CHAPTER ELEVEN

Yeast Expression Proteomics by High-Resolution Mass Spectrometry

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Contents

Abstract

Comprehensive analysis of yeast as a model system requires to reliably determine its composition. Systematic approaches to globally determine the abundance of RNAs have existed for more than a decade and measurements of mRNAs are widely used as proxies for detecting changes in protein abundance. In contrast, methodologies to globally quantitate proteins are only recently becoming available. Such experiments are essential as proteins mediate the majority of biological processes and their abundance does not always correlate

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well with changes in gene expression. Particularly translational and post-translational controls contribute majorly to regulation of protein abundance, for example in heat shock stress response. The development of new sample preparation methods, high-resolution mass spectrometry and novel bioinfomatic tools close this gap and allow the global quantitation of the yeast proteome under different conditions. Here, we provide background information on proteomics by mass-spectrometry and describe the practice of a comprehensive yeast proteome analysis.

1. Introduction

A major goal in analyzing yeast as a eukaryotic model is to understand how components of the system interact dynamically and to determine the ''wiring'' of the interacting parts. A prerequisite for such analysis is the ability to reliably and globally determine the composition of yeast cells under different conditions. While methods to determine RNA quantitatively and comprehensively, such as microarrays, have existed for more than a decade, the technology to globally determine changes of protein abundance is only recently becoming available. Proteins, however, constitute the majority of biologically active agents and information on their relative abundance is thus often crucial. Since changes in the amount of a protein are not always reflected in corresponding mRNA level changes (e.g., see Bonaldi *et al.*, 2008), it is essential for many experiments to measure them directly. This is particularly evident for regulatory processes that are mediated by posttranscriptional regulation affecting, for example, protein stability or production, such as heat stress.

For these reasons, many techniques to determine the relative abundance of proteins have been developed: Most notably, Western blot or fluorescence measurements of tagged proteins are routinely used and comprehensive libraries containing most yeast open reading frames fused to GFP or the TAP tag have been developed (for a global analysis using these resources see, e.g., Ghaemmaghami *et al.*, 2003; Huh *et al.*, 2003). However, in some cases, these tagging methods may interfere with protein function as they rely on altering the protein sequence by introducing tags. For example, C-terminally modified (e.g., by lipidation on a CaaX box) or tail-anchored proteins are not functional in these libraries as the tags are usually introduced at the C-terminus of proteins. In addition, assays relying on libraries of tagged proteins are not easily applied to global experiments, especially when the goal is to compare multiple experimental conditions, because one experiment for each gene or about 6000 individual experiments for all genes have to be performed for each condition.

In addition to these techniques, mass spectrometry (MS) is used to identify single proteins, for example, purified and resolved by denaturing

SDS polyacrylamide gel electrophoresis and this has become a standard tool in biochemistry. During the last 5 years, accelerating advances in MS technology, sample preparation, and computational proteomics led to the development of capabilities to comprehensively determine the relative amount of proteins in complex mixtures. Particularly the advent of precision proteomics due to new instrumentation, such as hybrid linear ion trap Fourier-transform mass spectrometers (e.g., the LTQ-Orbitrap) led to high mass resolving power (Mann and Kelleher, 2008). Resulting from these advances and the concomitant development of computational tools, "shotgun" approaches to sequence an increasingly high number of peptides from complex samples are now available (Cravatt *et al.*, 2007; Domon and Aebersold, 2006). The high mass resolution obtained in these experiments reduces the number of potentially false-positive assignments and increases the number of identifications from a single chromatographic run (Cox and Mann, 2008). Together these advances enabled us in 2008 to report the first determination of a complete eukaryotic proteome from *Saccharomyces cerevisiae* (de Godoy *et al.*, 2008).

To perform such global proteome quantitation, we used a state-of-the-art proteomics analysis setup consisting of sample preparation to separate peptides by isoelectric focusing (IEF), separation of peptides by liquid chromatography (LC), and online-injection of eluting peptides to the MS by electrospray ionization (ESI). In this chapter, we provide background on MS methods and describe the principles and practices of such a proteomic experiment. We also discuss how measurement time will be reduced drastically from our original report by new developments in sample preparation, novel MS instrumentation, and advanced computational methods. We expect that these developments together will in the future make expression proteomics of yeast a standard experiment for quantitative, comprehensive approaches.

2. Background, Methods, and Applications

2.1. The challenge

The principle challenge of proteomics is to reliably identify and quantitate proteins in dauntingly complex mixtures, for example, in a protein extract. In the case of yeast, at least 4200 proteins are expressed under normal growth conditions (de Godoy *et al.*, 2008; Ghaemmaghami *et al.*, 2003; Huh *et al.*, 2003). Some fraction of these proteins is posttranslationally modified, for example, by phosphorylation, acetylation, or glycosylation, and this further increases the chemical complexity of the polypeptide mixture in an extract.

At the moment not every protein with all modifications can be quantitated from a single experiment. However, several methods are successfully

employed to approach the problem. Initial experiments resolved proteins by sequential electrophoresis in two dimensions (2D-gel analysis), with each dimension fractionating the proteins based on a different principal characteristic of proteins (O'Farrell, 1975). Most commonly, IEF in one dimension and separation by size in denaturing SDS gels in the second dimension is used. The first "proteome-scale" experiments using this technology identified roughly 150 yeast proteins (Shevchenko *et al.*, 1996). However, this method often leads to multiple spots for the same protein, different, for example, in their modification(s) (Fountoulakis *et al.*, 2004). In addition, 2D gels are strongly biased toward only the most abundant proteins. Thus identification of more than a few hundred different proteins is generally not feasible. Moreover, accurate quantitation is often not possible because of overlaying spots (Campostrini *et al.*, 2005). A further complication is that the technology by itself does not allow the identification of proteins observed and therefore it is usually is combined with another analytical method, such as MS or Western blotting. For these combined reasons, 2D gels have not developed into a comprehensive proteomics technology.

In contrast, MS-based proteomics can unambiguously identify proteins in a very complex mixture with minimal prior separation. Because MS is a versatile tool that combines several unique capabilities; such as quantification of proteins from a cell or organism and characterization of important posttranslational modifications (PTMs) of proteins (e.g., phosphorylation) in addition to identification of individual proteins in a complex mixture, it has become the most important technology in proteomics today (Aebersold and Mann, 2003).

In an MS-based proteomic experiment, complex protein mixtures are usually digested by a protease, yielding a mixture of tens of thousands of peptides with a range of abundance over more than four orders of magnitude. Until recently, this complexity has limited the feasibility of "shotgun" approaches that aim to directly identify the peptides in the mixture. To deal with this problem, several methods to reduce the complexity of peptide mixtures for analysis were introduced. Traditionally, the starting material, that is, the yeast extract, is further fractionated using, for example, subcellular fractionation or denaturing SDS gel electrophoresis. Peptides resulting from the digest of such fractions are then separated by reversed-phase LC right before being analyzed "online" in the MS. Our recent experience, however, has been that extensive fractionation and separation at the protein level leads to rapidly diminishing returns in terms of the number of identified proteins (Bonaldi *et al.*, 2008; de Godoy *et al.*, 2008). Instead, several strategies for the fractionation of peptides after proteolytic digestion of protein mixtures have been designed. In one variation of this principle, termed *mu*lti*di*mensional *p*rotein *i*dentification *t*echnology or "MudPIT," the resulting peptides are separated by strong cation exchange chromatography (Washburn *et al.*, 2001). In addition, some variant techniques with

mixed-anion/cation beds also exist (e.g., see Motoyama *et al.*, 2007). Recently, an alternative method employing IEF of peptides in a combined stationary and liquid phase was developed and this was an important contribution to directly asses the protein composition of total yeast extract (de Godoy *et al.*, 2008; Hubner *et al.*, 2008). This protocol, which is described in detail below, uses immobilized p*I* strips to separate peptides by IEF and further resolves the peptides in the resulting fractions by LC to directly analyze them by MS.

2.2. Background on MS instrumentation for "shotgun" proteomics

MS is essentially a technique for weighing molecules, but the measurements are not performed with a conventional balance or scale. Instead, in MS gas phase ions of peptides are separated or filtered according to their mass-to-charge (m/z) ratio in a magnetic or electrostatic field and finally recorded by a detector. The resulting mass spectrum is a plot of the relative abundances of the produced ions as a function of their m/z ratio (see Fig. 11.2).

Because every peptide molecule and modification has a characteristic mass, MS is a very powerful and nearly universal tool in proteomics and can result in determination of the chemical composition when mass accuracy is sufficiently high. Peptides furthermore have distinct fragmentation patterns that provide structural information to identify their amino acid sequences and modifications.

MS instrumentation developments have greatly contributed to recent breakthroughs in proteomic research. Several types of MS are currently employed in proteomics. They are distinguished by the ionization method used to charge peptides and by the type of mass analyzer used to determine the mass-to-charge (m/z) ratio of the resulting ions.

As traditional ionization methods such as chemical ionization are often too harsh, "soft" methods that allow ionization of intact biomolecules are necessary for MS-based proteomics. The two ionization methods employed in proteomics are *m*atrix *a*ssisted *l*aser *d*esorption *i*onization (MALDI) and ESI. ESI, which is used most commonly, allows large, nonvolatile molecules such as peptides and proteins to be ionized nondestructively directly from a liquid phase, usually consisting of a mixture of volatile organic solvent and acidified water. In electrospray, a liquid is passed through a nozzle to which a high voltage is applied (Fenn *et al.*, 1989; Whitehouse *et al.*, 1985). The charged liquid becomes unstable as it is forced to hold more and more charges. Soon the liquid reaches a critical point and near the tip of the nozzle it blows apart into a cloud of tiny, highly charged droplets. These droplets rapidly shrink as solvent molecules evaporate from their surface increasing the electric field at the droplet surface. By a process of

''ion evaporation'' (Iribarne and Thomson model) or simple solvent evaporation (charged residue model), the ''naked'' biomolecule becomes a gas-phase ion (Iribane and Thomson, 1976).

The other ''soft'' ionization technique, MALDI, was also developed in the late 1980s (Hillenkamp and Karas, 1990; Hillenkamp *et al.*, 1991; Karas and Hillenkamp, 1988). In this technique, analyte molecules are cocrystallized with an UV- or IR-absorbing substance—termed the matrix—which is usually an organic carboxylic acid such as 2,5-dihydroxybenzoic acid (UV-absorbing) or succinic acid (infrared absorbing). The analytes are desorbed and ionized by a laser beam (pulsed laser irradiation) from the solid or liquid surface containing the organic matrix compound in approximately 1000-fold excess. A widely accepted view how the matrix assists the ionization is that neutral sample molecules are ionized by acid–base proton transfer reactions with the protonated carboxylic acid matrix ions in a dense phase just above the surface of the matrix.

Like ESI, MALDI is capable of efficiently ionizing large biomolecules such as peptides and proteins and is often used with *t*ime-*o*f-*f*light (TOF) MS (see below) due to the vacuum-compatibility and pulsing nature of the technique (the laser pulse frequency can easily be synchronized with the TOF extraction pulse). Both ESI and MALDI ionization allow introduction of biological molecules exceeding one million Daltons into MS, but they are by far most often used for analysis of peptides. For this purpose, the main difference between the two methods is that ESI predominately produces multiply charged ions, MH_n^{n+}. In contrast, MALDI almost exclusively generates singly charged peptide ions, MH^+, which can be difficult to sequence by the low-energy dissociation methods available on most proteomic mass analyzers; because when the single proton is fixed on the side chain of an arginine or lysine residue then there is no mobile proton available to induce peptide–amide bond fragmentations. 2D-gel-based proteomics is almost exclusively coupled to MALDI-TOF MS analysis, whereas most other areas of proteomics are increasingly based on ESI, since it is possible to integrate ESI with online LC-MS/MS.

After ionization, the mass of peptide ions is determined. The earliest analyzers used in MS-based proteomics combined a series of quadrupoles, each capable of selecting specific ions that can pass an applied electric field that deflects ions with other m/z ratios: In the first quadrupole, peptides are filtered (MS spectrum). In the second quadrupole, one filtered peptide at a time is fragmented at the peptide bond by collision with noble gases such as Argon or Helium and this is commonly called *c*ollision-*i*nduced *d*issociation (''CID''). Subsequently to the collisions, the resulting spectrum of the fragmented ions is filtered in the third quadrupole (MS/MS spectrum). Information about the sequence of the peptide analyzed is then contained in the mass difference between series of fragmented ions in this MS/MS spectrum.

As an alternative to CID, another fragmentation method, *e*lectron *t*ransfer *d*issociation (''ETD'') has been employed in the last few years. In this method, electrons are transferred from radical anions to the positively multiply charged peptide ions, which then fragment adjacent to the amino group of the peptide bond (for review, see Mikesh *et al.*, 2006). CID sometimes yields incomplete spectra, especially for very basic peptides. ETD often yields more uniformly fragmented peptides and in addition, the fragmentation is more specific for the peptide backbone and therefore PTMs, such as phosphorylations are less likely to be lost in the spectra of peptide-backbone-derived fragments. These different fragmentation methods can in principle be coupled with any type of MS instrument.

The classic setup consisting of triple quadrupoles described here is in principle very fast, but its mass accuracy (usually limited to 0.5 Da) and sensitivity are not high enough for experiments aimed at large-scale discovery of proteins in complex mixtures. Therefore, they are currently mainly employed in *m*ultiple *r*eaction *m*onitoring (MRM) experiments, where the fast MS/MS switching capability is used to detect a few preprogrammed fragmentation patterns for a number of predefined peptides, with the goal of accurate and targeted quantitation in complex mixtures (Anderson *et al.*, 2009).

As an alternative instrument type, TOF MS measure the travel time of ions to the detector after they have all been accelerated to the same kinetic energy. This time is directly proportional to the mass-to-charge ratio (m/z), which can be quite accurately measured. Drawbacks of these instruments include that very high resolution and sensitivity are difficult to achieve at the same time.

A mass analyzer type currently used more commonly in proteomics is the ion trap. The basic principle of ion traps is similar to that of quadrupoles, with the exception that selected ions are trapped in the electric field and can be accumulated over time. This makes this instrument highly sensitive, but similar to quadrupole analyzers, its resolution is quite limited, sometimes leading to a mass uncertainty of several daltons. To overcome some of these drawbacks of conventional 3D-ion traps, a new generation of ion traps with superior ion capacity, dynamic range, scan speed, and sensitivity has been introduced. These are the linear ion traps (or 2D-ion traps)—essentially segmented quadrupole mass filters—capable of trapping and detecting a factor hundred more ions than traditional 3D-ion traps (Hager and Yves Le Blanc, 2003; Schwartz *et al.*, 2002).

A major breakthrough in proteomics was the introduction of a ''hybrid'' MS, the LTQ-Orbitrap consisting of a linear ion trap and an orbitrap (Scigelova and Makarov, 2006; Fig. 11.1). The orbitrap is the first fundamentally new mass analyzer in more than 20 years. The instrument contains three components. It has a linear quadrupole ion trap (LTQ), in which it is possible to control and manipulate (e.g., accumulate and collisionally activate) ions in the subsecond time-scale. Detection can be achieved in two

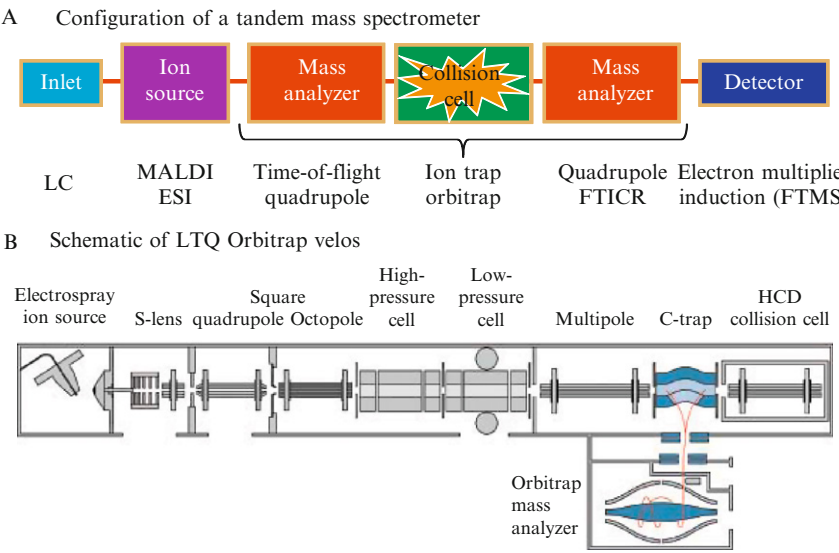

Figure 11.1 Schematic representation of typical tandem mass spectrometers for proteomics experiments. (A) Principal setup of a tandem mass spectrometer. Peptides are typically separated by liquid chromatography (LC) up-front and transferred to the gas phase in the ion source (by either MALDI or ESI). Peptide ions of interest can be separated or isolated in the first mass analyzer (either an ion trap or a quadrupole) and injected into the collision cell. The resulting fragmentation ions are analyzed in a second mass analyzer, for example, an ion trap or a TOF and recorded by electron multiplier detectors or by induction in Fourier-transform instruments. (B) Schematic overview of the LTQ-Orbitrap Velos. The front end of the instrument is a dual linear ion trap mass spectrometer capable of efficient ion accumulation, isolation, fragmentation, and detection of MS or MS^n ions. Accumulated ion populations are moved into the C-trap via an octapole ion guide, or for higher energy dissociation accelerates into the HCD collision cell and resulting fragments are subsequently moved back to the C-trap. In the C-trap, the motion of the ion population is damped by a residual pressure of nitrogen. Ions are then injected into the orbitrap in a short pulse and begin to circle the central electrode. The ion signals (peptide m/z values) are detected via a differential amplifier between the two halves of the outer orbitrap electrodes.

ways. In the linear ion trap, ions can be ejected radially through slits in the quadrupole rods and detected by two electron multiplier detectors. Alternatively, ions are ejected axially from the ion trap and transferred via octopole-ion guides into another ion trap (the C-trap) where they are collisionally cooled and focused, before they are orthogonally injected into the third component of the instrument, the Orbitrap mass analyzer, which operates in very high vacuum. The LTQ-Orbitrap instrument is particularly suitable for both qualitative and quantitative analysis of complex peptide mixtures, because of its high sensitivity, dynamic range, mass accuracy, and sequencing speed. This allows for sequencing of thousands of peptides by

high-resolution tandem MS in less than 1 hour of LC-MS/MS analysis time. Due to these advantages that allow collection of spectra with very high resolution (60,000) and routinely with low parts-per-million mass accuracy this LTQ-Orbitrap instrument is used for all MS analyses described below.

2.3. Quantitative proteomics

In many proteomic experiments, the goal is not only to test the presence of a specific protein, but to quantitate its abundance as well. To date, most approaches rely on relative quantitation between different conditions. In a "label-free" approach, the integrated intensity of peptide peaks is compared between different experiments and used as a measure of protein abundance. However at this point, methods that compare intensities of peptides in the same LC-MS run are more reliable. Several methods exist that specifically label peptides of one condition during the proteolytic cleavage, for example, by use of water containing "heavy" ^{18}O, which is incorporated at the C-terminus of each peptide (Yao *et al.*, 2001). Alternatively, chemical labeling of protein mixtures can be employed. Affinity tags with different mass are sometimes used and this technique was termed "*I*sotope-*c*oded *a*ffinity *t*ag" assay ("ICAT", Gygi *et al.*, 1999). Most commonly, thiol-reactive reagents are used to covalently link an isotope labeled affinity tag (e.g., biotin) to cysteine containing peptides. These are subsequently affinity purified and analyzed by MS. When samples are mixed after cross-linking with differently labeled reagents, they can be distinguished afterwards by their mass difference. Disadvantages of this method are that they introduce an additional processing step that may have different efficiency in different samples and that comparatively few tryptic peptides contain a cysteine that can be modified. For these reasons, ICAT is not generally used today. Instead, iTRAQ labeling of amine groups has become popular (Ross *et al.*, 2004). In this technique, peptides are labeled with up to eight different isobaric tandem mass tags, mixed and analyzed by LC-MS/MS. Each tag consists of a reporter and balance group, which has been designed such that it is very prone to fragmentation under CID. The technique is based on chemically tagging the free N-terminus and lysine-ε-amino group on peptides generated from protein digests that have been isolated from different cell states. The tagged samples are then combined and in the full scan spectra, peptides from the different conditions appear at the same m/z ratio. Upon fragmentation, however, different reporter ions are released from the different iTRAQ tags and their relative amount is then quantitated to calculate the relative contribution of peptides from each condition to the intensity signal of the protein.

For yeast cells, metabolic labeling is the preferred method for comparative proteomics since it is easily done with amino acids containing stable nonradioactive isotopes incorporated in a cell culture and results in highly uniform and efficient labeling, ("SILAC," Ong *et al.*, 2002). Conveniently,

amino acids are chosen that ensure that one and only one labeled residue is present in each peptide. Trypsin is a commonly used protease to digest protein mixtures and it cleaves at the carboxyl side of arginine and lysines. Thus, when proteins are labeled with these two amino acids (with [13C6/15N2] L-lysine and [13C6/15N4] L-arginine, respectively) and digested with trypsin, each resulting peptide contains one labeled amino acid. Analogously, the endoproteinase LysC that cleaves after lysines is used on proteins labeled only with lysine. For comparative proteomics, both the labeled and unlabelled proteins are treated the same way; in fact most easily, they are mixed together in equal ratio of total protein and processed together. The resulting peptides are easily distinguished in the MS by their characteristic mass difference and computational proteomics software automatically identifies and quantifies corresponding SILAC pairs, giving an accurate ratio of light and heavy peptides, which can then be averaged for protein ratios. This analysis is not limited to two different isotope forms of peptides and a third label is often used. However, each labeling increases the complexity of the mixture and therefore complicates the comprehensive analysis of all peptides contained in a mixture.

A complication of metabolic labeling of yeast by heavy isotope containing amino acids is potential conversion between each other due to coupled amino acid synthesis pathways. In practice, only conversion from arginine to proline is common (Ong *et al.*, 2003). Depending on the cell type and sequence of each peptide (the number of prolines contained) this can result in variable and complex patterns of SILAC peptide peaks, preventing accurate automated analysis. To avoid this problem, one possibility is to reduce the amount of labeled arginine added to the synthetic medium and another one is to omit arginine labeling altogether and to use LysC instead of trypsin for the digest. This latter approach has proven very powerful especially in yeast. It results in peptides of increased average length, but concomitantly reduces the complexity of the mixture. In addition, lysine labeling of yeast cells is easily achieved since many strain backgrounds are lysine auxotrophs. For example, the commonly used BY4739 strain used to derive the MAT alpha gene deletion collection bears the *lys2Δ0* allele, is lysine auxotroph and can therefore be directly labeled. (Giaever *et al.*, 2002; Winzeler *et al.*, 1999). Other commonly used lab strains, such as W303, can easily be made auxotroph by deleting *LYS2* and *ARG4* when double labeling with arginine and cleavage with trypsin is desired.

This *in vivo* labeling approach can be used to compare different conditions and Fig. 11.2 shows a representative workflow for such an experiment. In this way, we compared the complete proteome of haploid and diploid yeast cells. Of course the same logic can be applied to cells grown under different conditions, cells with a different genotype (e.g., harboring a deletion) or different biochemical fractions. For example, in a variation of this protocol, protein complexes purified from heavy lysine labeled cells by affinity chromatography are directly compared to background resulting

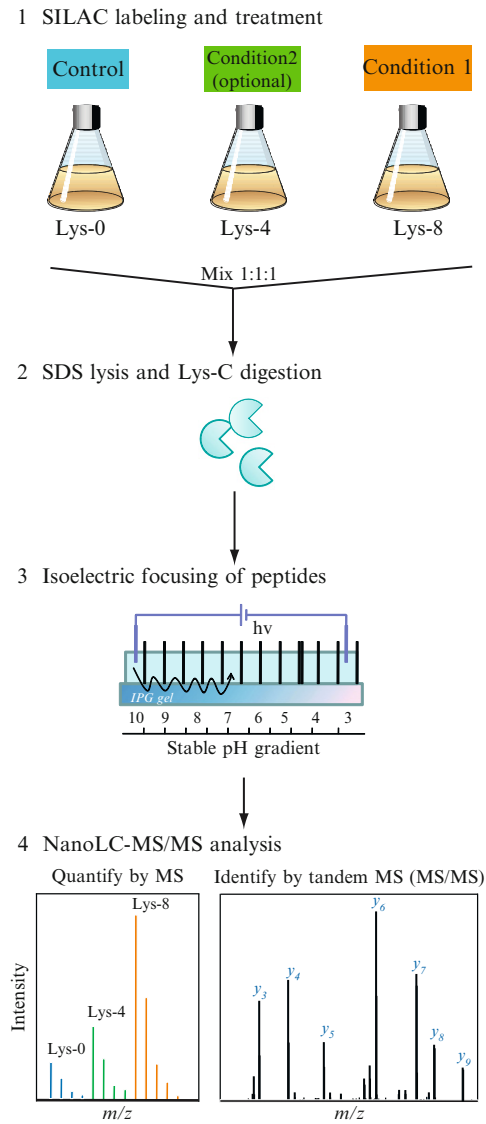

Figure 11.2 Workflow of a SILAC experiment in yeast. 1. Yeast cultures are grown in either light or heavy amino acid containing medium. 2. After harvesting, proteins are digested with a protease to yield peptides. 3. These are subsequently separated by an analytic method that is orthogonal to reversed-phase chromatography (such as isoelectric focusing or ion exchange chromatography methods) prior to LC-MS analysis. Here, isoelectric focusing on immobilized p*I* strips is shown as an example. 4. The fractions of this separation are then analyzed by LC-MS/MS. In full scan spectra, the same peptide from different cell populations is quantified by the relative difference in intensities between the SILAC labels. Peptides are subsequently sequenced and identified by MS/MS.

from unspecific binding of proteins from light labeled cells to a control matrix (Vermeulen *et al.*, 2008). Similarly, many other variations of this general principle are possible. Examples also include the measurement of turnover of proteins (Doherty *et al.*, 2009). For such an experiment, cells are switched from medium containing ''light'' amino acids to medium containing ''heavy'' amino acids for defined times. The change of ratio over time will then indicate the time course of protein turnover, measured individually for each protein.

2.4. Computational proteomics and data analysis

The streamlining of acquisition and the large amount of data necessitate efficient and automated evaluation of the resulting spectra. For example, in a typical proteomic experiment, at least 12 fractions from the IEF of peptides are analyzed, each by an LC run of at least 120 min, collecting a spectrum every second. Together, these runs result in at least 86,400 MS spectra of high mass accuracy that need to be evaluated. In addition, the most abundant peaks (usually the top five in abundance) of every MS scan are fragmented and the MS/MS spectra are collected, adding further data to be evaluated. A major breakthrough in computational proteomics was recently achieved in the MaxQuant software suite (Cox and Mann, 2008). The algorithms use correlation analysis and graph theory to detect peaks and isotope clusters in the MS, using the m/z ratio, intensity, and LC elution time as parameters. Figure 11.3 shows a plot with every detected peptide plotted as its m/z versus its elution time in green. Successful identifications are shown in purple. As can be easily appreciated, the identification rates are very high, usually resulting in identification of the majority of peptides. This information is automatically submitted to a commercial search engine (Mascot). An important consideration in computational proteomics is the control of the accuracy of peptide identification. Today, 99% certainty of protein identification is usually desired and this is controlled by monitoring the number of identifications in a ''nonsense'' or decoy database consisting of reversed protein sequences (Elias and Gygi, 2007). This search results in automatic identification of peptides in the mixture with high confidence. In the next step, peak intensities for each member of a SILAC pair are calculated from the isotope pattern. Multiple measurements for each peptide are integrated and statistically evaluated, resulting in a measurement of the abundance ratio of the proteins in each sample and a confidence estimate for that measurement.

2.5. Perspective and outlook

While total proteome quantitation is still a time-consuming experiment requiring advanced instrumentation and specialized know-how, several trends will make such experiments much more routine and available to

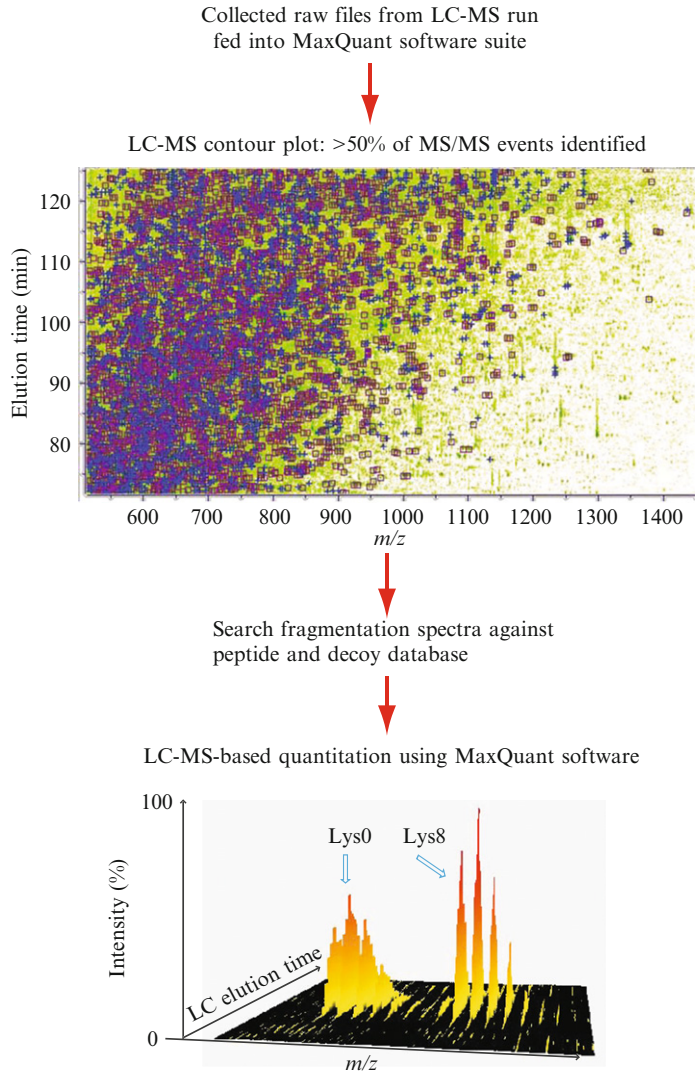

Figure 11.3 Workflow of the analysis of a proteomic experiment. Complex LC-MS Raw files containing MS and MS/MS spectra collected from the LTQ-Orbitrap is fed into the MaxQuant software package that automatically searches fragmentation patterns against a target/decoy protein database. Currently, identified peptide sequences are filtered based on their database score and accepted at a false discovery rate (FDR) of less than 1%. MaxQuant then automatically determines the peptide ratios of SILAC pairs and calculates the significance of regulation for all identified proteins.

molecular biology laboratories in the future. Among them, the development of a new generation of hybrid MS instruments named ''LTQ-Orbitrap Velos,'' which offers significantly higher sensitivity due to new ion optics systems that enhances the transfer of ions from the source to the MS by an order of magnitude (Olsen *et al.*, 2009). Similarly, this instrument enables faster scan cycle times at higher performance with a new dual pressure linear ion trap. Together this improves the scan speed more than twofold, which in effect will reduce MS measurement time requirements for yeast expression proteomics. In addition to developments in MS instrumentation, novel preparation methods (such as ''*f*ilter *a*ided *s*ample *p*reparation''; Wisniewski *et al.*, 2009) result in more uniform samples between experiments and therefore even higher reproducibility and identification rates. Finally, streamlined versions of MaxQuant and novel bioinformatic tools will result in faster evaluation of the data, a process that is still computationally intensive and time consuming.

With these enhancements, expression proteomics by MS is likely to continue to become more widespread and will soon be a common technique to comprehensively analyze the composition of yeast cells.

3. Protocols

In the following, we describe in detail how to grow SILAC labeled yeast for a proteomic experiment, how to process the samples for LC-MS, how to perform the measurements and how to analyze the resulting data.

3.1. Yeast strains for SILAC proteomics experiments

In principle, any yeast strain that is auxotroph for lysine and/or argenine is suitable for SILAC labeling, depending on which label is used. If a particular stain needs to be used, either *LYS2* or *ARG4* can be introduced either directly by transformation with a PCR product (Janke *et al.*, 2004) or by crossing with a deletion strain such as BY4739. Primers to delete *LYS2* using the system described by Janke *et al.* (2004) are Lys2-S1 (5′-atttcagtga aaaactgcta atagagagat atcacagagt tactcactaa tgcgtacgct gcaggtcgac-3′) and Lys2-S2 (5′-ctaattcat atttaattat tgtacatgga catatcatac gtaatgctca accttaatcg atgaattcga gctcg-3′); conversely, for *ARG4*, Arg4-S1 (5′-cgcaattgaa gagctcaaaa gcaggtaact atataacaag actaaggcaa acatgcgtac gctgcaggtc gac-3′) and Arg4-S2 (5′-gtcctagaag taccagacct gatgaaattc ttgcgcataa cgtcgccatc tgctaatcga tgaattcgag ctcg-3′) primers are used.

3.2. Media for SILAC labeling

Stock medium (any other drop out mix lacking arginine and lysine will work):

XYNB without amino acids	6.7	g/l
XGlucose	20	g/l

	Final concentration [mg/l]	Stock per 100 ml	Amount of stock [ml] for 1 l
Adenine sulfate	20	200 mg	10
Uracil	20	200 mg	10
L-Tryptophan	20	1 g	2
L-Histidine–HCl	20	1 g	2
L-Arginine–HCl[a]	20	1 g	2
L-Methionine	20	1 g	2
L-Tyrosine	30	200 mg	15
L-Leucine	60	1 g	6
L-Isoleucine	30	1 g	3
L-Phenylalanine	50	1 g	5
L-Glutamic acid	100	1 g	10
L-Aspartic Acid	100	1 g	110
L-Valine	150	3 g	5
L-Threonine	200	4 g	5
L-Serine	400	8 g	5

[a] ONLY for lysine labeling alone.

To prepare media ready to use (use light/medium/heavy amino acids as desired):

L-Lysine	30	mg/l
L-Argenine[a]	5	mg/l

[a] ONLY for double Lys/Arg labeling; concentration of arginine can be varied up to 20 mg/l; this low amount of 5 mg/l minimizes arginine to proline conversion.

SILAC amino acids (Isotec-Sigma):

Lys 4:	L-Lysine—4,4,5,5-d_4·Cl	Cat.# 616192
Lys 8:	L-Lysine—$^{13}C6$, $^{15}N_2$·Cl	Cat.# 608041
Arg 6:	L-Arginine—$^{13}C_6$·Cl	Cat.# 643440
Arg 10:	L-Arginine—$^{13}C_6$, $^{15}N_4$·Cl	Cat.# 608033

3.3. Growing yeast cultures for SILAC labeling

1. Grow preculture of a lysine auxotroph strain over night in SC medium
2. Inoculate SILAC medium (either heavy or light) with a 1:10,000 dilution of the preculture
3. Grow overnight at 30 °C
4. Check OD next morning (cells should reach OD = 0.5–0.7)
5. To harvest, spin down cells 10 min 4000 rpm 4 °C

3.4. Extract Preparation for SILAC experiments

1. Resuspend cells in buffer S (150 m*M* K acetate, 2 m*M* Mg acetate, 1× protease inhibitor cocktail (Roche), 20 m*M* HEPES, pH 7.4) at a density of 50 OD/ml (minimum buffer volume for the bead-mill (3 ml))
2. Slowly drop cell suspension in N2(l)
3. Grind cells in MM301 Ball Mill (Retsch), three cycles of 3 min at 10 Hz.
4. Thaw grinded cells
5. Detergent extraction (30 min; 1% TritonX 100, 4 °C rotating)
6. Spin down 10 min 1000 rpm 4 °C
7. Collect supernatant
8. Measure protein concentration by Bradford assay
9. Mix extracts from strains that are used for the comparison in a 1:1 ratio of protein amounts

3.5. In-solution digest of proteins for MS

1. Reduce proteins for 20 min at RT in 1 m*M* dithiothreitol (DTT)
2. Alkylate proteins for 15 min using 5.5 m*M* iodoacetamide (IAA) at RT in the dark
3. Digest with the endoproteinase Lys-C (Wako) using 1:50 w/w over night at RT (arginine and lysine labeled yeast proteins are digested with Lys-C in a similar manner)
4. Dilute the resulting peptide mixtures with Millipore water to achieve a final urea concentration below 2 *M*
5. *For argenine labeled cells*, add trypsin (modified sequencing grade, Promega) 1:50 w/w and digest overnight
6. Trypsin and Lys-C activity are quenched by acidification of the reaction mixtures with TFA to pH ~2.

3.6. Test of label incorporation

It is straightforward to calculate the SILAC incorporation efficiency of a peptide by high-resolution MS. To this end, a small aliquot of yeast cells grown in the presence of either [$^{13}C_6$]arginine or [$^{13}C_6$]lysine for several

generations is analyzed by MS. Proteins are extracted from the sample as described above and separated by one-dimensional gel electrophoresis (SDS-PAGE). The gel is Coomassie stained and a slice corresponding to 30–40 kDa protein size range is excised and digested *in situ* with LysC (or alternative trypsin for arginine labeling). The resulting peptide mixture is desalted and analyzed by nanoflow LC-tandem MS. The raw MS data can be analyzed in the MaxQuant software suite as described below. As an output, MaxQuant will generate a list of identified and quantified proteins. The median (H/L) peptide and protein ratio for all proteins directly reflects the SILAC amino acid incorporation rate. In order to allow accurate quantitation of ratios in the subsequent proteomic experiment, the ratio indicating labeling efficiency should be at least 99%.

3.7. Peptide IEF (Optional)

Alternative to the peptide isoelectric focusing described here, other peptide fractionation procedures yield good results. For example, anion exchange fractionation can be used as described in Wiśniewski *et al.* (2009).

1. To separate peptides according to their isoelectric point, 75 μg of in-solution digested peptides are fractionated using the Agilent 3100 OFFGEL fractionator (Agilent, G3100AA).
2. Set up the system according to the manual of the High Res Kit, pH 3-10 (Agilent, 5188-6424) but exchange strips by 24 cm Immobiline DryStrip, pH 3-10 (GE Healthcare, 17-6002-44) and ampholytes by IPG Buffer, pH 3–10 (GE Healthcare, 17-6000-87).
3. Rehydrate strips for 20 min with 20 μl rehydration buffer per well containing 5% glycerol and ampholytes diluted 1:50.
4. Prepare 150 μl of peptide solution containing 3.125 μg yeast digest, 5% glycerol, and ampholytes diluted 1:50.
5. Apply mixture to each well of the OFFGEL device.
6. Close wells with a silicon cover seal to prevent evaporation of liquid.
7. Focus peptides for 50 kVh at maximum current of 50 μA, maximum voltage of 8000 V and maximum power of 200 mW into 24 fractions.

Average run time is approximately 30 h.

8. Add 3% acetonitrile, 1% trifluoroacetic acid, and 0.5% acetic acid to acidify each peptide fraction.
9. Desalt and concentrate fraction on a reversed-phase C18 StageTip (Rappsilber *et al.*, 2007).

3.8. MS analysis

3.8.1. Equipment

We perform all MS experiments on a nanoflow high-performance liquid chromatography (HPLC) system (Agilent Technologies 1200, Waldbronn, Germany) connected to a hybrid LTQ-Orbitrap classic or XL (Thermo Fisher Scientific, Bremen, Germany) equipped with a nanoelectrospray ion source (Proxeon Biosystems, Odense, Denmark). The HPLC system consists of a solvent degasser nanoflow pump and a temperature controlled microautosampler kept constantly at 4 °C in order to reduce sample evaporation. The peptide mixtures are loaded onto a 15 cm analytical column (75 μm inner diameter) packed in-house with a methanol slurry of 3 μm reverse-phased, fully end-capped C18 beads (Reprosil-AQ Pur, Dr. Maisch) using a pressurized "packing bomb" operated at 50–60 bar. Mobile phases for HPLC consist of (A) 99.5% Milli-Q water and 0.5% acetic acid (v/v); (B) 19.5% Milli-Q water, 80% acetonitrile, and 0.5% acetic acid (v/v).

3.8.2. Procedure of sample preparation/injection

1. Prior to MS analysis elute all samples of the C18 StageTip directly into a 96 sample well plate (Abgene, UK) using two times 20 μl buffer B (80% acetonitrile, 0.5% acetic acid).
2. Concentrate samples in a "speed-vac" for 12 min in order to remove all organic solvent.
3. Adjust sample volume to approximately 8 μl by adding an appropriate volume of buffer A (0.5% acetic acid).
4. Load 5 μl of prepared peptide mixture onto the analytical column for 20 min in 2% buffer B at a flow rate of 500 nl/min followed by reverse-phased separation through a 90 min gradient ranging from 5% to 40% acetonitrile in 0.5% acetic acid.
5. Wash the column for 10 min with high concentration of organic solvent (90% buffer B) and equilibrate it for another10 min with buffer A (0.5% acetic acid) prior to loading of the next sample.
6. The eluted peptides from the HPLC column are directly electrosprayed into the MS for detection.

3.8.3. Mass spectrometry

We operate the MS instrument in data-dependent mode by automatically switching between full survey scan MS and consecutive MS/MS acquisition. Survey full scan MS spectra (mass range m/z 300–2000) are acquired in the orbitrap section of the instrument with a resolution of $R = 60{,}000$ at m/z 400 (after accumulation to a "target value" of 1,000,000 in the linear ion trap). The 10 most intense peptide ions in each survey scan with an ion

intensity above 500 counts and a charge state ≥ 2 are sequentially isolated to a target value of 5000 and fragmented in the linear ion trap by collisionally induced dissociation (CID/CAD).

All peaks selected for fragmentation are automatically put on an exclusion list for 90 s, which ensures that the same ion would not be selected for fragmentation more than once. For optimal duty cycle, the fragment ion spectra are recorded in the LTQ-MS "in parallel" with the orbitrap full scan detection. For all survey scan measurements with the orbitrap detector, a lock-mass ion from ambient air (m/z 391.284286, 429.08875, and 445.120025) is used for internal calibration as described ensuring an overall sub-ppm mass accuracy for all detected peptides (Olsen *et al.*, 2005).

For all MS experiments, data is saved in RAW file format (Thermo Scientific, Bremen, Germany) using the Xcalibur 2.0 with Tune 2.2 or 2.4. All data was loaded into the in-house written software MaxQuant and analyzed as described below.

3.9. Identification and quantitation of peptides and proteins

The data analysis is performed with the MaxQuant software as described in Cox and Mann (2008) supported by Mascot as the database search engine for peptide identifications. In addition, a step by step protocol for the analysis can be found in Cox *et al.* (2009). In short, peaks in MS scans are determined as three-dimensional hills in the mass-retention time plane. They are then assembled to isotope patterns and SILAC pairs by graph theoretical methods. MS/MS peak lists are filtered to contain at most six peaks per 100 Da intervals and searched by Mascot (www.matrixscience.com) against a concatenated forward and reversed version of the yeast ORF database (*Saccharomyces* Genome Database SGDTM at Stanford University—www.yeastgenome.org). Protein sequences of common contaminants, for example, keratins, were added to the database. The initial mass tolerance in MS mode was set to 7 ppm and MS/MS mass tolerance was 0.5 Da. Cysteine carbamidomethylation is searched as a fixed modification, whereas *N*-acetyl protein, *N*-pyroglutamine, and oxidized methionine are searched as variable modifications. Labeled arginine and lysine are specified as fixed or variable modifications, depending on the prior knowledge about the parent ion. The resulting Mascot.dat files are loaded into the MaxQuant software together with the raw data for further analysis. SILAC peptide and protein quantitation is performed automatically with MaxQuant using default settings for parameters. Here, for each SILAC pair, the ratio is determined by a robust regression model fitted to all isotopic peaks and all scans that the pair elutes in. SILAC protein ratios are determined as the median of all peptide ratios assigned to the protein. Absolute protein quantitation is based on extracted ion chromatograms (XICs) of contained peptides. To minimize false identifications all top-scoring peptide assignments made by Mascot are

filtered based on prior knowledge of individual peptide mass error, SILAC state, and the correct number of lysine and arginine residues specified by the mass difference observed in the full scan between the SILAC partners. Furthermore peptide assignments are statistically evaluated in a Bayesian model based on sequence length and Mascot score. We accept peptides and proteins with a false discovery rate (FDR) of less than 1%, estimated based on the number of accepted reverse hits.

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REFERENCES

Aebersold, R., and Mann, M. (2003). Mass spectrometry-based proteomics. *Nature* **422,** 198–207.

Anderson, N. L., Anderson, N. G., Pearson, T. W., Borchers, C. H., Paulovich, A. G., Patterson, S. D., Gillette, M., Aebersold, R., and Carr, S. A. (2009). A human proteome detection and quantitation project. *Mol. Cell. Proteomics* **8,** 883–886.

Bonaldi, T., Straub, T., Cox, J., Kumar, C., Becker, P. B., and Mann, M. (2008). Combined use of RNAi and quantitative proteomics to study gene function in *Drosophila*. *Mol. Cell* **31,** 762–772.

Campostrini, N., Areces, L. B., Rappsilber, J., Pietrogrande, M. C., Dondi, F., Pastorino, F., Ponzoni, M., and Righetti, P. G. (2005). Spot overlapping in two-dimensional maps: A serious problem ignored for much too long. *Proteomics* **5,** 2385–2395.

Cox, J., and Mann, M. (2008). MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. *Nat. Biotechnol.* **26,** 1367–1372.

Cox, J., Matic, I., Hilger, M., Nagaraj, N., Selbach, M., Olsen, J. V., and Mann, M. (2009). A practical guide to the MaxQuant computational platform for SILAC-based quantitative proteomics. *Nat. Protoc.* **4**(5), 698–705.

Cravatt, B. F., Simon, G. M., and Yates, J. R. III (2007). The biological impact of mass-spectrometry-based proteomics. *Nature* **450,** 991–1000.

de Godoy, L. M., Olsen, J. V., Cox, J., Nielsen, M. L., Hubner, N. C., Frohlich, F., Walther, T. C., and Mann, M. (2008). Comprehensive mass-spectrometry-based proteome quantification of haploid versus diploid yeast. *Nature* **455,** 1251–1254.

Doherty, M. K., Hammond, D. E., Clague, M. J., Gaskell, S. J., and Beynon, R. J. (2009). Turnover of the human proteome: Determination of protein intracellular stability by dynamic SILAC. *J. Proteome Res.* **8,** 104–112.

Domon, B., and Aebersold, R. (2006). Mass spectrometry and protein analysis. *Science* **312,** 212–217.

Elias, J. E., and Gygi, S. P. (2007). Target-decoy search strategy for increased confidence in large-scale protein identifications by mass spectrometry. *Nat. Methods* **4,** 207–214.

Fenn, J. B., Mann, M., Meng, C. K., Wong, S. F., and Whitehouse, C. M. (1989). Electrospray ionization for mass spectrometry of large biomolecules. *Science* **246,** 64–71.

Fountoulakis, M., Tsangaris, G., Oh, J. E., Maris, A., and Lubec, G. (2004). Protein profile of the HeLa cell line. *J. Chromatogr. A* **1038,** 247–265.

Ghaemmaghami, S., Huh, W. K., Bower, K., Howson, R. W., Belle, A., Dephoure, N., O'Shea, E. K., and Weissman, J. S. (2003). Global analysis of protein expression in yeast. *Nature* **425,** 737–741.

Giaever, G., Chu, A. M., Ni, L., Connelly, C., Riles, L., Veronneau, S., Dow, S., Lucau-Danila, A., Anderson, K., Andre, B., Arkin, A. P., Astromoff, A., *et al.* (2002). Functional profiling of the *Saccharomyces cerevisiae* genome. *Nature* **418,** 387–391.

Gygi, S. P., Rist, B., Gerber, S. A., Turecek, F., Gelb, M. H., and Aebersold, R. (1999). Quantitative analysis of complex protein mixtures using isotope-coded affinity tags. *Nat. Biotechnol.* **17,** 994–999.

Hager, J. W., and Yves Le Blanc, J. C. (2003). Product ion scanning using a Q-q-Q linear ion trap (Q TRAP) mass spectrometer. *Rapid Commun. Mass Spectrom.* **17,** 1056–1064.

Hillenkamp, F., and Karas, M. (1990). Mass spectrometry of peptides and proteins by matrix-assisted ultraviolet laser desorption/ionization. *Methods Enzymol.* **193,** 280–295.

Hillenkamp, F., Karas, M., Beavis, R. C., and Chait, B. T. (1991). Matrix-assisted laser desorption/ionization mass spectrometry of biopolymers. *Anal. Chem.* **63,** 1193A–1203A.

Hubner, N. C., Ren, S., and Mann, M. (2008). Peptide separation with immobilized pI strips is an attractive alternative to in-gel protein digestion for proteome analysis. *Proteomics* **8,** 4862–4872.

Huh, W. K., Falvo, J. V., Gerke, L. C., Carroll, A. S., Howson, R. W., Weissman, J. S., and O'Shea, E. K. (2003). Global analysis of protein localization in budding yeast. *Nature* **425,** 686–691.

Iribane, J. V., and Thomson, B. A. (1976). On the evaporation of small ions from charged droplets. *J. Chem. Phys.* **64,** 2287–2294.

Janke, C., Magiera, M. M., Rathfelder, N., Taxis, C., Reber, S., Maekawa, H., Moreno-Borchart, A., Doenges, G., Schwob, E., Schiebel, E., and Knop, M. (2004). A versatile toolbox for PCR-based tagging of yeast genes: New fluorescent proteins, more markers and promoter substitution cassettes. *Yeast* **21,** 947–962.

Karas, M., and Hillenkamp, F. (1988). Laser desorption ionization of proteins with molecular masses exceeding 10,000 daltons. *Anal. Chem.* **60,** 2299–2301.

Mann, M., and Kelleher, N. L. (2008). Precision proteomics: The case for high resolution and high mass accuracy. *Proc. Natl. Acad. Sci. USA* **105,** 18132–18138.

Mikesh, L. M., Ueberheide, B., Chi, A., Coon, J. J., Syka, J. E., Shabanowitz, J., and Hunt, D. F. (2006). The utility of ETD mass spectrometry in proteomic analysis. *Biochim. Biophys. Acta* **1764,** 1811–1822.

Motoyama, A., Xu, T., Ruse, C. I., Wohlschlegel, J. A., and Yates, J. R. III (2007). Anion and cation mixed-bed ion exchange for enhanced multidimensional separations of peptides and phosphopeptides. *Anal. Chem.* **79,** 3623–3634.

O'Farrell, P. H. (1975). High resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.* **250,** 4007–4021.

Olsen, J. V., de Godoy, L. M., Li, G., Macek, B., Mortensen, P., Pesch, R., Makarov, A., Lange, O., Horning, S., and Mann, M. (2005). Parts per million mass accuracy on an Orbitrap mass spectrometer via lock mass injection into a C-trap. *Mol. Cell. Proteomics* **4,** 2010–2021.

Olsen, J. V., Schwartz, J.C., Griep-Raming, J., Nielsen, M. L., Damoc, E., Denisov, E., Lange, O., Remes, P., Taylor, D., Splendore, M., Wouters, E. R., Senko, M., *et al.* (2009). A dual pressure linear ion trap orbitrap instrument with very high sequencing speed. *Mol. Cell Proteomics.* **8**(12), 2759–2769. [Epub 2009 Oct 14].

Ong, S. E., Blagoev, B., Kratchmarova, I., Kristensen, D. B., Steen, H., Pandey, A., and Mann, M. (2002). Stable isotope labeling by amino acids in cell culture, SILAC, as a simple and accurate approach to expression proteomics. *Mol. Cell. Proteomics* **1,** 376–386.

Ong, S. E., Kratchmarova, I., and Mann, M. (2003). Properties of 13C-substituted arginine in stable isotope labeling by amino acids in cell culture (SILAC). *J. Proteome Res.* **2,** 173–181.

Rappsilber, J., Mann, M., and Ishihama, Y. (2007). Protocol for micro-purification, enrichment, pre-fractionation and storage of peptides for proteomics using StageTips. *Nat. Protoc.* **2,** 1896–1906.

Ross, P. L., Huang, Y. N., Marchese, J. N., Williamson, B., Parker, K., Hattan, S., Khainovski, N., Pillai, S., Dey, S., Daniels, S., Purkayastha, S., Juhasz, P., *et al.* (2004). Multiplexed protein quantitation in *Saccharomyces cerevisiae* using amine-reactive isobaric tagging reagents. *Mol. Cell. Proteomics* **3,** 1154–1169.

Schwartz, J. C., Senko, M. W., and Syka, J. E. (2002). A two-dimensional quadrupole ion trap mass spectrometer. *J. Am. Soc. Mass Spectrom.* **13,** 659–669.

Scigelova, M., and Makarov, A. (2006). Orbitrap mass analyzer—overview and applications in proteomics. *Proteomics* **6**(Suppl. 2), 16–21.

Shevchenko, A., Jensen, O. N., Podtelejnikov, A. V., Sagliocco, F., Wilm, M., Vorm, O., Mortensen, P., Shevchenko, A., Boucherie, H., and Mann, M. (1996). Linking genome and proteome by mass spectrometry: Large-scale identification of yeast proteins from two dimensional gels. *Proc. Natl. Acad. Sci. USA* **93,** 14440–14445.

Vermeulen, M., Hubner, N. C., and Mann, M. (2008). High confidence determination of specific protein–protein interactions using quantitative mass spectrometry. *Curr. Opin. Biotechnol.* **19,** 331–337.

Washburn, M. P., Wolters, D., and Yates, J. R. III (2001). Large-scale analysis of the yeast proteome by multidimensional protein identification technology. *Nat. Biotechnol.* **19,** 242–247.

Whitehouse, C. M., Dreyer, R. N., Yamashita, M., and Fenn, J. B. (1985). Electrospray interface for liquid chromatographs and mass spectrometers. *Anal. Chem.* **57,** 675–679.

Winzeler, E. A., Shoemaker, D. D., Astromoff, A., Liang, H., Anderson, K., Andre, B., Bangham, R., Benito, R., Boeke, J. D., Bussey, H., Chu, A. M., Connelly, C., *et al.* (1999). Functional characterization of the *S. cerevisiae* genome by gene deletion and parallel analysis. *Science* **285,** 901–906.

Wisniewski, J. R., Zougman, A., Nagaraj, N., and Mann, M. (2009). Universal sample preparation method for proteome analysis. *Nat. Methods* **6,** 359–362.

Wiśniewski, J. R., Zougman, A., Mann, M. (2009). Combination of FASP and StageTip-based fractionation allows in-depth analysis of the hippocampal membrane proteome. *J. Proteome Res.* **8**(12), 5674–5678.

Yao, X., Freas, A., Ramirez, J., Demirev, P. A., and Fenselau, C. (2001). Proteolytic 18O labeling for comparative proteomics: Model studies with two serotypes of adenovirus. *Anal. Chem.* **73,** 2836–2842.