. R. Amin, Z. Chen, Z. G. Chen and D. M. Shin, *Cancer Prev Res (Phila)*, 2014, **7**, 65-73.
146. E. K. Chow and D. Ho, *Sci Transl Med*, 2013, **5**, 216rv214.
147. H. Lu, L. Y. Koo, W. M. Wang, D. A. Lauffenburger, L. G. Griffith and K. F. Jensen, *Anal Chem*, 2004, **76**, 5257-5264.
148. J. H. Kang, Y. C. Kim and J. K. Park, *Lab Chip*, 2008, **8**, 176-178.
149. P. J. Hung, P. J. Lee, P. Sabounchi, R. Lin and L. P. Lee, *Biotechnol Bioeng*, 2005, **89**, 1-8.

150. K. R. King, S. Wang, A. Jayaraman, M. L. Yarmush and M. Toner, *Lab Chip*, 2008, **8**, 107-116.
151. R. Gomez-Sjoberg, A. A. Leyrat, D. M. Pirone, C. S. Chen and S. R. Quake, *Anal Chem*, 2007, **79**, 8557-8563.
152. S. Mohammed, K. Kraiczek, M. Pinkse, S. Lemeer, J. Benschop and A. Heck, *Journal of Proteome Research*, 2008, **7**, 1565-1571.
153. J. Srbek, J. Eickhoff, U. Effelsberg, K. Kraiczek, T. van de Goor and P. Coufal, *Journal of Separation Science*, 2007, **30**, 2046-2052.
154. C. Zhao, Z. Wu, G. Xue, J. Wang, Y. Zhao, Z. Xu, D. Lin, G. Herbert, Y. Chang, K. Cai and G. Xu, *Journal of Chromatography A*, 2011, **1218**, 3669-3674.
155. A. Manz, N. Graber and H. M. Widmer, *Sensors and Actuators B: Chemical*, 1990, **1**, 244-248.
156. T. Thorsen, S. J. Maerkl and S. R. Quake, *Science*, 2002, **298**, 580-584.
157. V. Lecault, A. K. White, A. Singhal and C. L. Hansen, *Curr Opin Chem Biol*, 2012, **16**, 381-390.
158. W. Wei, Y. S. Shin, C. Ma, J. Wang, M. Elitas, R. Fan and J. R. Heath, *Genome Med*, 2013, **5**, 75.
159. J. M. de Oliveira and L. H. de Graaff, *Appl Microbiol Biotechnol*, 2011, **89**, 225-237.
160. R. A. Laursen, *Eur J Biochem*, 1971, **20**, 89-102.
161. P. H. O'Farrell, *J Biol Chem*, 1975, **250**, 4007-4021.
162. M. Karas and F. Hillenkamp, *Anal Chem*, 1988, **60**, 2299-2301.
163. J. B. Fenn, M. Mann, C. K. Meng, S. F. Wong and C. M. Whitehouse, *Science*, 1989, **246**, 64-71.
164. A. Manz, D. J. Harrison, E. M. J. Verpoorte, J. C. Fettinger, A. Paulus, H. Lüdi and H. M. Widmer, *Journal of Chromatography A*, 1992, **593**, 253-258.
165. M. Mann and M. Wilm, *Analytical Chemistry*, 1994, **66**, 4390-4399.
166. J. A. Loo, *Mass Spectrom Rev*, 1997, **16**, 1-23.
167. M. Bantscheff, M. Boesche, D. Eberhard, T. Matthieson, G. Sweetman and B. Kuster, *Mol Cell Proteomics*, 2008, **7**, 1702-1713.
168. S. Wiese, K. A. Reidegeld, H. E. Meyer and B. Warscheid, *Proteomics*, 2007, **7**, 340-350.
169. S. Di Palma, D. Stange, M. van de Wetering, H. Clevers, A. J. Heck and S. Mohammed, *J Proteome Res*, 2011, **10**, 3814-3819.
170. K. T. Rigbolt, T. A. Prokhorova, V. Akimov, J. Henningsen, P. T. Johansen, I. Kratchmarova, M. Kassem, M. Mann, J. V. Olsen and B. Blagoev, *Sci Signal*, 2011, **4**, rs3.
171. D. Ishii and T. Takeuchi, *TrAC Trends in Analytical Chemistry*, 1990, **9**, 152-157.
172. A. Jovic, B. Howell and S. Takayama, *Microfluidics and Nanofluidics*, 2009, **6**, 717-729.
173. K. K. Sanford, W. R. Earle and G. D. Likely, *J Natl Cancer Inst*, 1948, **9**, 229-246.
174. S. C. Terry, J. H. Jerman and J. B. Angell, *Electron Devices, IEEE Transactions on*, 1979, **26**, 1880-1886.
175. D. J. Harrison, A. Manz, Z. Fan, H. Luedi and H. M. Widmer, *Analytical Chemistry*, 1992, **64**, 1926-1932.
176. D. C. Duffy, J. C. McDonald, O. J. A. Schueller and G. M. Whitesides, *Analytical Chemistry*, 1998, **70**, 4974-4984.
177. J. Olofsson, H. Bridle, J. Sinclair, D. Granfeldt, E. Sahlin and O. Orwar, *Proc Natl Acad Sci U S A*, 2005, **102**, 8097-8102.
178. Y. Lee, E. K. Lee, Y. W. Cho, T. Matsui, I. C. Kang, T. S. Kim and M. H. Han, *Proteomics*, 2003, **3**, 2289-2304.
179. J. Skommer, D. McGuinness and D. Wlodkowic, *Biotech Histochem*, 2011, **86**, 174-180.
180. N. J. Panaro, P. K. Yuen, T. Sakazume, P. Fortina, L. J. Kricka and P. Wilding, *Clin Chem*, 2000, **46**, 1851-1853.
181. A. G. Hadd, D. E. Raymond, J. W. Halliwell, S. C. Jacobson and J. M. Ramsey, *Anal Chem*, 1997, **69**, 3407-3412.

1

Figure 1. Landmarks for proteomics and microfluidics.





* MRM/SRM are also used for relative quantification.

Figure 3. Hypothetical design of a microfluidic chip for quantitative phosphoproteomics.

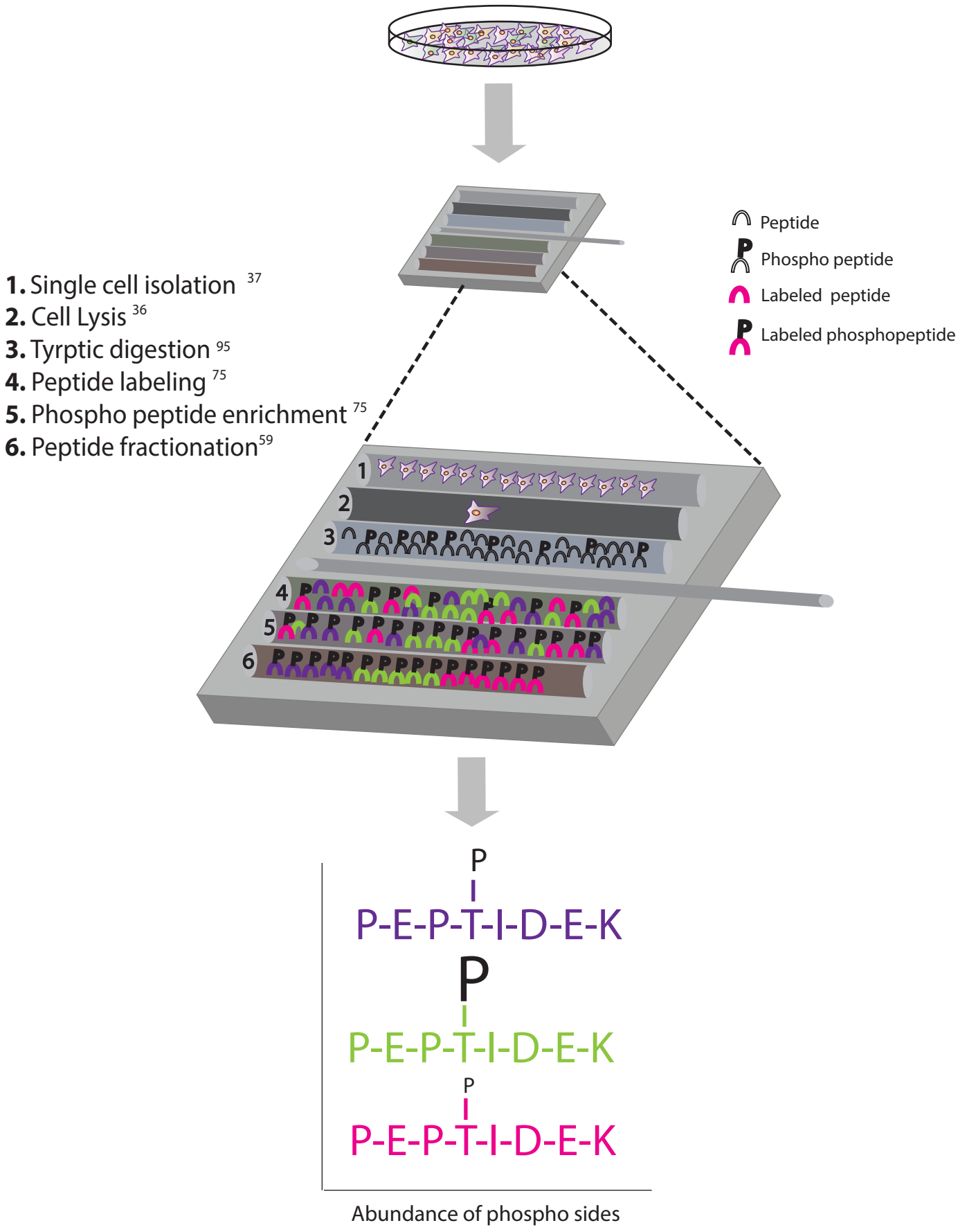


Figure 4. Analysis of phosphorylation sites in a single cell versus mixed cell population

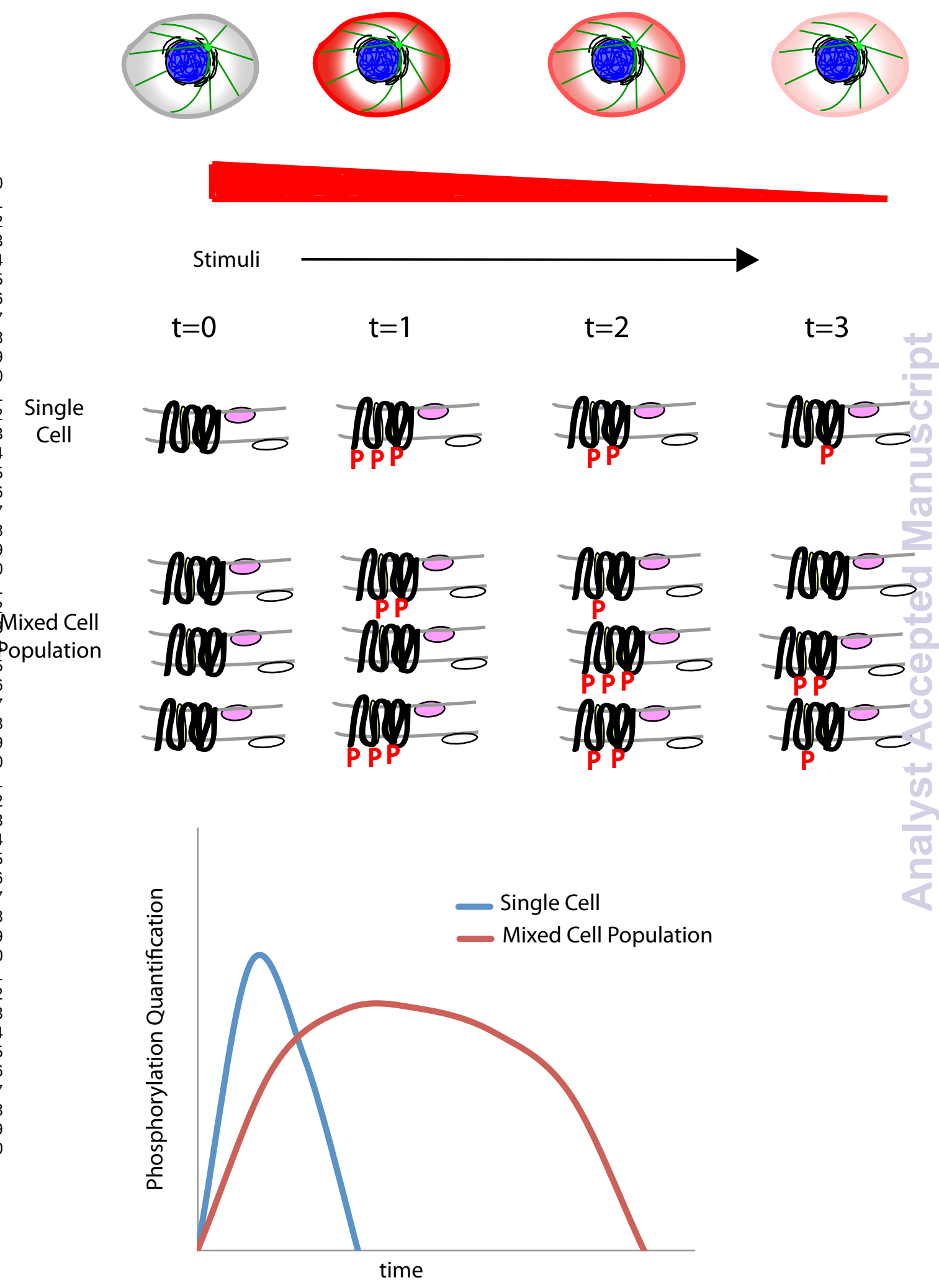


Table1. A review of the potential applications of the current phosphoproteomics methods to microfluidics.

a. *Each enrichment method's pros and cons and their compatibility with future adaptations of the nano/microfluidic systems are rated as low, moderate or high with respect to their current trends and applications.*

Method	Pros	Cons	Compatibility
SCX, SAX, WAX	✓ Large scale applicable ✓ Easily scalable ✓ Robust for LC fractionation	× Need to be used in combination with other strategies for high enrichment efficiency × Low resolution separation × Peptide loss during desalting	**HIGH**
ERLIC,HILIC	✓ No desalting required ✓ Highly orthogonal with ion exchange	× Requires a combined workflow with other enrichment methods	**HIGH**
IMAC, TiO_2, Ti-IMAC	✓ High selectivity ✓ µg to mg scale ✓ Online LC applications possible	× Non-selective binding of acidic peptides × Highly sensitive to buffer conditions ph, salt concentration etc. × Biased for multi phosphorylated peptides	**HIGH**
Antibody purification (IP)	✓ Performed both peptide and protein level ✓ The most efficient system for pTyr	× Not preferable for pSer and pThr peptides × Low selectivity for complex mixtures × Low reproducibility through antibody batches and protocols × Enrich only specific pool of peptides	**LOW**
Chemical derivatization	✓ Low cost for pTyr enrichment ✓ Extensive washing steps allowed for the removal of non-phosphopeptides	× Major sample loss and low efficiency due to many reaction steps harsh conditions × Occurrence of non-specific reactions × Does not work efficiently except pSer residues	**MODERATE**

b. *Each quantification method's pros and cons and their compatibility to future adaptations of the nano/microfluidic systems are rated as low, moderate or high with respect to their current trends and applications.*

Method	Pros	Cons	Compatibility
Metabolic labelling	✓ Label introduced during protein synthesis minimizes variation ✓ Extendable to label whole organisms	× High cost × Potential metabolic conversion of arginine to proline × Up to five channel labelling	**HIGH**
Chemical labelling	✓ Mostly introduced at peptide level ✓ Extendable to ten channels for labelling ✓ Applicable to all samples type of samples	× iTRAQ and TMT requires pre-optimization and not all MS are compatible with this method × Co-elution of peptides	**MODERATE**
Label free quantification	✓ No limitation for the number of samples ✓ No-additional steps for sample preparation ✓ Samples from all sources can be analyzed	× Stable LC systems and robust sample preparation protocols are required × Elaborate and time consuming × Highly dependent on data processing	**HIGH**
SRM/MRM PRM	✓ No label required ✓ A highly robust method	× Stable LC systems and robust sample preparation protocols are required × Requires discovery experiments and optimization for each target	**HIGH**