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CHAPTER 3

Methods for proteomics in neuroscience

Nilesh S. Tannu* and Scott E. Hemby

Department of Physiology and Pharmacology, Wake Forest University School of Medicine, Winston-Salem, NC 27157, USA

Abstract: Proteomics reveals complex protein expression, function, interactions and localization in different phenotypes of neuron. As proteomics, regarded as a highly complex screening technology, moves from a theoretical approach to practical reality, neuroscientists have to determine the most-appropriate applications for this technology. Even though proteomics compliments genomics, it is in sheer contrast to the basically constant genome due to its dynamic nature. Neuroscientists have to surmount difficulties particular to the research in neuroscience; such as limited sample amounts, heterogeneous cellular compositions in samples and the fact that many proteins of interest are hydrophobic proteins. The necessity of exclusive technology, sophisticated software and skilled manpower tops the challenge. This review examines subcellular organelle isolation, protein fractionation and separation using two-dimensional gel electrophoresis (2-DGE) as well as multi-dimensional liquid chromatography (LC) followed by mass spectrometry (MS). The methods for quantifying relative gene product expression between samples (e.g., two-dimensional difference in gel electrophoresis (2D-DIGE), isotope-coded affinity tag (ICAT) and iTRAQ) are elaborated. An overview of the techniques used currently to assign post-translational modification status on a proteomics scale is also evaluated. The feasible coverage of the proteome, ability to detect unique cell components such as post-synaptic densities and membrane proteins, resource requirements and quantitative as well as qualitative reliability of different approaches is also discussed. While there are many challenges in neuroproteomics, this field promises many returns in the future.

Keywords: proteomics; brain; 2-DGE; 2D-DIGE; MuDPIT; ICAT; iTRAQ; mass spectrometry

Introduction

The comprehensive sequencing of human and other important genomes has immensely enhanced our insight of the cellular machinery of higher organisms. This has been largely accomplished by the innovations in large-scale analysis of mRNA expression, viz. microarrays, serial-analysis of gene expression (SAGE) and differential display into gene expression (Venter et al., 2001). It is desirable to complement the global gene expression analyses with studies examining the corresponding proteomes. The hypotheses-based approach has always been foremost to an unbiased approach to determine organized changes encompassing the expression of entire proteome. The early proteomic research, ~6000 scientific publications, have been directed toward cataloging proteins and constructing protein databases (Anderson et al., 2001). This stepping-up has made us understand that higher organisms have many differences in the controlling mechanisms of cellular function. The most obvious insight has been the understanding that a plethora of proteins are produced by a single gene in higher organisms. These rapid advancements have improved our understanding of the cellular machinery within the brain and its

*Corresponding author. Tel.: +1-336-716-8589; Fax: +1-336-716-8501; E-mail: ntannu@wfubmc.edu

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role in health and disease. The dynamic nature of proteome; changing nature of protein-expression profiles during cell cycle and with the intra and extracellular stimuli, alternative splicing and the post-translational modifications (PTM) has made a paradigm shift in the neuroscience field to analyze cellular proteomes by various proteomics methodologies. To realize the elaborate neuroadaptive mechanism in health and disease, it has become elementary to determine the global alternations in proteins by simultaneous assessment of all proteins in a cell (Freeman and Hemby, 2004).

Proteins carry out the greater part of biological events in the cell, even though certain mRNAs can act as effector molecules. mRNA expression may not directly associate with protein expression as mRNA is not the working endpoint of gene expression (Anderson and Seilhamer, 1997; Gygi et al., 1999b). Also, there has been sufficient evidence recently that over 50% of all genes are subject to transcriptional variation by RNA splicing and editing accountable for production of specific isoforms in various cell/tissue types. Sufficient evidence also points toward significant transcriptional and PTM of proteins controlling cellular functions (Roberts and Smith, 2002). Nearly 30–50% of mammalian genes are expressed in the central nervous system (CNS). Proteomic analysis of brain regions may be useful to study the differential patterns of gene expression in order to investigate the complexity of CNS disorders (Fountoulakis, 2004). It is necessary to emphasize the fundamental difference in the study of proteomics as compared to genomics. Proteins have no base pairing and consequently there is no technique of protein amplification like the polymerase chain reaction (PCR) (Mullis, 1990) or antisense RNA (aRNA) (Van Gelder et al., 1990). Nucleic acid hybridization relies on base pairing and the construction of probes that will recognize a specific nucleic acid sequence of interest. However, for proteomic research the recognition of specific proteins is more difficult. The average protein concentration is 10^2–10^8 copies/cell; on the other hand, the rapid turnover of mRNAs is responsible for their average concentration to range from 10^{-4} to 10^2 copies/cell. Thus, proteomics technology has an inherent advantage over genomics for investigating small number of cells (Holland, 2002; Godovac-Zimmermann et al., 2005).

The expansion of proteomic technologies can be ascribed to the rapid development of mass spectrometry (MS), bioinformatics and the current accessibility of vast information from genome sequencing of many organisms. Rather than traditional approaches which examined one or a few proteins at a time, in a few samples, proteomics attempts to concurrently examine large numbers (thousands) of proteins in mutliple samples. Proteomics, a technology-driven science, involves the study of each and every protein in a biological system with respect to structure, change in expression level, protein–protein interactions, PTM as well as the study of multi-protein complexes, coined as structural-, functional- and expression-proteomics, respectively. Most of the initial effort in proteomics was directed toward protein identification and determination of relative abundances. The most-widely developed field, possible at large-scale in automatic mode, is of expression proteomics and is by default the preferred approach of most studies involving proteomics. However, changes in protein abundance exclusively do not define protein function as many of their vital activities are brought about by PTM and do not have the ability to resolve regulatory mechanisms that affect protein abundance and function such as protein–protein interactions and subcellular distribution. Therefore, it is unrealistic that a single strategy will suffice to unravel all the protein complexities in a tissue or cell type.

In addition to the classical approach of expression proteomics to study the various aspects of global proteomics, it is necessary to use variety of differing strategies The development of innovative strategies has been ongoing in neuroproteomics in particular for studying the PTM, mapping of proteins from multi-protein complexes and mapping of organelle proteomes (Dreger, 2003b). Significant innovations in MS continue to have a major impact as well as further the field of neuroproteomics. Routine unequivocal and high-throughput protein identification has been made possible by the nano-electrospray combined with the hybrid quadrupole time-of-light mass spectrometer tandem mass analyzer (ESI Q-TOF MSMS)

as well as by matrix-assisted laser desorption-ionization (MALDI) Q-TOF MSMS tandem MS and MALDI-TOF-TOF tandem MS. To know the identities of thousands of different proteins in neurons along with their expression levels, their PTMs as well as protein–protein interaction maps would revolutionize neurobiology and medicine by detecting novel drug targets and diagnostic biomarkers (Fountoulakis, 2004). The present review will analyze applications to familiarize neuroscientists with the available tools for proteome research.

Subcellular fractionation

Biological samples subjected to proteomic analysis in neuroproteomics encompass cell populations, tissues and CSF. These samples are extremely complex as the protein constituents vary in charge, molecular mass, hydrophobicity, PTM and occurrence in complex and subcellular location. The coding genes for CNS oscillate between 25,000 and 30,000 (Southan, 2004). This added complexity of neuroproteome will be overwhelming if we hypothesize that each protein on an average has 10 splice variants, cleavage products and PTM leaving us to analyze almost 250,000–300,000 protein forms. Currently, there are no proteomic methods, which have the capacity to segregate and identify this many proteins at a time. Therefore, fractionation of the entire proteome into distinct fractions to be analyzed separately for content and phenotypic differences is of paramount importance. Each neuron has proteins, which are compartmentalized, providing distinct environments for biological processing such as protein synthesis, degradation, energy production, DNA replication, etc. Therefore, protein localization is normally linked to its function and subcellular fractionation reduces the complexity of the neuroproteome to be analyzed by segregating proteins based on their cellular locations. Tannu et al. (2004a) and many other groups have documented that the subcellular fractionation enables the potential enrichment of lower-abundance proteins (such as signaling molecules) by allowing higher starting amount (at least 3–8 times) than whole-cell proteome analysis.

Most of the organelles in the neuron have been initially characterized by subcellular fractionation and microscopy; however, a complete registry of the proteins in each organelle is yet to be made. Even in physiological states some proteins are translocated between different compartments, such as shuttling between nucleoplasm and cytoplasm. In many diseases the change in gene expression is preceded by translocation events which do not alter the overall abundance of proteins in the entire neuron. A very opportune example for a neuroscientist will be the shuttling of neurotransmitter receptors between the membrane and the cytoplasmic pools in a synapse, which have been associated with synaptic plasticity (Malinow and Malenka, 2002). From a neuroscience point of view, studying differing amounts of protein in different compartments has functional significance as compared to the study of total amount in a neuron. In studies involving subcellular proteomics, fractionation strategies are of prime importance with respect to the accuracy of the proteomics data which assigns new gene products to a particular subcellular location.

The most important caveats for subcellular fractionation are (1) the varying extent of enrichment, (2) differential isolation of cytoskeleton components with organelles and (3) current fractionation techniques which enrich one particular subcellular structure. An ideal proteome study involves monitoring multiple subcellular structures in parallel (Yates et al., 2005). Post-analyses methods, high-throughput prediction tools accessible though Internet are routinely used to validate the subcellular location. These are based on the in-silico analyses of the primary structure of unknown gene products giving cues to credible protein functions and subcellular locations. The programs routinely used and available publicly are: MitoProt (http://ihg.gsf.de/ihg/mitoprot.html) for mitochondrial, SignalP (http://www.cbs.dtu.dk/services/SignalP/) for signal sequences and TargetP (http://www.cbs.dtu.dk/services/TargetP/) and/or PSORT (http://psort.ims.u-tokyo.ac.jp/) for general subcellular location prediction (Dreger, 2003b). Notwithstanding the caveat, the study of neuroproteomics by subcellular fractionation-proteome analysis will help in mining low abundance and membrane

proteins and at the same time assign most of the specific proteins to specific subcellular structures and provide valuable insight into the physiological mechanisms and the molecular basis of pathological events. Most of the fractionation strategies are similar for tissues as well as the cell populations. However, the CSF proteins are in a different environment, as compared to the tissues and cell populations, and their segregation strategies will be discussed separately (Dreger, 2003a; Swatton et al., 2004; Righetti et al., 2005; Yates et al., 2005; Wang and Hanash, 2005). It must be noted at this point that the discussion will include the studies involving non-neuronal tissues for instances where no data exists for neuronal tissues. One should also consider that detailed fractionation conditions are valid for particular tissues and may/may not work on different tissue. However, the outlined strategies should serve as an important starting point to standardize the protocol for tissue under consideration. The goal of neuroproteomics development is to gain the ability to simultaneously study nuclear envelope (NE), nuclear pore complex (NPC), nucleolus, golgi apparatus, mitochondria, perioxisomes, cytoplasm, membrane, synapse and post-synaptic density (PSD) fractions of different phenotypical neurons (Schirmer and Gerace, 2002; Schirmer and Gerace, 2005). The fractionation strategies which remain unique for brain tissue are for the synaptosomes, PSD and the CSF. These will be discussed separately to stress their importance in neuroscience.

Cerebrospinal fluid

Even though CSF, secreted by the choroid plexus in the lateral ventricles, is mostly found in the four ventricles it is an important determinant of the extracellular fluid (ECF) surrounding neurons and glia in the CNS. The changes in the brain which affect the proteins involved in biochemical pathways may be reflected in CSF (e.g., change in the CSF levels of total-tau protein and the light subtype of the neurofilament proteins (NF-L) after acute ischemic stroke (Hesse et al., 2001) and the elevation of the tau and phosphorylated tau in the CSF of Alzheimer's disease (AD) patients (Tapiola et al., 1997)). CSF is also found in the subarachnoid space flowing down the spinal canal as well as upwards over the brain convexities. The CSF composition is in steady state with the brain ECF and, therefore, has an important function of maintaining a constant external environment for neurons and glia. It also functions to remove harmful brain metabolites, provide mechanical cushion, and serve as a lymphatic system and as a conduit for peptide hormones secreted by hypothalamus. CSF contains proteins, peptides, enzymes, small molecules and salts which play an important role in many physiological processes e.g., CSF pH is an important regulator of pulmonary ventilation and cerebral blood flow. Even though most of the proteins in CSF are derived either directly from neuronal cells or actively transported by pinocytosis across the blood–brain barrier (BBB) some proteins are synthesized intrathecally e.g., prostaglandin D2 synthase and cystatin C. CSF is not simply an ultrafiltrate of serum but a highly specific repository of cellular byproducts, metabolites, neurotransmitters and proteolytic fragments making analysis of its proteome crucial for understanding neurobiology and neuropathology (Wildenauer et al., 1991; Zheng et al., 2003).

While the proteomic studies of neuronal tissue has the challenges of dealing with deterioration of the BBB in postmortem tissues (PMT) or perform invasive biopsies from antemortem tissues, CSF proteomics is amenable for serial analysis by minimal invasive lumbar puncture either ante or postmortem. The serial evaluation of CSF proteome in context of disease progression and treatment efficiency is a potential clinical application of proteomics that can be used from bench to bedside. A change in protein expression may yield important insight into various CNS diseases by improving our understanding of the molecular basis of disease as well as providing early-stage biomarkers. The CSF has a low protein concentration ($\sim$150–450 μg/ml) and a high salt concentration ($>$150 mmol/l). Despite the low total protein concentration, the concentration of albumin ($\sim$60% of the total CSF protein) (Hammack et al., 2003) and immunoglobulin is extremely high in the CSF. First, the salt concentration should be adjusted in

the CSF in a way so that the final concentration of salts should be <10 mmol/l in rehydration buffer. Second, the high-abundance proteins, albumin and immunoglobulin, should be depleted so that the lower-abundance proteins can be resolved. We briefly describe the strategies that have been tested to circumvent the above problems.

Desalting

Until recently, studies using desalting techniques have reported varying degrees of success with respect to the recovery of the proteins from CSF. A brief summary of the techniques used till date follow.

Protein precipitation: Protein precipitation has been undertaken using ice-cold ethanol, trichloroacetic acid (TCA) in acetone and acetone. Hansson et al., (2004) showed 100% recovery of CSF proteins using $>70\%$ ice-cold ethanol for 2 h at -20°C. Ice cold 80% acetone precipitation for 2 h at -20°C, on the other hand showed mixed reproducibility of 40–50% (Sickmann et al., 2000) and 94% (Yuan et al., 2002 recovery. However, precipitation with TCA in acetone (4:10%:1, v/w/v) for 45 min at -20°C as well as chloroform/methanol (4:8:3, v/v/v) at room temperature for 2 h showed very low recovery of 23% CSF proteins (Yuan et al., 2002; Hansson et al., 2004).

Bio-Spin column (Bio-Rad): Bio-Spin polyacrylamide micro-column with a M_W cutoff of 6 kDa gave less recovery as compared with the ethanol precipitation for the Hansson group (Hansson et al., 2004). On the other hand, the study conducted by (Yuan 2002; Yuan et al., 2002), and Terry (Terry and Desiderio, 2003) showed a recovery of 91% and 99%, respectively.

Ultra filtration: The Ultrafree MC with a cutoff of 5 kDa and Centricon with a cutoff of 3 kDa (Millipore, Bedford, MA, USA) was used by Sickmann et al. (2000) and Hammack et al. (2003), respectively, for desalting of the CSF followed by protein concentration. This approach tested by them showed a recovery of more than 70% CSF proteins by both the groups.

Dialysis: This method was compared directly by Hammack et al. (2003) with the ultra filtration. There was a non-specific loss of 40–60% proteins using MWCO dialysis tubing (Spectrum, Rancho Dominguez, CA, USA) for 12 h. From a range of methods applied for desalting of CSF, 70% ice-cold ethanol shows the most promise. The protein recovery and the separation of proteins on a 2D-gel are crucial factors that decide the efficiency of the above methods. The ultrafiltration, which losses most of the protein due to their adsorption to the filter has a better protein recovery than dialysis. On the other hand, even though the acetone precipitation had a higher recovery of proteins, the vertical and horizontal streaking on the 2D-gels marred the image analysis quality. The Bio-Spin column has an overall better recovery of proteins with the image quality of 2D-gels well preserved.

Protein depletion

To have a realistic opportunity of analyzing the low-abundance CSF proteins, it becomes crucial to deplete the CSF sample from albumin and immunoglobulin which constitute 50–60% of the CSF protein concentration.

Affinity removal: There is a loss of albumin-binding proteins during albumin depletion. This was shown during depletion study of albumin and immunoglobulin using a Cibacron Blue F3G-A (Blue Sepharose 6 Fast Flow) and protein G (Prosep-G), respectively by Raymackers et al. (2000) and Hammack et al. (2003). The loss of proteins bound to albumin can be minimized by segregating them by a separate experiment. Another problem encountered was the low binding of lipoproteins and enzymes to Cibacron Blue F3G-A. As the above kits had been specifically designed for serum, preconcentration of the CSF is recommended. Recently highly specific immobilized anti-albumin and anti-immunoglobulin antibodies were developed by Pierce (ProteoSeek™) and Sigma (ProteoPrep®), which claim the depletion of approximately 90–95% of albumin and immunoglobulin. More recently ProteoPrep® 20 has been developed by Sigma to deplete the 20-most abundant proteins

which constitute approximately 97% of the total protein amount.

Liquid-phase isoelectric focusing: Prefractionation method in which CSF proteins are segregated into different fractions based on charge. Each of these fractions is then run separately on a 2-D gel for further segregation. This technique has shown to facilitate detection of less-abundant protein components, reduce sample complexity, increase the protein load and the protein amount in each gel spot for MALDI-MS analysis as compared to unfractionated CSF by Davidsson et al. (2002).

Solid-phase extraction: This method utilizes the differential hydrophobic nature of proteins to separate CSF proteins into three different fractions using a solid-phase extraction cartridge (Yuan and Desiderio, 2005). This technique showed the enrichment of low-abundance CSF proteins at the same time resulting in the ability to resolve them well from high abundance proteins (Schirmer and Gerace, 2005).

Synaptosomes and post-synaptic density

Synapses can be purified in vitro and are called as synaptosomes. Synaptosomes constitute the entire pre-synaptic terminal (including mitochondria and synaptic vesicles) and portions of the post-synaptic terminal (including post-synaptic membrane and PSD) and are considered as highly specific intercellular junctions responsible for transmission of signals within CNS. The study of the proteomes of synapse as well as the PSD is an important starting point in neuroscience to understand complex brain functions.

Synaptosomes are subcellular membranous structures formed during mild disruption of brain tissue. The shearing forces cause the nerve endings to break off and subsequent resealing of the membranes form the synaptosomes. Synaptosomes have a complex structure equipped with components of signal transduction, metabolic pathways and organelles, as well as structural components required for vesicular transport. Schrimpf et al. (2005) characterized 1131 proteins from synaptosomal fractionation belonging to the following categories: proteins involved in exo- and endocytosis; guanine nucleotide-binding proteins and their regulators; synaptic adhesion molecules and ligands; PSD proteins; cytoskeletal proteins; enzymes involved in transmitter synthesis and degradation; transporter proteins; receptor proteins. Synaptosomes can be isolated from brain homogenate by differential and density-gradient centrifugation (Schrimpf et al., 2005). Briefly, brain tissue is homogenized in 5 mM HEPES and 320 mM sucrose (pH 7.4) using a Potter–Elvehjem homogenizer (800 rpm, 12 passes). The cell debris and nuclei are removed by centrifugation of the homogenate twice for 5 min at $1000 \times g$, and the combined supernatant is centrifuged for 20 min at $12000 \times g$. The resulting pellet consists of a colored layer comprising the mitochondria and a white pellet composed of the synaptosomes. On whirl mixing the synaptosomal pellet gets resuspended whereas the mitochondrial pellet remains. This is repeated twice and the suspended synaptosomal pellet is layered on a Ficoll gradient comprising of 4.8 ml of 12% Ficoll overlaid with 4.8 ml of 7.5% Ficoll. Centrifugation at $6{,}8999 \times g$ for 1 h, the synaptosomal fraction is enriched at the junction of 7.5%/12% Ficoll as a cream-colored layer. The synaptosomes are then recovered by aspiration, resuspended in Krebs' solution. The suspended synaptosomes are centrifuged for 20 min at $12{,}000 \times g$ to be recovered as a pellet.

The PSD is a disk-like structure with a thickness of ~30–40 nm and width of ~100–200 nm.The most important structures associated with the PSD are the cytoskeletal proteins, regulatory enzymes, and neurotransmitter receptors and associated proteins. There has been more than one fractionation method used for the segregation of PSD in proteomic studies. Recently, two groups successfully characterized 244 and 374 proteins, respectively from PSD fractions. Trinidad et al. (2005) as well as Peng et al. (2004) found proteins know to be in the PSD, NMDA receptor subunits NR1A and NR2A as well as the associated PSD-95, to be highly enriched whereas the pre-synaptic protein synaptophysin to be undetectable. The proteins identified in the PSD fractions belonged to the

following groups: scaffold and adaptor proteins; signaling proteins; cytoskeletal and interacting proteins; phosphoproteins; proteins involved in trafficking; proteins involved in energy production and transfer; ubiquitination system; the receptors, ion channels and adhesion proteins; kinases, phosphotases and regulators. These studies were successful in characterization of many proteins that had not been previously associated with PSD, opening up possibilities of their involvement in synaptic morphology and signal transduction. A major concern of these studies was the contamination from subcellular structures unrelated to PSD such as pre-synaptic proteins, housekeeping proteins, mitochondrial proteins, glial cytoskeleton and myelin sheaths. It is imperative to follow up such studies with more focused experiments to confirm whether these are bona fide PSD proteins. As the methods are similar, for brevity we will discuss the method used by Peng et al. (2004). Briefly, the isolated adult rat forebrain was homogenized using Teflon homogenizer (12 passes) in buffer A comprising of 5 mM HEPES (pH 7.4), 1 mM $MgCl_2$, 0.5 mM$CaCl_2$, 1 mM NaF, 1 mM β-glycerophosphate, 0.1 mM PMSF, 1 μg/ml aprotonin, 1 μg/ml leupeptin, 1 mM benzamidine, 0.1 mM pepstatin and the phosphatase inhibitor mixture I (Sigma). All the purification steps were performed at 4°C. This homogenate was centrifuged for 10 min at 1400 × *g* to obtain supernatant (S1) and pellet (P1). The pellet P1 was homogenized again with the Teflon homogenizer (5 strokes). After centrifugation at 700 × *g*, the supernatant (S1″) was pooled with S1 to be centrifuged for 10 min at 13800 × *g* to collect pellet P2. A P2 suspension was created in buffer B (0.32 M sucrose, 6 mM Tris (pH 8.0), 1 mM NaF, 1 mM β-glycerophosphate, 0.1 mM PMSF, 1 μg/ml aprotonin, 1 μg/ml leupeptin, 1 mM benzamidine, 0.1 mM pepstatin) using 5 strokes of Teflon homogenizer. A discontinuous sucrose gradient comprising of 0.85 M/1 M/1.15 M sucrose solution in 6 mM Tris (pH 8.0) was loaded with P2 suspension and centrifuged at 82500 × *g* for 2 h using SW-41 rotor. The synaptosomal fraction at the junction of 1 and 1.15 M sucrose was collected using a syringe and needle and was made up to 4 ml using the buffer B. An equal volume of buffer constituting of 6 mM Tris (pH 8.1) and 1% Triton X-100 was added to the above suspension mixed for 15 min and centrifuged for 20 min at 32,800 × *g* using Ti70.1 rotor. The pellet was brought up in buffer made of 6 mM Tris (pH 8.1) and 0.5% Triton X-100, and centrifuged for 1 h at 201,800 × *g*. The resulting pellet is the PSD fraction used for further proteomic analysis.

Nuclei, mitochondria, cytoplasm and membrane

Several recent proteomics studies have employed fractionation methods that allow collection of multiple cellular components from one tissue source (Fountoulakis, 2004; Tannu et al., 2004a). The major benefit of these studies has been the ability to compare by enriching the low-abundance proteins, which were not detectable by analysis of the whole-cell proteome. This has enabled the analysis of important signaling molecules. The coverage of the proteome analyzed is also increased as the proteins spots from different fractions have an additive effect toward the whole proteome. As the fractions are from the same cellular subset they minimize the experimental variability. The crucial drawback has been the overlap of the proteins between fractions. It is important to differentiate between the proteins that are cross-contaminants. A general approach to segregate the above-mentioned fractions in a single experiment is schematized in Fig. 1 and described in detail previously (Tang et al., 2003; Tannu et al., 2004a; Fountoulakis, 2004). Briefly, tissue samples are dounce homogenized in 10 mM HEPES, 10 mM NaCl, 1 mM KH_2PO_4, 5 mM $NaHCO_3$, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 5 mM EDTA and the following protease inhibitors (PI): 1 mM PMSF, 10 mM benzamidine, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 μg/ml pepstatin and centrifuged at 9645 × *g* for 5 min. Supernatant (cytosol and membrane) is removed and the pellet (nuclei and debris) resuspended in 20 mM Tris HCl, 1 mM EDTA (pH = 8.0) with PIs and centrifuged at 9645 × *g* for 5 min. This procedure is repeated twice and the pellet is resuspended in the solution and stored at −20°C (nuclear fraction). The supernatant is then centrifuged at 107 170 × *g* for

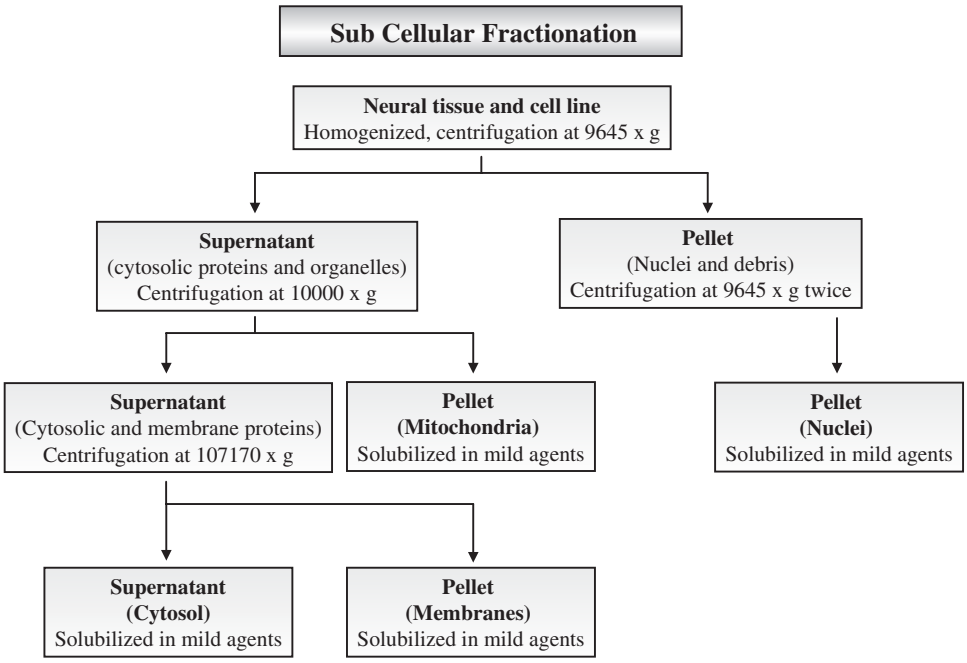

Fig. 1. Schematic representation of subcellular fractionation technique commonly used prior to neuroproteomic analysis (elaborate description in text). Adapted with kind permission from Wiley (Fountoulakis, 2004).

30 min at 4°C. Following, the supernatant containing the cytosolic fragment is removed and stored at −20°C (cytosolic fraction). The pellet is resuspended in 10 mM Tris (pH = 7.5), 300 mM sucrose, 1 mM EDTA (pH = 8.0), 0.1% NP40 and PIs and centrifuged at 4287 × *g* for 5 min at 4°C. The supernatant is discarded and the pellet resuspended in the buffer and washed three times before resuspension in the buffer and protease inhibitors and storing the samples at −20°C (membrane fraction).

Mitochondria

The mitochondrion is a complex structure involved in fundamental processes, such as the TCA cycle, β-oxidation of fatty acids, urea cycle, electron transport, oxidative phosphorylation, apoptosis and heme synthesis, dysfunction of which is observed in many neurological degenerative diseases (Alzheimer, Parkinson and Huntington Disease). For example, mitochondria in frontal and temporal cortex of AD are decreased 25% of normals. Neuroproteomic analysis of the mitochondria has focused on the abundance of the mitochondrial proteins in different brain regions (Lovell et al., 2005; Zhang et al., 2005). Cataloging mitochondrial proteomes from different species and tissues have documented 400–700 mitochondrial-associated proteins (Mootha et al., 2003; Sickmann et al., 2003; Taylor et al., 2003). These datasets will enable scientists to compare protein orthologues between species to better understand the mitochondrial machinery. Mootha et al. (2003) identified 591 mitochondrial proteins of which approximately 170 proteins had not been previously linked to mitochondria. This important study enabled the identification of a single candidate gene (LRPPRC) for the French-Canadian-type of Leigh syndrome by integration of the proteomics and the RNA-expression data with the genotype data. We briefly present a method used by Mark A Lovell to fractionate mitochondria from primary rat cortical neuron cultures. A FractionPREP cell fractionation kit (BioVision, Mountain View, CA, USA) was used to isolate the mitochondria. The cell pellet lysed in 500 µl lysis buffer (on ice for 15 min) was centrifuged twice at 2000 × *g* for 5 min at 4°C to pellet nuclei

and cell membranes. The supernatant was further centrifuged for 30 min at 11,000 × *g* (4°C). The mitochondria were then rinsed in ice-cold PBS (3–5 times) and resuspended in 200 µl distilled/deionized water (DW) and homogenized using micro-Dounce homogenizer on ice.

Membranes

Based on the recent sequencing information from several genomes, membrane proteins constitute 20–30% of all the cellular proteins. Plasma membrane proteins are involved in various important cellular processes in the brain including signal transduction, cell adhesion, exocytosis and metabolite and ion transport (Stevens et al., 2000). Even though the lipids form an important constituent of the membrane, the membrane-spanning proteins confer unique functions to the membranes forming the means of communication between separated structures. As the membrane proteins are amphipathic, their hydrophobic nature makes them difficult to study and necessitates different strategies for analysis as compared to the rest of the cellular proteins. Therefore, while great strides have been made toward the analysis of soluble cellular proteins, membrane proteins reported in majority of the proteomic analyses have been under-represented (Wilkins et al., 1998). The importance of characterization of membrane proteins cannot be over emphasized; they account for approximately 70% of all the drug targets and present an enormous challenge for the modern proteomics initiative (Wu et al., 2003). Even though some reports showed improved solubilization using different buffers (zwitterionic detergent MEGA 10 (decanoyl-N-methylglucamide), zwitterionic lipid LPC (1-lauroyl lysophosphatidylcholine)) and detergent (1, 2-diheptanoyl-*sn*-glycero-3-phosphatdiyl choline (DHPC)), the traditional proteomic approach of two-dimensional gel electrophoresis (2–DGE) has many limitations for analyzing membrane proteins (Churchward et al., 2005). The most important limitations are that most hydrophobic proteins are insoluble in non-detergent isoelectric focusing (IEF) sample buffer, the ones which are insoluble are precipitated at their isoelectric point (pI), they are in low abundance compared to hydrophilic proteins; the pIs of hydrophobic proteins are generally alkaline and even the use of extended pH gradients are difficult to resolve well at the basic ends. The carrier ampholytes inhibit the interaction between the hydrophobic proteins and the immobiline. This prevents the precipitation and the subsequent streaking of the basic end of the gels. Irrespectively, the liquid chromatography (LC)-MS methods have an ascendancy in resolving the issues of the hydrophobic membrane protein separation. To overcome the problems of 2-DGE associated with resolving membrane proteins, several studies have used SDS-PAGE followed by LC-MS to catalog membrane proteins (Ferro et al., 2002; Galeva and Altermann, 2002). The principle issue faced by this approach is the inaccessibility of cleavage sites of the membrane-spanning domains to trypsin and the limited sequence coverage, confidence, with which proteins are characterized.

An attractive alternative is shotgun/multidimensional protein identification technology (MuDPIT) proteomics where proteins are first digested by proteases and the complex peptide *mixture* is analyzed by LC-MS-based methods, although significant computing recourses are required for analysis. This strategy has greatly enhanced sensitivity for detecting mass changes due to covalent modifications making it amenable for detection of PTMs as well as giving insights into protein topology. The methods recently employed to address the issue of solubility of membrane proteins have used detergents (Navarre et al., 2002), organic solvents (Blonder et al., 2002) and organic acids (Washburn et al., 2001).

Organic acid: This method utilized 90% formic acid in the presence of cyanogen bromide to solubilize yeast membrane-enriched fraction. The concentrated formic acid provides the solubilization agent. The cyanogen bromide on the other hand is active in this acidic ambiance, when it is able to cleave the embedded membrane proteins. The fragments were further digested serially by proteinase LysC and trypsin followed by peptide characterization by MuDPIT. MuDPIT allows peptide separation by 2-DLC(separation based on

charge and hydrophobicity) and identification by in- or off-line tandem MS. This qualitative method was successful in identifying 131 integral membrane proteins (Washburn et al., 2001).

Detergents: The detergent is used to solubilize the membrane proteins. Microsomal membrane-enriched fraction was boiled using 0.5% SDS. Labeling with isotope-coded affinity tag (ICAT) was followed by dilution of SDS, so that the SDS concentration was compatible with trypsin digestion. The peptide mixture was separated by sequential cation exchange chromatography, avidin affinity chromatography and reverse-phase microcapillary HPLC for MS analysis resulting in identification of 491 proteins. This method offers the ability to quantitatively analyze the sample while at the same time improving the recovery of low-abundance integral-membrane proteins.

Organic solvents: The organic solvents can also be used to solubilize the membrane proteins. Blonder et al. (2002) thermally denatured and sonicated membrane-enriched fraction of *Deinococcus radiodurans* in 60% methanol in presence of trypsin. This method was also used for quantitation as well as detection of low-abundance proteins by coupling with ICAT (Goshe et al., 2003).

High pH: The high pH method prevents the resealing of the membrane structures after mechanical agitation thereby maintaining the native topology, as against the methods of organic acids, solvents and detergents (Goshe et al., 2003). The sample is incubated at 37°C for 3 h at a high pH (200 mM Na_2CO_3, pH 11) favoring the formation of membrane sheets and proteinase K (hpPK, 5 μg) cleaving the membrane protein hydrophilic domains. A MuDPIT analysis of the lysate identified 1610 proteins with the cellular proportion of membrane to soluble being close to 1:2.5.

Building on the initial analyses of membrane proteins from other tissues, recent studies have attempted to map the brain plasma membrane proteome. Nielsen et al. (2005) used the conventional plasma membrane isolation approach. A stepwise depletion of mouse brain cortex to remove the non-membrane proteins using high salt, carbonate and urea washes was performed. This was followed by treatment with sublytic concentrations of digitonin and density gradient fractionation. The enriched membrane fraction was lysed by endoproteinase LysC to identify about 1000 membrane proteins. Schindler et al. (2005) described a membrane fractionation protocol compatible with small amounts of brain tissue (e.g., cerebellum of a single rat) so that distinct functional and anatomical regions of brain from model animals can be studied (Schindler et al., 2005). Affinity-based partitioning of microsomes in an aqueous two-phase (polyethylene glycol (PEG) and dextran) system was used to enrich the plasma membrane fraction. Membranes from different subcellular fractions were separated based on their charge and hydrophobicity on the two-phase system. The plasma membranes have higher affinity for PEG phase and were separated on the top. Close to 500 proteins were characterized by LC-MS/MS, of which 197 (42%) proteins were bona fide plasma membrane proteins. Besides the proteins from plasma membrane such as transporters, channels and neurotransmitter receptors e.g., cerebellum-specific GABA receptor GABAR6, proteins belonging to the mitochondrial (1.2%) as well as endoplasmic reticulum (1%) membrane were also identified, signifying the cross-contamination between cellular compartments.

Nucleus

The nucleus has a high degree of organization, consisting of structurally and functionally distinct compartments; nucleolus, nuclear speckles, NPC and the nuclear envelope. The nucleus is a highly organized organelle consisting of domains fundamental for preserving the homeostasis of the cellular milieu.

Nuclear envelope

NE is a double-membrane system (inner and outer) continuous with the endoplasmic reticulum,

perforated with NPCs and lined by nuclear lamina. Over the last few decades, various integral NE proteins have been characterized by biochemical and genetic approaches. However, a single proteomics study has been able to detect many of these along with numerous novel components (Dreger et al., 2001). The inner nuclear membrane (INM) comprising of distinct transmembrane proteins connects the INM to a polymer of intermediate filaments (lamins) to form the lamina. The outer nuclear membrane (ONM) is continuous with the endoplasmic reticulum and is functionally similar to it, making the proteomic analysis of NE a challenging task. There have been a few studies which have attempted to decipher the NE proteome exlusively. One of the studies undertaken by Dreger et al. (2001) assumed that NE proteins will have similar biochemical extraction characteristics as the proteins from lamina. With the above assumption, the nuclei from cultured neuroblastoma cells (5–8 mg of protein) were suspended in 40 ml of ice-cold TP buffer (10 mM Tris HCl (pH 7.4), 10 mM NaH_2PO_4, Na_2HPO_4 (pH 7.4), 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin) containing 250 μg/ml heparin, 1 mM Na_3VO_4, 10 mM NaF, and 400 units of Benzon Nuclease (Merck). After stirring for 90 min at 4°C, nuclear envelopes were sedimented by centrifugation ($10{,}000 \times g$) for 30 min at 4°C and resuspended in STM 0.25 buffer [20 mM Tris HCl (pH 7.4), 0.25 M sucrose, 5 mM $MgSO_4$, 1 mM Na_3VO_4, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin). The NE proteins were extracted with 4 M urea in 0.1 M sodium carbonate generating an insoluble fraction rich in integral proteins. The known lamina-associated INM proteins from the non-ionic detergent-insoluble as well as salt-insoluble fractions were used for comparison. Proteins deemed likely candidates for novel INM proteins comprised the chaotrope pellet, which were also found in the detergent- or salt-extracted pellets. Most, but not all of the previously known INM were characherized along with four novel proteins. The second study was performed using subtractive approach. The proteins from the endoplasmic reticulum also present in the NE fraction were excluded (Schirmer et al., 2003).

Nuclear pore complex

The first comprehensive proteomic study of the yeast NPC by Rout et al. (2000) identified 40 proteins, comprising of previously known and unknown gene products, of which 11 were transport factors and 29 nucleoporins. More recently, Cronshaw et al. (2002) performed a proteomic analysis of rat liver NPC to identify ~30 proteins. Briefly, pelleted nuclei are resuspended with constant vortexing at a final concentration of 100 U/ml by drop-wise addition of buffer A (0.1 mM $MgCl_2$, 1 mM DTT, 0.5 mM PMSF, 1 μg/ml leupeptin/pepstatin/aprotinin) supplemented with 5 μg/ml DNase I and 5 μg/ml RNase A. After resuspension, nuclei are immediately diluted to 20 U/ml by addition of buffer B (buffer A + 10% sucrose, 20 mM triethanolamine, pH 8.5) with constant vortexing. After digestion at room temperature for 15 min, the suspension is underlayed with 4 ml ice-cold buffer C (buffer A + 30% sucrose, 20 mM triethanolamine, pH 7.5) and centrifuged at 3500 *g* for 10 min in a swinging bucket rotor (Sorvall SH-3000). The pellet is then resuspended in ice-cold buffer D (buffer A + 10% sucrose, 20 mM triethanolamine, pH 7.5) at a final concentration of 100 U/ml. The suspension is diluted to 67 U/ml with buffer D + 0.3 mg/ml heparin, and then immediately underlayed and pelleted as above. The heparin pellet is resuspended in ice-cold buffer D (100 U/ml), diluted to 67 U/ml with buffer D + 3% Triton X-100, 0.075% SDS, and then pelleted as above. The resultant pellet (the NPC-lamina fraction) is resuspended in buffer D + 0.3% Empigen BB (final concentration of 100 U/ml). After incubation on ice for 10 min, the insoluble lamina is separated from soluble nucleoporins by centrifugation in a microfuge at 16,000 *g* for 15 min. The NPC proteins in this study were separated by C4 reverse-phase chromatography followed by SDS-PAGE. The individual protein bands from SDS-PAGE were subjected for tandem MS analysis. The examination of the NPC has also been undertaken in some studies involving the brain; in hippocampus neurons of AD subjects and in the hypothalamic ventromedial neurons of rats post-exposure to high estrogen to name a few.

Nucleolus

The nucleolus coordinates the synthesis and assembly of ribosome's and has been implicated in cell growth, cell-cycle, apoptosis, senescence as well as the stress responses (Coute et al., 2005). Surprisingly not many studies have been conducted to document the changes in nucleolar proteome in neuroscience. The studies that will benefit the most by means of this approach will be the various studies on brain tumors. Before the year 2002, 121 human proteins were known to be located in the nucleolus based on publications, which used biochemical and subcellular localization techniques such as antibody staining and/or fluorescent tagging. Andersen et al. explored the proteome of human HeLa cells to identify 271 (of which 82 were novel nucleolar proteins) and 667 proteins in successive studies (Andersen et al., 2002, 2005). Proteins from important functional groups were revealed; ribosomal structural proteins as well as proteins involved in their synthesis and assembly; chromatin structural proteins; transcriptional and splicing factors; mRNA metabolism; translation factors; chaperones; DNA repair, replication; mitosis and cell-cycle regulation; ubiquitination and protein degradation; nucleocytoplasmic transport; kinases and phosphatases; enzymes; unpredictable function. Briefly, aliquots (250 µl) containing $\sim 1 \times 10^8$ nuclei were washed three times with PBS, resuspended in 5 ml buffer A (10 mM HEPES-KOH (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT), and Dounce homogenized 10 times using a tight pestle. Dounced nuclei were centrifuged at $228 \times g$ for 5 min at 4°C. The nuclear pellet was resuspended in 3 ml 0.25 M sucrose, 10 mM $MgCl_2$, and layered over 3 ml 0.35 M sucrose, 0.5 mM $MgCl_2$, and centrifuged at $1430 \times g$ for 5 min at 4°C. The clean, pelleted nuclei were resuspended in 3 ml of 0.35 M sucrose, 0.5 mM $MgCl_2$ and sonicated for 6×10 s using a microtip probe and a Misonix XL 2020 sonicator at power setting 5. The sonicate was checked using phase contrast microscopy, ensuring that there were no intact cells and that the nucleoli were readily observed as dense, refractile bodies. The sonicated sample was then layered on 3 ml 0.88 M sucrose, 0.5 mM $MgCl_2$ and centrifuged at $2800 \times g$ for 10 min at 4°C. The pellet contained the nucleoli, while the supernatant consisted of the nucleoplasmic fraction. The nucleoli were then washed by resuspension in 500 µl of 0.35 M sucrose, 0.5 mM $MgCl_2$, followed by centrifugation at $2000 \times g$ for 2 min at 4°C. The protein identification was performed by LC-MS/MS.

Expression proteomics

As mentioned previously, expression proteomics is the workhorse of current neuroproteomics initiative. Gel-based and MuDPIT approaches have enabled important advances in the measurement of protein expression alterations in normal and disease states. Important consideration for expression proteomics is the accuracy and reproducibility of each technique. It is widely believed that the two techniques offer complimentary data and it is critical to understand the advantages as well as the challenges inherent to them (Choe et al., 2005). The factors that are crucial for the successful implementation of proteomics technique are low detection limits, optimal signal-to-noise ratio and a wide dynamic range.

Two-dimensional gel electrophoresis

2-DGE is a widely used separation method in which the application of an electrical potential is applied across a solid-based gel whereby proteins are first separated by IEF and then by molecular mass in the second dimension. The basic principles of 2-DGE have remained the same since its introduction in 1972 by O'Farrell (1975) and Klose (1975). Recent technological improvements have enabled more reliable and reproducible IEF on strips that are gels that can be sued with the second-dimension slab gels and improved image analysis software that more accurately matches staining profiles across different gels. In general, approximately 1000–2000 proteins spots can be visualized on a gel depending upon the visualization technique, the pI range of the first dimension as well as the size of the 2-DGE. These usually represent the most-abundant housekeeping proteins and the more interesting proteins (signaling molecules and receptors) from a biological point of

view, which are in lower abundance, remain obscured by the most-abundant proteins. Enrichment of such low-abundance proteins can be well achieved by subcellular fractionation (Fig. 1). Some of the inherent caveats/disadvantages associated with 2-DGE are (a) many protein spots are likely comprised of multiple proteins, (b) individual proteins may migrate as multiple spots based on differential digestion, (c) labor-intensive image analysis requires gel matching and manual removal of artifacts, (d) poor spot resolution at higher pIs, (e) difficulty in electrophoresing large and hydrophobic proteins in the first-dimension separation and (f) extreme acidic and basic proteins are not well represented (Van den Bergh et al., 2003). The technical variability seen with 2-DGE is due to sample preparation, sources of reagent, staining methods, image analysis software and individual experimenter variability accounting for a coefficient of variation of 20–30% (Molloy et al., 2003).

Sample preparation

Like any experiment, the quality of the results is dependent on the quality of the preparatory material. Adequate coverage of the intricacies of protein isolation would be difficult to address in current review; however, a few points of critical importance for proteomic experiments are discussed. Protein stability and purity, as well as prevention of protein degradation and modification, are of critical importance during sample preparation for proteomic approaches. Rapid removal of brain tissue, dissection and freezing are obvious imperatives for the maintenance of the proteome state in the animal. Human postmortem studies pose unique challenges, but can and should be undertaken with careful documentation of postmortem interval, brain pH and agonal state (Hynd et al., 2003). Specific proteins such as dihydropyrimidinase-related protein-2 has been putatively identified as a marker of *postmortem* interval and temperature (Franzen et al., 2003), highlighting the need for careful selection of controls in human brain postmortem studies (Fountoulakis, 2004). Protease and phosphatase inhibitors are used to help prevent degradation and dephosphorylation of proteins during protein preparation (Olivieri et al., 2001); however, care should be taken such that adducts and charge trains are not introduced by these inhibitors.

Purification of protein from other cellular substances is also necessary. Lipids and specific proteins (e.g., albumin and immunoglobulin isoform) are particularly abundant in the brain and, along with nucleic acids, must be eliminated from the protein sample. The most common methods of protein purification rely on selective precipitation. Acetone, TCA and other precipitation methods can be performed and a number of commercially available kits make this a routine procedure (Polson et al., 2003). In some instances though, pure protein may not be sufficient since proteins such as IgGs or albumin constitute the vast majority of protein concentrations in cells. Selective elimination of these proteins improves detection of less highly expressed proteins (Lollo et al., 1999).

Isoelectric focusing

IEF separates proteins according to their pI. The pI of a protein is primarily a function of its amino acid sequence although PTM can also contribute to the pI. Proteins are amphoteric molecules, capable of acting as either an acid or base. The side chains of the amino acids in proteins have acid or basic buffering groups that are protonated or deprotonated, depending on the pH of the solution in which the protein is present. IEF takes advantage of this property by placing proteins in a pH gradient and applying a potential such that the protein will migrate toward the anode or cathode, depending on the net charge. At the pI, the protein will reach the point in the pH gradient where the net charge of the protein is zero and stop migrating.

Initially, the preparation and use of the pH gradients needed for IEF was very difficult and inconsistent. pH gradients were often in the form of tube gels with carrier ampholytes, but the introduction of chemistries (Husi et al., 2000) to immobilize the pH gradient into the gel matrix and the popularization of this technique (Bjellqvist et al., 1982) was a significant step in making IEF more widely accessible. Most current applications

of IEF use immobilized gradients in dedicated instruments which control both potential and temperature (Gorg et al., 1995). Exceedingly high potentials (e.g., 8000 V) are needed to focus proteins and consistent focusing requires close control of the temperature (Gorg et al., 2000). Commercial suppliers are producing IEF gels with narrow pH ranges, which when used in an overlapping fashion (pH 4–5, 4.5–5.5, 5–6, 5.5–6.7 and 6–9) enable the separation and detection of thousands of proteins (Gorg et al., 1991). IEF is rarely used on its own and is usually followed by applying the IEF strip to SDS-PAGE gel for electrophoresis in the second dimension based on molecular weight (described below).

Solution IEF

Solution IEF operates on the same principle as normal IEF except that proteins are separated into pI range bins, in solution (Wildgruber et al., 2000). Proteins can then be electrophoresed on standard IEF gels with a narrow pI range, the same as the pI range of the bin. One of the reasons for performing solution IEF is that when loading a whole-cell lysate onto a narrow pI range IEF gel, proteins outside the IEF range precipitate and can pull out proteins from within the range of the IEF gel. Solution IEF can increase the number of proteins observed, the amount of sample loaded and resolution. The drawback to this approach is the addition of another experimental step that can result in the sample loss; however, with further development, this technology has great potential.

SDS-PAGE

2-DGE along with MS are the two most commonly used techniques in proteomics, namely the separation of proteins by IEF (first dimension) followed by SDS-PAGE (second dimension) which involves the separation by molecular weight, of proteins that have already been separated by IEF. In general, the IEF gel or strip is equilibrated with SDS and placed on top of the SDS gel. The equilibration step is necessary to allow the SDS molecules to complex with the proteins and produce anionic complexes that have a net negative charge roughly equal to the molecular weight of the protein. The SDS gel is then electrophoresed and the proteins migrate out of the IEF gel and into the SDS gel, where they separate according to molecular weight. While most applications use denaturing SDS-PAGE, native approaches have also been used. Conventional SDS-PAGE instruments, such as those used for Western blotting and special purpose apparatuses can be used for this step.

Traditional stains. Coomassie brilliant blue (CBB), silver nitrate, and negative staining are common post-electrophoresis methods available for the 2-D gel-based proteomics analysis. The sensitivity of these stains ranges from 100 ng (e.g., Coomassie) down to 1 ng (e.g., silver) for individual protein spot detection (Neuhoff et al., 1990; Scheler et al., 1998). The organic dye CBB available in two modifications, Coomassie R-250 and Coomassie G-250, is one of the most widely used stains for expression analysis. In acidic medium, the dye binds to amino acids by electrostatic and hydrophobic interactions. However, it is not reproducible and reliable for quantitation as some of the proteins release the dye during the background destaining procedure. As a rule of thumb, naked-eye visualization of a spot by CBB stain infers adequate protein for mass spectrometric characterization. Colloidal CCB staining is more reproducible and has higher sensitivity, has quantitative protein binding and is lower in price. CCB is compatible with MS as complete destaining can be achieved using bicarbonate.

Silver staining method is the other traditional staining technique most widely followed for quantitative analysis because its sensitivity is as low as 1 ng per spot. The popularity of this stain can be gauged by extensive use of this method represented by 150 modifications of silver staining protocols published to date (Rabilloud et al., 1994). However, in principle, silver staining detects the proteins primarily at the gel surface. As it is not an endpoint procedure, there is a high degree of variability of staining intensities for particular spots and thus is unreliable for quantitation. A common glitch encountered with this method in the detection of abundant protein spots is the formation of yellow center, which result in concave peaks that are problematic for quantitative analysis. Despite

its excellent sensitivity, silver staining lacks reproducibility, has a limited linear dynamic range, a subjective judgment of the staining end-point and interferes with the MS compatibility, resulting in a much lower sequence coverage compared to CBB (Mortz et al., 2001). Even though silver staining is still used currently, there has been an ever-increasing trend in the scientific community to use the new generation fluorescent stains especially for broad-scale proteomics analyses.

Other traditional staining, though less popular, methods employ copper and negative imidazol SDS zinc. The negative imidazol initially showed immense promise for further analysis of proteins after quantitation as it stains the background without modifying the protein. The protein spots were visualized against dark background and the detection sensitivity stayed between CBB and silver. Even though it was documented to show quantitative analysis, it was disputed because only the background was detected directly and not the protein (Ferreras et al., 1993).

Fluorescent stains. In general, the fluorescence-based detection methods are more sensitive than the absorbance-based methods because the detected wavelength is different to the incident wavelength. Prevailing over various issues limiting the traditional gel staining techniques, fluorescence-based gel stains are recently becoming widely accepted (White et al., 2004). SyproRuby™ dye (Molecular Probes, Eugene, OR), among the first of the fluorescent stains for proteins, is part of a stable organic complex composed of ruthenium which interacts non-covalently with the basic amino acids in proteins (Berggren et al., 1999). The stain can be visualized using a wide range of excitation sources commonly used in image analysis system and has a sensitivity which approximates silver staining while maintaining a broad linear dynamic range (three orders of magnitude). The fluorescent stain does not contain or require chemicals such as glutaraldehyde, formaldehyde and Tween-20 that normally impede with peptide mass fingerprinting (Berggren et al., 2000). A recent study showed an enhanced recovery of peptides from in-gel digests of SyproRuby™ stained proteins compared to silver staining using MALDI-TOF MS (Lopez et al., 2000); however, important drawbacks include the tendency to induce speckling and high background staining which blemish the visual appraisal of gel images, as well as concerns of stain disposal. Recently, DeepPurple™ (GE Healthcare, Piscataway, NJ), a fluorophore epicocconone from the fungus *Epicoccum nigrum* which interacts non-covalently with SDS and protein, has been introduced for protein gel staining. As sensitive as SyproRuby™, Deep Purple has a dynamic linear range over four orders of magnitude, shows no speckling and has a low background staining intensity (Mackintosh et al., 2003; Smejkal et al., 2004). Tannu et al. (2006a) in recent study concluded that the Deep-Purple™ stain results in an increased peptide recovery from in-gel digests compared to SyproRuby™ stain and also improves MALDI-TOF based identification of lower intensity proteins spots by increasing sequence coverage. The additional peptides seen from Deep Purple™ stained proteins were attributed to incomplete cleavage or modified (primarily with respect to methionine oxidation) forms of peptides already present in the spectrum. Incomplete cleavage by trypsin was attributed to the binding of epicocconone to the lysine residue, one of its primary cleavage sites (Coghlan et al., 2005). This study opens up the possibility of confident identification of low-abundance proteins for identification which have been evasive until now in spite of reliable quantitative data by the fluorescent dyes. With the availability of more sensitive stains, the challenge to acquire reasonable mass spectra for identification of lower-abundance proteins is crucial. It becomes important to confirm that dyes do not interfere with MS since dye interference can cause ion suppression with a resultant lower recovery of peptides or a reduction of signal intensities. In spite of this caveat, the majority of information currently available about protein gel staining involves comparing the efficacy of the staining techniques with little information regarding their comparative MS compatibilities.

2D-DIGE. One of the most-recent technical advances in 2-DGE has been the multiplexing fluorescent 2D-DIGE (Unlu et al., 1997). This

method relies in direct labeling of the lysine groups on proteins with cyanine (Cy) dyes prior to IEF. The critical aspect of the use of 2D-DIGE technology is the ability to label 2–3 samples with different dyes and electorphorese all samples on the same 2-D gel. This ability reduces spot pattern variability and reduces the number of gels in an experiment making spot matching much more simple and accurate. The single positive charge of the CyDye replaces the single positive charge present in the lysine at neutral and acidic pH keeping the pI of the protein relatively unchanged. A mass of approximately 500 Da is also added by the CyDye to the labeled protein. The most popularized experimental design has been the use of a pooled internal standard (sample composed of equal aliquots of each sample in the experiment) labeled with the Cy2 dye and labeling the control and the diseased/treatment groups with either Cy3 or Cy5 dyes swapped equally across the samples, respectively (Fig. 2). Minimal labeling is performed to tag only one lysine residue in each protein to prevent the vertical train of spots due to the added mass of each fluorophores and prevent the protein precipitation due to increased hydrophobicity. The individual protein data from the control and diseased/treatment (Cy5 or Cy3) samples are normalized against the Cy2 dye-labeled sample, Cy5:Cy2 and Cy3:Cy2. These log abundance ratios are then compared between the control and diseased/treatment samples from all the gels using statistical analysis (*t*-test and ANOVA) (Tonge et al., 2001, Alban et al., 2003; Tannu).

This method has the advantage of being able to quantify the protein spots that are uniquely present in one group due to the presence of internal standard. The accuracy of quantitation as well as the statistical confidence obtained for the differentially regulated gene products is significantly higher using the experimental design of 2D-DIGE (Alban et al., 2003; Knowles et al., 2003; Tannu). CyDye-labeled proteins are scanned by Typhoon™ variable mode imager. Sequential scanning of Cy2, Cy3 and Cy5-labeled proteins is achieved by the following lasers/emission filters; 488/520 nm, 532/580 nm and 633/670 nm, respectively. Scanned images of fluorescence-labeled proteins are sequentially analyzed by differential in-gel analysis (DIA; performs Cy5/Cy3: Cy2 normalization) followed by biological variation analysis (BVA; performs inter-gel statistical analysis to provide relative abundance in various groups). The 2D-DIGE approach offers great promise and has been used increasingly by researchers to address a wide range of neuroscience questions from e.g. Alm et al. studying the neurodevelopment toxicity of PBDE-99 and Tannu et al. studying the altered phenotype of nucleus accumbens of human cocaine overdose victims (Prabakaran et al., 2004; Swatton et al., 2004; Beckner et al., 2005; Roelens et al., 2005; Sitek et al., 2005; Tannu). 2D-DIGE offers the most reliable quantitation of any 2-DGE method, is comparable in sensitivity to silver staining method and compatible with the downstream MS protein characterization (as majority of the lysine residues remain untagged and accessible for tryptic digestion).

The major drawback of this technique is that it is proprietary to GE Healthcare and requires expensive labeling dyes as well as specific equipment such as a three-laser fluorescent scanner and robotic spot picker including dedicated software. Also due to the prolonged scanning times, the protein diffusion affects the eventual protein spot quantitative analysis across the larger set of gels. However, Tannu et al. have recently documented the expediency of protein spot fixation prior to the scanning of gels in a large-scale 2D-DIGE experiment. This study circumvented the problem of protein spot diffusion at the same time maintaining the original protein spot quantitative analysis (Koichi Tanaka et al., 1988; Tannu and Scott, in press: 2006b). Also should be noted is that proteins with high percentage of lysine residues are possibly labeled more efficiently compared with the proteins with few/no lysines. Therefore, the possibility remains that a high-abundant protein spot in the conventional 2DGE can be a medium or even low-abundant protein in 2D-DIGE due to low lysine content. A modification of 2D-DIGE in which Cy dyes that label all of the cystine residues of proteins are labeled has recently been introduced. The detection limit for saturation labeling is 0.1 ng or protein per spot as opposed to 1 ng protein per spot thereby reducing the amount of protein sample required for analysis (Shaw et al., 2003).

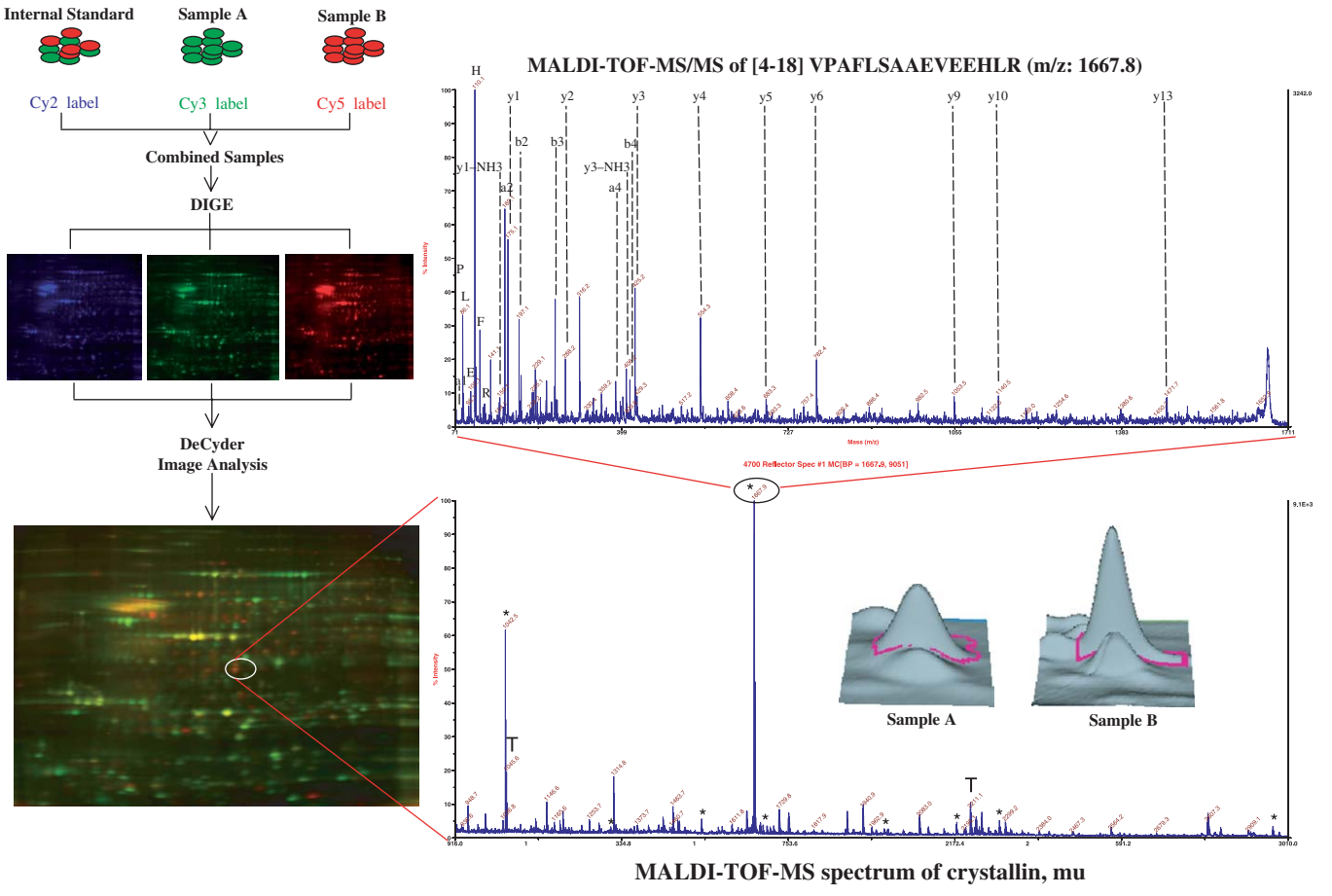

Fig. 2. Typical 2D-DIGE workflow for comparative expression proteomics. The samples are labeled by fluorescent dyes (Cy 2, 3 and 5) and combined together prior to the IEF. The scanned fluorescent images are analyzed by DeCyder™ analysis software using the normalization by Cy2-labeled pool sample. The protein spots (e.g., crystallin mu) with statistical significance that express different expression profiles between the two groups are further analyzed by MALDI-TOF-MS. The peptide mass fingerprint of crystallin (mu) shows the peptides marked with '*' matched to MASCOT search against the NCBInr *primate* database. The *x*- and *y*-axes show the mass to charge (m/z) ratio and the % abundance of the tryptic peptide fragments, respectively. Also depicted in the inset for crystallin (mu) mass spectrum is the representative 3-D protein-expression profiles for the two groups. Typically the most-abundant peptide fragment is sequenced by, additive series of the y- and b-ions generated as well as the immonium ions, MALDI-TOF-TOF (e.g., peptide fragment with m/z of 1667.8) to confirm the identification of protein and characterize the sites of PTM.

They provide a very attractive alternative for performing quantitative 2D-DIGE when dealing with low sample amounts, typical of neuroscience, even though only two saturation dyes are currently available (Cy3 and Cy5). A caveat, low protein amount complicates MS-based characterization of differentially regulated proteins analyzed by saturation labeling (Zhou et al., 2002a).

Quantitative image analysis

The image analysis in a classic 2-DGE-based proteomic approach encompasses the analysis of thousands of proteins simultaneously across different groups to reveal differentially regulated proteins. It becomes mandatory to use powerful algorithms to analyze these large data sets and a number of software packages are currently available (Marengo et al., 2005). These image analysis software packages are uniquely designed for either 2-DGE or 2D-DIGE, except Progenesis™ that has the capability of handling either method. The precision with which image analysis software performs spot detection, matching and normalization dictates the quality of data generated. The state-of-the-art commercial products have been automated with respect to these requirements; however, require some manual confirmations even with the higher-end products. Much software available has been compared to evaluate their individual pros and cons (Rosengren et al., 2003; Arora et al., 2005; Fodor et al., 2005; Marengo et al., 2005). PDQuest™, one of the most popular software for image analyses of 2-DGE ($\approx$70 studies), requires manual setting of appropriate selection parameters for spot detection. The sensitivity, size scale, minimum peak are determined based on manual selection of a faintest, smallest, large spot and a large representative section of the image containing spots, streaks, and background gradations to make corrections for noise filter. The typical normalization method used for each protein spot detected by PDQuest™ is a ratio of its raw spot intensity and the cumulative total intensity from each protein spot detected on the entire gel based on absorbance. Also to get the best-image matching between multiple gels, manual land-marking encompassing the entire gel is imperative. Progenesis™ Workstation ($\approx$8 studies) on the other hand is designed for automatic spot detection with no requirement for manual intervention and is by far the most high-throughput as well as the high-end image analysis software available for 2-DGE as well as the 2D-DIGE analysis. An iterative method is used in the spot detection, image warping and matching by the Progenesis™. DeCyder™ ($\approx$12 studies) spot detection is based on initial detection of the protein spots in the Cy2 image of pooled sample followed by application of similar spot boundaries to the remaining images (Cy3 and Cy5). The 3-D spot viewer which has been incorporated in most of the software program's recent version's have been very helpful for the purposes of manual land marking, confirming detection of true spots as well as the spot matching across the entire sub-set of gels in an experiment. A study comparing the softwares PDQuest™ and Progenesis™ for 2-DGE showed comparable accuracy of protein spot quantitation for well-resolved areas of gels (Arora et al., 2005). The integrated interpretation of results for studies using different image analysis softwares is a difficult task, and requires a common platform with integrated software addressing the weaknesses of individual programs and at the same time incorporating the strengths into a single user-friendly workstation.

Multi-dimensional separation of proteins

The coupling of efficient chromatographic and electrophoretic separation methods with high-performance MS hold great promise for qualitative and quantitative characterization of highly complex protein mixtures. The advances in chemical tagging and isotope labeling techniques have made possible the quantitative analysis of proteomes, and the specific isolation strategies have enabled the analysis of PTM. The multi-dimensional separation is typically based on using $\geqslant$two physical properties of peptides (size, charge, hydrophobicity and affinity) to fractionate complex peptide mixture into individual components. The methods employed to fractionate peptides based on their corresponding physical and chemical properties are ultracentrifugation (density), capillary

electrophoresis (size and charge), IEF (pI), size-exclusion chromatography (Stoke's radius), ion-exchange chromatography (charge), hydrophobic interaction chromatography (hydrophobicity), reverse-phase chromatography (hydrophobicity) and affinity chromatography (biomolecular interaction).

The drawback of 2-DGE to detect low-abundant proteins as well as the proteins with extreme pI, molecular weight and hydrophobicity has been the promise offered by the multi-dimensional chromatographic approach for proteomic analysis (Gygi et al., 2000; Washburn et al., 2001; Peng et al., 2003a). A caveat, no single chromatographic or electrophoretic method used in different combinations employed by multi-dimensional separations has been successfully devised to separate, detect and quantify all proteins in a given proteome. In most multi-dimensional approaches the proteins are digested into peptides prior to separation. The digestion of proteins produces complex mixture of peptides, however, at the same time it increases the overall solubility by eliminating non-soluble extremely hydrophobic peptides. This is extremely critical in neuroscience for studying the synaptic and PSD proteins, typically insoluble in aqueous buffers, involved in signal transduction (neurotransmitter receptors and G-proteins), molecular transport (carriers and voltage-gated ion channels) and cell–cell interactions. The peak capacity of multi-dimensional separation is the product of the peak capacities of its component one-dimensional methods. As mass spectrometer can perform mass measurements on several but not all coeluting peptides, fractionation is a critical aspect for mass spectral identification of peptides. The MS/MS cycle times of all conventional mass spectrometers are limited by the number of peptides that can be selected by collision-induced dissociation (CID).

Wolters et al. (2001) have initially showed that MuDPIT was reproducible within 0.5% between two analyses. Furthermore, a dynamic range of 10,000 to 1 between the most-abundant and least-abundant proteins/peptides in a complex peptide mixture was also demonstrated. The comprehensive proteome analysis requires the ability of a system to detect variation in protein abundance in $\geqslant$six orders of magnitude to detect a potential biological significance (Corthals et al., 2000). The LC techniques currently used successfully in neuroscience have been ion-exchange (cation as well as anion), reverse-phase (RP) and affinity and will be elaborated here. Most RP-HPLC separations are carried out using acetonitrile (ACN) in combination with ion-pairing agent (formic acid or trifluoroacetic acid (TFA); depending on the downstream mass spectrometer to be used) to improve the selectivity. The ion-exchange chromatography is performed using salts (sodium chloride, potassium chloride, ammonium acetate/formate) at different concentrations at a gradient format. It is important to select the salt, its concentration and the buffer composition in such a way as to not affect the second-dimension separation in terms of resolution.

Quantitative analysis

Several strategies have been developed for relative quantitation of protein expression between samples. The labeling of proteins or peptides for quantitation followed by MS is currently rapidly advancing approach. The important steps (Fig. 3) in which this technique is practiced are: (1) isotopic labeling of separate protein mixtures, (2) combined digestion of the labeled proteins followed by multi-dimensional liquid chromatographic separation, (3) automated MS/MS of the separated peptides and (4) automated database search to identify the peptide sequences and quantify the relative protein abundance based on the MS/MS.

Isotope-coded affinity tags (ICAT and iT-RAQ). This is the prototypical and the most popular method for quantitative proteome analysis based on stable isotope affinity tagging and MS (Gygi et al., 1999a). The ICAT reagent is a sulphydryl-directed alkylating agent composed of iodoacetate attached to biotin through a short oligomeric coupling arm (d0). The substitution of 8 deuterium atoms for hydrogen atoms in the coupling arm produces a heavy isotope version of the reagent (d8). Thus the reagent comprises of a cysteine-reactive group, a linker containing the heavy or light isotopes (d8/d0) and a biotin affinity

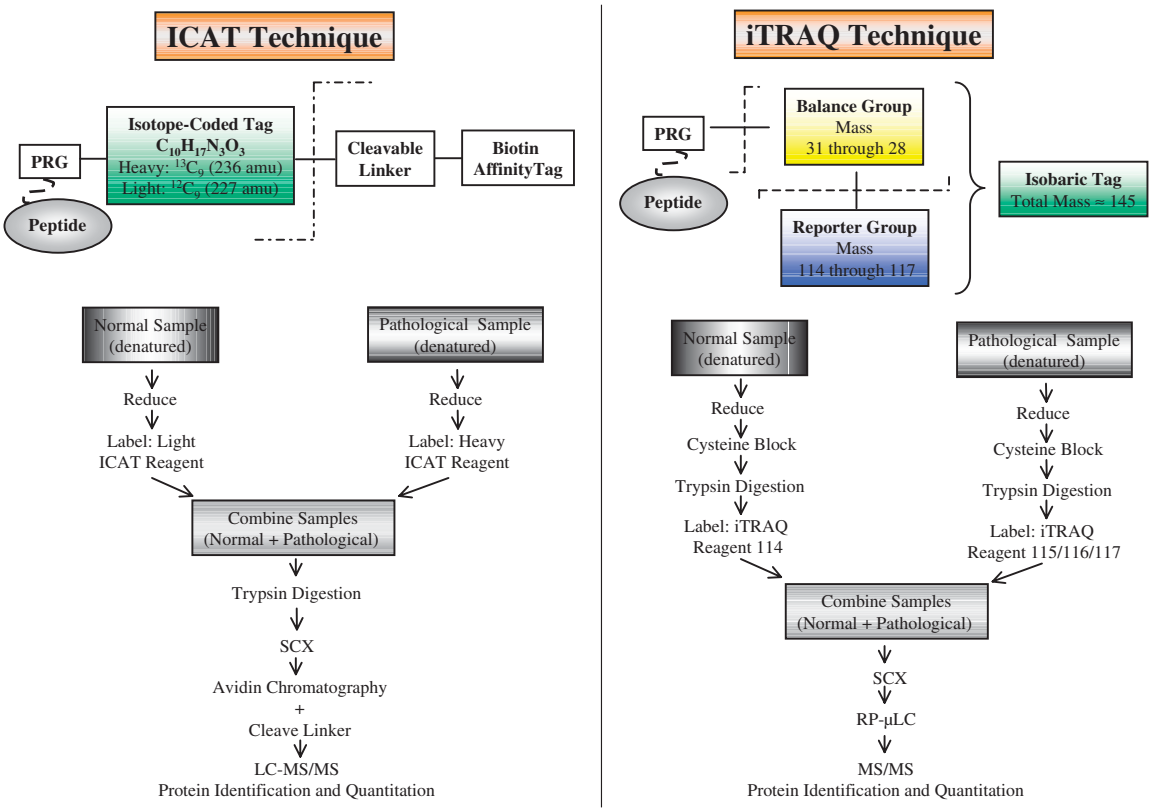

Fig. 3. Non-gel-based approach for analysis of expression proteomes. The most commonly used, commercially available, quantitation methods coupled with the MDLC approach of comparative proteomics are depicted. The figure shows the ICAT™ and iTRAQ™ reagent structures. The peptide-reactive group (PRG) covalently links iTRAQ™ as well as the ICAT™ reagent isobaric tag with the lysine side chain and N-terminal group of peptide. The fragmentation ( ) occurs as shown during MS/MS. The balance group of the iTRAQ™ reagent undergoes neutral loss, and the resultant reporter group (114–117) peaks in the low-mass region are used for relative quantitation. The elaborate description of the ICAT™ and iTRAQ™ workflow is in the text.

tag (Fig. 3). This method involves in vitro derivatization of cysteine residues in protein with d0 or d8 followed by enzymatic digestion of the combined sample. All the cysteine residues thus tagged with biotin are selectively separated by avidin column. The cysteine-containing peptides are further separated by reverse phase followed by MS analysis. In the process the complexity of the peptide mixture decreases significantly as only the cysteine-containing residues are enriched selectively. In humans, databases indicate that the occurrence of cysteine and the frequency of cysteine-containing peptides is ~90% and 17%, respectively. The isotopically tagged peptides give quantitative MS analysis based on the relative peak intensities/areas of d0- and d8-labeled peptides (Gygi et al., 1999a). Another advantage is the ability to analyze peptides with molecular weight more than 3000 Da easily because the mass difference between the coded isoforms is sufficiently large.

The major limitation of ICAT is that it can only be used to examine the concentration or structural changes in cysteine-containing peptides (10–20% of the peptides). The resolution is greatest in the case of smaller peptides where the d8/d0 ratio is higher and with peptides that have multiple cysteine residues (Regnier et al., 2002). Another limitation of the technique is that the biotin affinity tag remains linked to the peptides throughout the analysis causing shifts in chromatographic separation, shifts in m/z and changes to MS/MS spectra relative to the unlabelled peptides

complicating the manual or computer-assisted interpretation (Gygi et al., 1999a; Ferguson and Smith, 2003). To address the issues of the first-generation ICAT technique, several second-generation chemistries for cysteine-specific isotope tagging have been developed. Zhou et al. used a solid-phase capture-and-release method and a photo- and acid-cleavable linker, an isotope-tag transfer group and a specific reactive group (Fig. 3) (Qiu et al., 2002; Zhou et al., 2002b). The major advantage provided by this method is that the isolation of cysteine-containing peptides and incorporation of the stable isotopes are achieved in single step, making it simpler, faster and easier to automate. There is also improved selective enrichment of cysteine-containing peptides as well as increased sensitivity by this method. The second-generation ICAT undergoes similar procedure as the original except that the biotin group is cleaved off before MS analysis of the tagged peptides. The isotope tag introduced with this reagent consists of $^{13}C_6$ instead of deuterium, which is relatively small. The peptides labeled with $^{13}C_6$ or $^{12}C_6$ have virtually the same retention time during the RP-HPLC (Zhang et al., 2001). The only chemical modification remaining on the peptides at the time of LC-MS/MS is an isotopically labeled leucine residue. The added advantage are, reduced sample handling and facilitation of extensive sample washing protocols prior to peptide elution (Zhou et al., 2002b).

Most of the analyses based on ICAT technology have coupled strong cation exchange (SCX) LC with reverse-phase microcapillary LC coupled on-line (RP-μLC) with MS and MS/MS (Link et al., 1999; Washburn et al., 2001; Gygi et al., 2002). Data-dependent software is used to select specific mass/charge (m/z) peptides for CID, alternating MS and MS/MS scans for collecting qualitative and quantitative data. The on-line LC-ESI-MS/MS has the drawback due to the requirement of continual sample consumption and the 'on the fly' selection of precursor ions for sequencing. The use of MALDI-MS/MS offers significant advantage that the temporal constraints of an on-line detection are eliminated as the peptides separated by the μLC are deposited on the MALDI sample plate before the MS analysis (Medzihradszky et al., 2000; Krutchinsky et al., 2001). Alternative strategies such as per-methyl esterification of carboxylic acid groups (Goodlett et al., 2001), specific labeling of lysine residues(Peters et al., 2001) and peptide N-termini (Munchbach et al., 2000) have also been probed recently. The quantification softwares such as XPRESS (http://www.systemsbiology.org/research/software/proteomics/) and ProICAT™ (Applied Biosystems, Foster City, CA) have been developed which can assemble a composite ratio for a protein based on the calculated expression ratio from all the peptides from a single protein. The data obtained from the above softwares can be analyzed collectively using INTERACT for multiple experiments (Han et al., 2001).

The iTRAQ™ technique capable of multiplexing samples is primarily based on the ICAT technique and compared in detail in Fig. 3. The iTRAQ™ technique uses four isobaric reagents (114, 115, 116 and 117) allowing the multiplexing of four different samples in a single LC-MS/MS experiment. The multiplexing capability of iTRAQ™ allows a control sample to be compared with different points in time of a disease state (e.g., acute, sub-acute, chronic and relapse) as well as with respect to different drug treatments. One of the major advantages of this technique over the ICAT™ is its ability to label multiple peptides per protein, which increases the confidence of identification as well as quantitation. As shown in the Fig. 3, each isobaric iTRAQ™ reagent constitutes of a reporter group, a balance group and a peptide-reactive group (PRG). Multiple peptides in a protein are labeled by covalent linking of the PRG with each lysine side and the N-terminal group of a peptide. The MS spectra of combined samples bear a resemblance to that of an individual sample. The balance group (31 to 28) makes it possible to display all the iTRAQ™ reagents (114–117) to be displayed at same mass. A neutral loss of the balance group occurs during the MS/MS, and the reporter group ion peaks appear in the low mass region. The area under the curve for each reporter ion peak represents the quantitation for that particular peptide. An average quantity can be assigned to a protein after incorporating the quantitative information from all the peptides that were identified for a particular protein. The

relative amounts of protein from different samples are then the ratios of the average quantity obtained as above. A recent study compared the 2-DGE and iTRAQ™ technique using the *Escherichia coli* for consistent measurements showing an average CV of 0.24 for isobaric tagging to 0.31 for 2-DGE. Also a greater range of expression ratios was demonstrated by the proteins quantified by the isobaric tagging as compared with the 2-DGE (Choe et al., 2005). A more recent study by Wu et al. (2006) systematically compared the techniques of DIGE, ICAT™ and iTRAQ™. The DIGE technique was amenable for compromised quantitation due to partial/complete comigration of proteins. The global tagging iTRAQ™ was found to be more sensitive than the ICAT™ which was as sensitive as the DIGE. The complimentary nature of these techniques was confirmed by the limited overlap of the proteins characterized (Wu et al., 2006).

Peptide labeling with $H_2^{16}O/H_2^{18}O$. The samples to be compared are separately digested in either $H_2^{16}O$ or $H_2^{18}O$. The oxygen atom derived from the aqueous solvent is incorporated into the newly formed C-terminus acid functional group in each peptide, providing an effective isotope tag for relative quantitation (Mirgorodskaya et al., 2000; Stewart et al., 2001; Yao et al., 2001). The requirement of this technique to separately digest the protein samples to be compared has the potential to be imprecise due to separate sample handling. The difference of 4 Da between the ^{16}O and ^{18}O has limited usefulness for larger peptides, where mass spectral isotopes of labeled and unlabeled peptides separated by only 4 Da begin to overlap.

Tandem ion exchange /reverse-phase chromatography

The method of choice for multi-dimensional separation of peptides has been the SCX for the first-dimensional separation of peptides followed by the microcapillary RP chromatography. The negative charges at the carboxyl groups and the C-terminus are neutralized due to complete protonation at pH < 3. This leaves the arginine, lysine and histidine residues as well as the N-terminus contributing to a net positive charge of the peptide. The SCX chromatography fractionates the fully protonated peptides. On the other hand for anion exchange chromatography completely deprotonated basic residues by pH > 12 are required. A mixed mode effect is commonly exerted by most ionic exchangers, principal for the ionic interactions during tandem ion exchange (IEX), due to their hydrophobic influence (Zhu et al., 1991). Recently a biphasic column combining SCX and RP chromatography (direct analysis of large protein complexes: DALPC) has shown to have a improved resolution, loading capacity and the ability to detect low-abundance proteins as compared to a single-dimension column (Link et al., 1999). The SCX has four times greater loading capacity than the RP, greatly increasing the number of digested proteins that can be analyzed. DALPC has been shown to detect novel components of splicing, transcription and RNA processing as well as protein kinases which were not detected by earlier studies such as the 2-DGE analyses (Ohi et al., 2002; Sanders et al., 2002). Recently this method was optimized by the use of volatile salts to elute peptides, automated and combined to sensitive MS to be recoined as multidimensional protein identification technology identifying approximately 1500 proteins in single analysis (Washburn et al., 2001; Wolters et al., 2001; VerBerkmoes et al., 2002). The MuDPIT has been combined with ICAT™ for quantitative proteomic analyses in neuroscience as well (Li et al., 2004; Lovell et al., 2005; Schrimpf et al., 2005).

The peptides are separated by SCX using a LC system (Ultimate 3000, Dionex, Sunnyvale, CA, USA; Ettan, GE Healthcare, Uppsala, Sweden). Typically 4.6 × 200 mm Polysulphoethyl A™ column (PolyLC, Columbia, MD, USA), a silica-based column having a hydrophilic anionic polymer (poly(2-sulfoethly aspartamide)), is used for separation by 200 µl/min flow rate. The buffers generally used are 'A' 10 mM KH_2PO_4, ACN 30% (pH 3.0) and 'B' 10 mM KH_2PO_4, 350 mM KCl ACN 30% (pH 3.0). The gradient used is usually optimized for a particular tissue. A typical gradient run consists of 0–50% B over 30 min, 50–100% B from 30–31 min, remain at 100% B up to 36 min, return to 100% A and equilibrate for 20 min before the next run. The fractions collected vary

from as low as 10 to as high as 62 depending on the sample complexity and the gradient used. The fractions are neutralized at this stage and loaded into 4 × 15 mm avidin column equilibrated in 2 × phosphate-buffered saline. The ICAT™-labeled peptides are eluted with three column volumes of 30% ACN 0.4% TFA, dried and reconstituted in cleavable reagent to cleave the biotin portion of the tag from the labeled peptides. The fraction is dried and dissolved in the mobile phase for RP separation of peptides.

Since most of the multidimensional separation techniques are interfaced with MS, RP chromatography is the choice of second-dimension because the samples eluted from it are in most desirable form for injection into the mass spectrometer. The separation efficacy of the RP is dependent on the particle size, pore size, surface area, stationary phase as well as the chemistry of the substrate surface. The C_{18}-bound phase has been the most popular as it offers retention and selectivity for a wide range of compounds containing different polar and non-polar groups on their surface. To enhance mass transfer, silica monolith columns have been introduced recently (Minakuchi et al., 1997; Ishizuka et al., 2000; Premstaller et al., 2001). These columns are comprised of continuous rod of silica-based gel, which is made of highly interconnected network of large and small pores. The macropores (2 μm) allow fast flow of the eluent and the fine pores (13 nm) offer the surface area required for the separation process. The monolith material has a total porosity of over 80% that facilitates high permeability, good surface area. It also enhances mass transfer due to convection and not diffusion. The combination of effects results in practically no loss in peptide resolution, peak elution volume and concentration of analyte with flow rates (10 ml/min) 10 times higher than conventional rates (1 ml/min). Each salt fraction from the SCX is subjected to RP gradient of 60–90 min on a Ultimate 3000™ equipped with a Famos Micro Autosampler and Switchos Micro Column Switching Module (Dionex, Sunnyvale, CA, USA) using the buffers; A (0.1% TFA) and B (80% ACN, 0.08% TFA) at 0.4 μl/min. Typically the Ultimate 3000™ (Dionex, Sunnyvale, CA, USA) elutes the peptides through 5 mm C18 PepMap100 trapping column (300 μm i.d.) and a 15 cm C18 PepMap100 resolving column (75 μm i.d.) at 0.4 μl/min. A typical gradient run consists of 0–80% B over 60 min, 80–100% B from 60–61 min, remain at 100% B up to 71 min, return to 100% A and equilibrate for 20 min before the next run. The eluent is monitored at 214 nm and mixed with matrix (7 mg/ml CHCA in 70% ACN, 0.1% TFA spiked with 0.15 mg/ml dibasic ammonium citrate and 0.25 fmol/ ml ACTH clip 18–39 (ratio of 1:2)) every 4 s via a micro-tee fitting of Probot Micro Fraction Collector (Dionex, Sunnyvale, CA, USA) on to a MALDI plate for MALDI-TOF-TOF analysis (Li et al., 2004; Peng et al., 2004; Lovell et al., 2005; Schrimpf et al., 2005; Trinidad et al., 2005).

Top–down proteomics

The above-described technique (bottom–up proteomics) is critically based on consistent enzymatic conversion of proteins to peptides. It is customary to accurately make mass measurements by a MS/MS of lower molecular weight peptides rather than higher molecular weight intact proteins. The bottom–down approach increases the sample complexity and the entire sequence coverage for proteins is rarely achieved. This seriously limits site-specific PTM analysis of proteins from a biological context of view. These limitations of the bottom–up approach have renewed interest in the top–down proteome characterization strategies. This technique characterizes the individual proteins by MS without prior enzymatic cleavage. Capillary isoelectric focusing (CIEF) coupled with Fourier transform-ion cyclotron resonance (FTICR) MS has been the first report to analyze complex protein mixture using top–down approach (Jensen et al., 1999; Valaskovic and Kelleher, 2002). A two-dimensional display, pI and molecular weight, similar to the conventional 2-DGE, however, with a higher resolution are seen on both the axes. The mass measurement accuracy can be enhanced by isotope depletion of proteins. The major limitation of this technique is that this level of information is not always sufficient for confident protein identification due to the possibilities of point mutations, PTM and the

presence of ORFs having high sequence homology. This problem can be solved to some degree by incorporation of isotopically labeled amino acids into the cellular proteins of unicellular model organisms. The partial amino acid content information obtained combined with CIEF-FTICR, enables identification of proteins from genome databases without the MS/MS information (Jensen et al., 1999; Martinovic et al., 2002). The top–down approach using FTICR-MS/MS demonstrated localization of PTM and site-specific mutations in bovine carbonic anhydrase providing 100% sequence coverage for the protein. Simple protein mixtures can be analyzed by prior 1-D separation of proteins as well as 2-D separation followed by infrared multiphoton dissociation (IRMPD)-MS/MS by tandem quadrupole-FTICR. Besides requiring large amount of sample (1 g of yeast cells) the method is not high-throughput and not amenable for automation, much needed for analysis of complex protein mixtures.

Surface-enhanced laser desorption ionization

The surface-enhanced laser desorption ionization (SELDI) technique comprises of ProteinChip arrays, a mass analyzer and the data analysis software. ProteinChip array-based technology consists of spots with chromatographic surfaces. These surfaces are either preactivated for capture of protein molecules or have certain physiochemical properties such as hydrophobic, hydrophilic, cationic, etc. The technique requires an incubation of the sample (1–10 μg) on the spot, followed by washing of the unbound proteins as well as the salts. Matrix solution is added to the sample adsorbed on the spot to be analyzed by laser desorption ionization TOF-MS. The intensities of the different sample components plotted on y-axis against the m/z (x-axis) are used for differential mapping. The similar m/z components are clustered and the clusters are compared to give statistical significant p-value for a given profile.

SELDI-TOF-MS has shown application in proteomic profiling from pre-frontal cortex of schizophrenia and bipolar disorder subjects; CSF of frontotemporal dementia subjects; CSF of rat models of cerebral ischemia; CSF of glioma subjects; sera of neuroblastoma subjects and CSF of AD subjects. The technology was found to be well suited for generating differential maps of protein regulation; however, the major drawback is its inability to characterize the proteins of interest in succession. The identification needs subsequent purification and/or enrichment, followed by proteolytic digestion and peptide mass fingerprinting. The second major drawback is the inability to analyze all the proteins unlike most of the currently available proteomics techniques. It has also been noted that the higher molecular weight proteins (>30 kDa) are not well resolved. The technique also runs into problem due to a narrow dynamic linear range for purposes of quantitation. The diagnostic potential of SELDI technique-derived proteomic maps justifies further studies.

Functional proteomics

This field of proteomics monitors and analyzes the spatial and temporal properties of molecular network of proteins. The functional proteomics analyzes a large set of proteins for PTM critical to the function of proteins in signaling, their localization and turnover, and protein interactions. MS is a general method used to determine the PTM due to its ability to accurately measure the change in molecular weight. Some of the important PTM in neuroscience are: phosphorylation, glycosylation, acetylation, methylation, sulfation, ubiquitination and tyrosyl nitration. The most popular of the above PTM will be discussed with an emphasis on diverse ways to identify them on a proteomics scale.

Phosphorylation

Analysis of phosphorylation, conventionally regarded as the most imperative PTM, includes identification of phospho-proteins and localization of exact phosphorylated residue(s). Phosphorylation of serine, threonine and tyrosine residues is recognized as a key regulator for a wide range of biological functions and activities in eukaryotic

cells, such as enzyme activity, signal transduction, transcriptional regulation, cell division, cytoskeletal rearrangement, cell movement, apoptosis and differentiation (Krebs, 1983; Hunter, 1998; Yan et al., 1998) affecting approximately one-third of all proteins at any given time (Zolnierowicz and Bollen, 2000). The various techniques currently available for phosphopeptide detection are schematized in Fig. 4. The classical method, detects phosphoproteins by autoradiography, by incorporating ^{32}P or ^{33}P by protein kinases into cultured cells or subcellular fractions. This approach is limited to specimens amenable to radio-labeling and poses certain safety and disposal problems (Guy et al., 1994; Wind et al., 2001). Immunoblotting is also used for phosphoprotein detection with antiphosphoserine, antiphosphotyrosine and antiphosphothreonine antibodies. In spite of the availability of high quality antibodies to phosphotyrosine residues, antiphosphoserine and antiphosphothreonine antibodies have inconsistent reproducibility. Immunoblotting also complicates subsequent use of the protein for sequencing by MS (Kaufmann et al., 2001).

Specific fluorescence-based detection methods such as Pro-Q Diamond stain devised recently have been rigorously established for staining phosphoproteins (Martin et al., 2003a; Schulenberg et al., 2003; Steinberg et al., 2003). Even though autoradiography is considered the most sensitive detection method for phosphorylation a recent study failed to show any significant differences in the number of proteins spots detected by autoradiography as compared to the Pro-Q Diamond stain (Wu et al., 2005). The Pro-Q Diamond staining method avoids the culture artifacts during culturing of cells with ^{32}P and is feasible with tissue from animal models as compared to the radioisotopes. This stain has a detection sensitivity of 1–16 ng of phosphoprotein and can be used to stain IEF gels, SDS-PAGE and 2-DGE for detection of phosphorylated serine, threonine and tyrosine residues (Martin et al., 2003b). This is a very convenient and less time consuming technique for preliminary assignment of phosphorylation status to hundreds of protein spots, from a total of thousands present on a 2-DGE map. This allows focusing on a small number of protein spots of interest stained by Pro-Q diamond to be later analyzed for protein content and characterization of the phosphorylation residues by MS/MS. Recently, Tannu et al. (Tannu) undertook the first proteomic scale phosphoproteome analysis of primate brain tissue, to throw light on some novel membrane-, receptor- and cytoskeletal-associated proteins, by coupling 2 DGE/Pro-Q Diamond staining with MS, to be involved after cocaine self-administration (Tannu). The use of both 2-DGE and LC-based approaches enables us to achieve greater proteome coverage (Collins et al., 2005a).

In spite of the importance of phosphorylation, identification of phosphorylation site(s) is still a challenge. There are several reasons which complicate phosphoprotein analysis: (1) only a small fraction of the intracellular proteins is phosphorylated at a given time, (2) the phosphorylated sites on proteins might vary (a protein can exist in many different phosphorylated forms), (3) most of the signaling molecules are present at low abundance intracellularly and (4) phosphotases can dephosphorylate a protein unless appropriate precautions are taken during the preparative stages of cell lysates. A general approach to overcome this challenge consists of phosphopeptide isolation from the enzymatic digest of proteins using the immobilized metal affinity chromatography (IMAC) columns, followed by derivatization of phosphoprotein and/or phosphopeptide prior to its analysis by MS/MS to confirm the identification as well as localizing of the phosphorylation site (Trinidad et al., 2005; Collins et al., 2005a). This method exploits the high affinity of phosphate groups toward a metal-chelated stationary phase such as Fe^{3+} (Posewitz and Tempst, 1999; Zhou et al., 2000; Stensballe et al., 2001; Trinidad et al., 2005). IMAC generally enriches for phosphoserine, phosphotyrosine and phosphothreonine residues as they are negatively charged groups. The affinity is extended over to aspartic acid and glutamic acid, and histidine (electron donor). Virtual elimination of the non-specific binding to IMAC column can be achieved (Ficarro et al., 2002). The phosphopeptides are first derivatized to corresponding peptide methyl esters rendering the IMAC selective for phosphopeptide and eliminating the confounding binding through

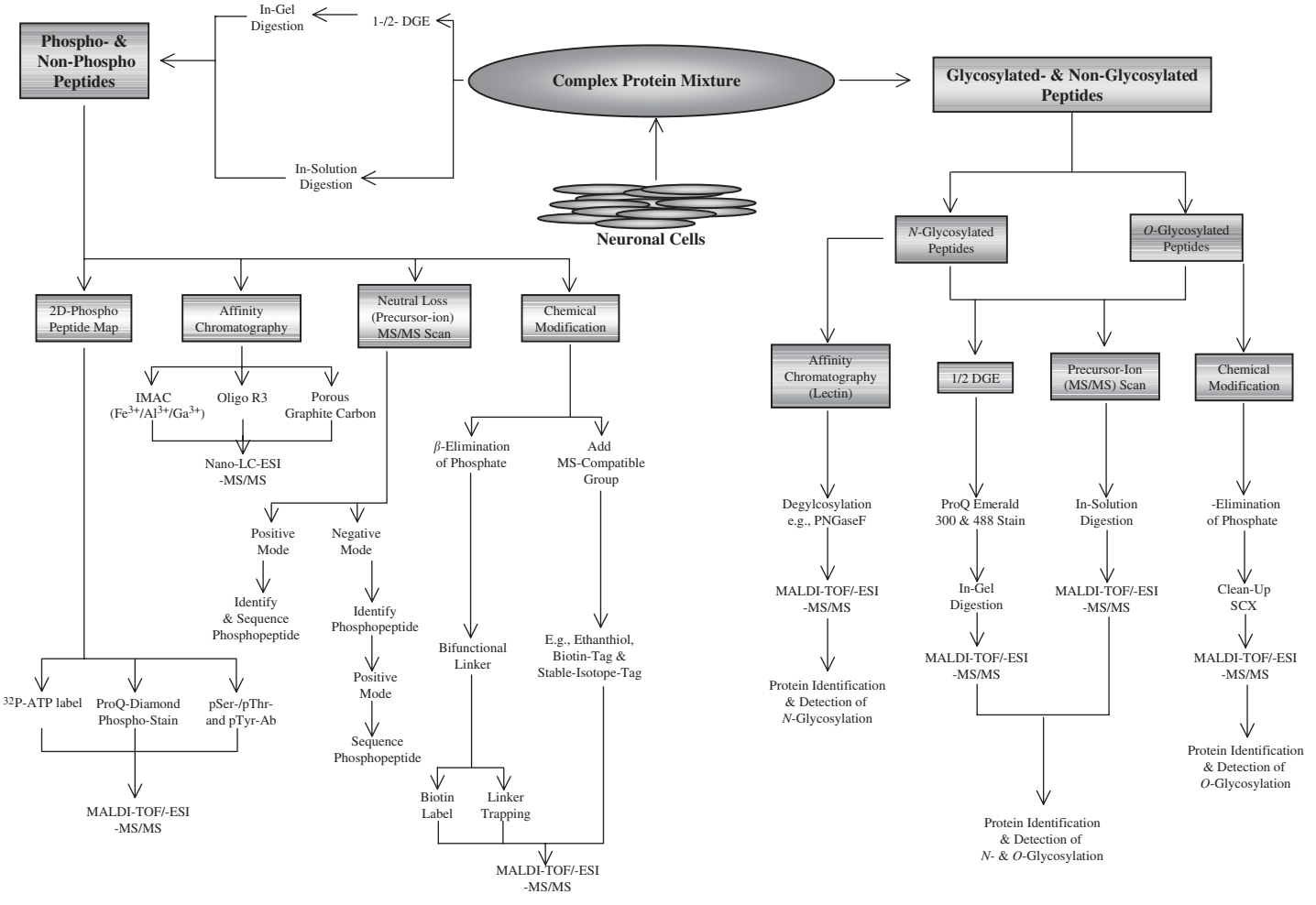

Fig. 4. Graphic description of the different detection methods routinely practiced for analysis of phosphorylation and glycosylation events at proteomics-scale (elaborate description in text). Adapted and reprinted with kind permission from Wiley-VCH Verlag GmbH & Co KG, and Elsevier (Mann et al., 2002; Reinders et al., 2004).

the carboxylate groups. The use of IMAC enriches for phosphopeptide, however, other alternatives such as purification on a polymer-based reverse-phase perfusion chromatography resin (oligo R3) (Matsumoto et al., 1997; Neubauer and Mann, 1999) or alternatively a porous graphite carbon (PGC) (Chin and Papac, 1999) are also beneficial. The chemical modifications derivatizing the phosphopeptides by 1% phosphoric acid (Kjellstrom and Jensen, 2004), ethanethiol (Resing et al., 1995), ethanedithiol (Oda et al., 2001), DTT (Amoresano et al., 2004) and diammonium citrate (Asara and Allison, 1999) have also shown to enhance the phosphopeptide ion signal in mass spectra.

There are some challenges which present during the MS stage of the phosphoproteome analysis: (1) the phosphopeptides are negatively charged whereas the MS is generally performed in the positive ion mode, (2) phosphopeptides being hydrophilic do not bind well to the columns which are routinely used for purification of peptides before analysis and (3) there is a strong possibility of ionic suppression by non-phosphopeptides making the phosphopeptides to be observed as less intense peaks in the mass spectra. The peptides containing phosphoserine or phosphothreonine residues when subjected to CID, undergo a gas-phase β-elimination reaction, resulting in neutral loss of phosphoric acid (-98 Da:H_3PO_4 loss) or are de-phosphorylated (-80 Da:HPO_3) (Bennett et al., 2002). However, phosphotyrosine is usually more resistant to this loss. As the mass spectrometer measures m/z values, doubly and triply charged peptide ions show an apparent loss of 49 and 32.66 Thompson (Th) in the mass spectrum (Covey et al., 1988; Schlosser et al., 2001). In the positive ion mode of MALDI-TOF-TOF, the serine and threonine residues show predominant neutral loss as compared to 80 Da loss, and are differentiated from tyrosine residue which generally shows only 80 Da loss (Annan and Carr, 1996). The suppression effect of the phosphopeptides in the mass spectra can be overcome by elimination of phosphate group to generate dehydroalanine from phosphoserine and dehydroamino-2-butyric acid from phosphothreonine; on the other hand, phosphotyrosine undergoes no elimination due to aromatic nature of the side chain. In the MS/MS spectrum, a spacing of 69 Da (dehydroalanine) or 83 Da (dehydroaminobutyric acid) indicates the exact location of phosphorylated serine and threonine residues respectively. There have also been reports where specific treatment with phosphatase was used to specifically identify phosphopeptides based on characteristic shift of mass due to the loss of phosphate after the treatment (Yip and Hutchens, 1992; Liao et al., 1994).

Specific MS scans methods such as parent ion scanning (precursor ion scanning operated in negative ion mode) as well as the neutral loss scanning have been used for analysis of phosphorylation sites (Carr et al., 1996; Schlosser et al., 2001; Steen et al., 2001). The negative mode of MALDI-MS has been recently shown to produce somewhat more intense signals from phosphopeptides as compared with the positive mode, an avenue which can be furthered (Ma et al., 2001). The fragments in the CID of triple quadrupole MS specifying for phosphate groups serve as reporter ions for precursor ion scanning by tandem MS. Quite a few large-scale analyses of synaptic phosphoproteome mapping have been undertaken using the above-discussed multiple complimentary approaches at the level of protein extraction, phosphopeptide enrichment and the analysis by MS and MS/MS (Collins et al., 2005b; Trinidad et al., 2005).

Glycosylation

Glycosylation, attachment of glycans/carbohydrates to proteins, is one of the most extensive and complex PTM. This modification is confounded by variable modifications such as phosphorylation, sulfation, methylation and acetylation of the glycans residues. It has been documented to play an important role in cell growth and development, cell–cell interaction, carcinogenesis, neurodegeneration and AD (Adamo et al., 1989; Lowe and Marth, 2003; Takeuchi et al., 2004; Lue et al., 2005; Moreira et al., 2005; Robbe et al., 2005). The brain is affected mostly to a severe degree in 10 of the 11 known congenital disorders of N-linked glycosylation (Jaeken and Matthijs, 2001). The branched glycans present as

N- and O-linked glycosylation have great linkage diversity as well as structural complexity presenting a significant challenge for characterization of glycosylation at global scale. Typically, O-linked glycosylation occurs on serine or threonine residues, while the N-linked glycosylation (most widely analyzed in the eukaryotes) occurs on asparagines residue. The protein glycosylation, particularly the N-linked glycosylation is encountered in extracellular proteins (Roth, 2002). The various techniques currently available for detection of glycosylation events in cellular proteins are represented in Fig. 4. Recently gel-based strategies have been developed, which do not require the breakdown of the glycoprotein, and can be integrated in the general proteomics workflow (Packer et al., 1999; Raju, 2000; Hart et al., 2003). The staining with Pro-Q Emerald 300 is based on reacting carbohydrate groups by a periodate/Schiff's base (PAS) mechanism. The oxidation of carbohydrate groups to aldehydes is followed by conjugation with chromogenic (acid fuschin, Alcian Blue) or fluorescent (Pro-Q Emerald 300 and 488) substrates. The glycoproteins segregated by 2 DGE and stained by Pro-Q Emerald 488 are scanned by fluorescent scanners and the images analyzed by image analysis softwares.

Another promising strategy is the coupling of lectin-affinity technology with LC-MS/MS (Hirabayashi et al., 2002). The general strategy used by this technique is the selective capture of glycopeptides from proteolytic protein products by lectin. This is followed by the determination of N-glycosylation sites using peptide-N-glycosidase F (PNGaseF) in presence of $H_2^{18}O$ (isotope-coded glycosylation site-specific tagging: IGOT) and identification of the protein by LC-MS/MS (Gonzalez et al., 1992; Hirabayashi, 2004). This technique has been able to successfully reveal 400 N-glycosylation sites in 250 proteins (Kaji et al., 2003). This technique is limited for its incapability to analyze O-glycopeptides by and large due to the unavailability of a universal glycosidase for release of O-glycans from glycoproteins. Typically, chemical modification of the O-linked glycosylated residues undergoing β-elimination (reducing conditions such as sodium borohydride) are used for assigning this modification for analysis by MS/MS. A broader capture of glycoproteins based on the conjugation of glycoproteins to a solid support using hydrazide chemistry, stable isotope labeling of glycopeptides and the specific release of N-linked glycosylated peptides by PNGaseF has also been successfully applied to identify membrane and extracellular glycoproteins (Zhang et al., 2003). The above study also made a reasonable quantitative assessment of the glycopeptides by isotopically N-terminal labeling with d0 (light) and d4 (heavy) forms of succinic anhydride after C-terminal lysine residues were converted to homoarginines. The precursor ion scans in tandem mass spectrometers identify glycopeptides based on characterization of one or more of the daughter ions with m/z of 204 Da ($[HexNAc + H]^+$), 274 Da ($[NeuAc\text{-}H_2O + H]^+$), 292 Da ($[NeuAc + H]^+$) and 366 Da ($[Hex\text{-}HexNAc + H]^+$). A comprehensive N-glycoproteome analysis at global scale has been recently attempted by coupling the above technique with 2D LC-MS/MS (FTICR) to identify 303 non-redundant *N*-glycoproteins having 639 N-glycopeptides (Liu et al., 2005).

Ubiquitination

The 2004 Nobel Prize in chemistry was awarded to Hershko, Ciechanover and Rose for the central importance of ubiquitin in regulating protein degradation (Kirkpatrick et al., 2005). This PTM involves modification of protein substrates by a highly conserved 76 amino acid polypeptide, ubiquitin (Kaiser and Huang, 2005). The C-terminal glycine of ubiquitin covalently links through an isopeptide bond to the side chain of lysine(s) within the substrate as mono-, multi- or poly-ubiquitination. A family of ubiquitin-like (Ubl) proteins is also known to form similar covalent PTM. Ubiquitin typically is a degradation signal, while Ubl modulates exclusively non-proteasomal endpoints. Ubiquitination plays a central role in protein stabilization, localization, interactions as well as functional activity for many protein substrates (Finley et al., 2004). Mono-ubiquitination is linked to protein transport and poly-ubiquitination initiates the proteolysis of substrates; on the other hand, both regulate the protein function

directly without affecting their stability (Hershko and Ciechanover, 1998; Hicke, 2001; Colledge et al., 2003; Pickart, 2004; Pickart and Eddins, 2004; Pickart and Fushman, 2004; Kato et al., 2005). Recent direct evidence has been established between oxidative damage to the neuronal ubiquitination/deubiquitination machinery and the pathogenesis of sporadic AD and Parkinson's disease (Choi et al., 2004). The enzymes involved in the multi-step transfer of ubiquitin are E1 enzymes (activating), E2 enzymes (conjugating) and E3 enzymes (ligases) (Kirkpatrick et al., 2005). The highly dynamic process of ubiquitination is balanced by the deubiquitinating enzymes.

The importance of ubiquitination in the cell physiology and disease necessitates a global approach to study cellular ubiquitination at large scale. This is difficult because the modification is large, 8 kDa, and due to the rapid turnover of ubiquitinated proteins the steady-state levels are characteristically low. A tryptic signature peptide at the ubiquitination site consists of two-residue remnant (glycine–glycine) derived from the C terminus of ubiquitin that remain attached by an isopeptide bond to the target lysine residue. This signature peptide shows a mass shift at the lysine residue of 114.1 Da. A caveat, because of the missed cleavage at the ubiquitin modification site the –GG signature peptides become too large for standard MS analyses and require alternate digestion strategy. The protein conjugates which have been detected by present technique are only a subset of all ubiquitin conjugates. Each ubiquitin-like protein modifier potentially will leave its own signature remnant peptide bound to its target and detected by MS. The substrates purified via an N-terminal epitope tag [DK 10] fused to ubiquitin, digested by trypsin and separated by LC-MS/MS identified as many as thousand proteins with ubiquitination along with 110 precise ubiquitination sites (Peng et al., 2003b; Kirkpatrick et al., 2005). The use of high mass accuracy Fourier transformion cyclotron resonance mass spectrometer (FTICR) have been beneficial for identifying the ubiquitination sites (Cooper et al., 2004).

The phosphorylation of some proteins is prerequisite for ubiquitination and subsequent substrate degradation (Ciechanover et al., 2000). Non-tagging strategies for enriching targets like ubiquitin-binding proteins as well as in vitro systems can be used effectively to identify targets for ubiquitin and Ubl proteins (Gocke et al., 2005; Mayor and Deshaies, 2005; Mayor et al., 2005). The amino-terminal labeling of −GG signature peptides by modification with fluorous affinity tags has also been used (Brittain et al., 2005). A double-affinity purification procedure is routinely used to increase the stringency of ubiquitin and Ubl substrates characterized by shotgun proteomics approach (Mayor and Deshaies, 2005; Mayor et al., 2005). Coupling the ICAT™ technique for quantitation of ubiquitinated proteins promises a unique analytical benefit for overcoming the huge excesses of peptides from ubiquitin for large-scale characterization (Kirkpatrick et al., 2005). Even though there has been a recent progress in global characterization of mono-ubiquitination, to develop a methodology for global analysis of poly-ubiquitination still remains a challenge.

Nitration

The chemical modifications exerted by nitric oxide having biological significance are through interactions with transition metals, free radicals, redox regulators and thiol groups such as in cysteine (Martinez-Ruiz and Lamas, 2004a, b). The S-nitrosylation of cysteine residues (addition of NO to sulfur atom to form S-NO bond) and nitration of tyrosine residues (addition of nitro group to position 3 of phenolic ring of tyrosine residue) are the primary PTMs by NO. The S-nitrosylation is implicated in cellular signal transduction pathways (Bolan et al., 2000; Lane et al., 2001; Martinez-Ruiz and Lamas, 2004b). The tyrosine nitration modifies protein function and causes irreversible protein damage due to oxidative stress as well as neuronal differentiation (Cappelletti et al., 2003). Neurodegenerative as well as inflammatory diseases viz. Parkinson's, Alzheimer's, familial amyotrophic lateral sclerosis and Huntington's have been associated with tyrosine nitration (Giannopoulou et al., 2002).

Tyrosine nitration is a relatively stable modification. The strategies used for separation, detection

and quantitation of nitrotyrosine residues are, anti-3-NT antibodies, HPLC coupled to ESI-MS and GC-MS. Large scale nitro-tyrosine residues can be affinity tagged by reducing nitrotyrosine into aminotyrosine followed by biotinylation (Nikov et al., 2003). The affinity-tagged enrichment can be complemented to the 2-DGE and Western blotting proteomic methods for identification of nitrated proteins (Miyagi et al., 2002). Also chromatographic methods after tryptic digestion of proteins can be used for analysis of nitrotyrosine residues. Typically, the specific nitrotyrosine residues resolved by 2-DGE or HPLC are identified by N-terminal micro sequencing and mostly MS (Δ mass: +45 Da) (Marcondes et al., 2001; Haqqani et al., 2002). Large-scale proteomic approach for detection of global nitrotyrosine residues using the above techniques have been successfully employed recently (Miyagi et al., 2002; Zhan and Desiderio, 2004; Casoni et al., 2005). S-nitrosylation of protein cysteine residues to give nitrosothiols is a reversible (reduced by ascorbic acid, GSH and thioredoxin) modification. S-nitrosylated proteins can be detected by 2-DGE. This method derivatized SH with biotinylated thiol reagent and several derivatized proteins were identified by immunoblotting and/or immunoaffinity (Jaffrey et al., 2001). However, there are only few studies where techniques to detect nitrotyrosine and S-nitrosylation have been applied to large-scale proteomic analysis.

Multi-protein complex (protein–protein interactions)

The express and transitory associations in large protein complexes play an important role in modulating protein functions in various molecular mechanisms in neuron (Krapivinsky et al., 2004; Kim and Sheng, 2004; Soosairajah et al., 2005; Teng and Tang, 2005). The affinity-based techniques implementing the affinity tags and ligands; Poly-His (Ni2+), Biotin (Streptavidin), Calmodulin-binding peptide (Calmodulin), GST (Glutathione), and specific epitope such as FLAG, c-myc and HA (Monoclonal Ab) are commonly used for isolation of multi-protein complexes. The protein of interest is expressed with suitable tag to be used as bait, with the above antitag systems immobilized on agarose–sepharose supports, to isolate the entire multi-protein complex from cellular extract. The proteins in the multi-protein complex are further separated by SDS-PAGE followed by LC-MS/MS for protein characterization. The success of the affinity-based approach depends on non-specific binding which in turn depends on the specificity of the bait partners' recognition. The drawbacks of this technique include extensive pre-cleaning and the protein–protein interactions are essentially in vitro interactions (Monti et al., 2005). Most of the drawbacks of the affinity-based approaches can be overcome by immunoprecipitation strategies (Whetstone et al., 2004). The gene coding for the bait tagged with the epitope is expressed after transfection into appropriate cell line. The protein complexes formed in vivo are immunoprecipitated with antitag monoclonal antibodies. The multi-protein complex is further characterized by SDS-PAGE coupled with LC-MS/MS. The major disadvantage of this method is the cross-recognition of non-specific antigens and non-specific binding of proteins to antibodies and the peptide tags leads to false positives. The above problem has been largely overcome by tandem affinity purification (TAP) tag system (Anders et al., 1999; Puig et al., 2001). The TAP technique is based on combining two different tags (such as *Staphylococcus aureus* protein A (ProtA) and calmodulin-binding peptide (CBP)) on the same protein usually spaced by an enzyme-cleavable linker sequence (TEV protease cleavable site) (Wine et al., 2002; Hurst et al., 2004). The TAP technique has been recently employed in neuroscience (Borch et al., 2005; Davey et al., 2005; Gingras et al., 2005; Gottschalk et al., 2005; Swanson and Washburn, 2005).

Once the interacting partners are identified, to map the interaction site is usually desirable. Many techniques are currently available for studying protein–protein interactions; X-ray crystallography, multi-dimensional NMR, phage display, yeast two-hybrid screens and protein microarrays. Modern MS has increased the scope of these tools as well as improved the ability to investigate complex protein–protein interactions. The MS analysis can be simplified by digesting and

isolating the cross-linked peptide, aided by incorporation of fluorescent probes or affinity handles into traditional cross-linkers, for determining the specific site of interaction (Trakselis et al., 2005). The most widely used fluorescent cross-linkers are bromobimanes and sulfosuccinimidyl-2-(7-azido-4-methylcoumarin-3-acetamido)-ethyl-1, 3′-dithiopropionate, and affinity handle as biotin specifically isolated using monomeric avidin or Streptavidin (Kim et al., 1995; Wine et al., 2002; Trester-Zedlitz et al., 2003). After tryptic digestion the fluorescent/affinity-purified protein fragments are separated by SDS-PAGE to be analyzed by tandem MS for interaction sites (Heck and Van Den Heuvel, 2004). The composition of the cross-linked peptide can be deciphered by comparison with the known sequences of the interacting proteins. The coupling of these techniques enables complete protein complex analysis in response to various physiological as well as pathological stimuli.

Mass spectrometry

The sequence analysis of peptides and proteins along with the PTM separated by electrophoresis and chromatography has been a major application of MS in proteomics (Aebersold and Mann, 2003). The mass spectrometer consists of three major units: ion source, mass analyzer and the ion-detection system (Fig. 5). A large variety of MSs are based on coupling of MALDI and ESI with different types of mass analyzers. MS is based on separating the ionized proteins or peptides based on mass to charge ratio (m/z). The tandem MS (MS/MS) on the other hand couples two MSs in time and space and has revolutionized the field of expression and functional proteomics (Smith, 2002). The MS/MS involves mass selection, fragmentation and mass analysis (in two stages) (Fig. 5). In the first stage of mass analysis (MS1) the precursor ion produced by the ion source gets selected for fragmentation in the CID. The fragmentation results in the production of product ions to be analyzed in the second stage of mass analysis (MS2). The inconvertible link between the precursor ion and the product ions is responsible for the unique molecular specificity of MS/MS.

All of these MS techniques can be applied to complex protein samples, i.e., mixtures of hundreds or thousands of proteins. It is important to separate the use of MS instruments to separate proteins from the MS used for protein identification as will be described later. For separation MS has great capabilities but also limitations. As described below, quantitative analysis by MS is limited to techniques like ICAT™ and iTRAQ™. For researchers looking to profile the expression of proteins in a large number of samples, MS can be problematic and requires a great deal of time on expensive instruments.

Ion source

A number of ionization technologies exist including: fast ion bombardment (FAB) (Barber et al., 1981), MALDI (Karas and Hillenkamp, 1988), and ESI (Fenn et al., 1989; Wilm and Mann, 1996). MALDI and ESI are the techniques of choice for most proteomic applications of neuroscience research.

MALDI

MALDI operates based on irradiation by an intense laser beam of the protein mixture with matrix. There occurs a transfer of large amount of energy absorbed by the crystallized matrix to the sample molecules, desorbing and ionizing them into a gas phase plume by proton-transfer (Fig. 5). This is usually done on MALDI plates such as stainless steel and AnchorChip coated with a hydrophobic material (Tannu et al., 2004b). The most-popular fragmentation types utilized are, ion-source decay (source-accelerating region fast fragmentations) and post-source decay: field-free region metastable fragmentation process). The current proteomic applications overwhelmingly couple MALDI to TOF instrument as the pulsed nature of the laser beam matches well with the pulsed mode of TOF. MALDI-TOF MS is normally used for analysis of simple peptide mixture. Many matrix–laser combinations have been tried; however, for proteomics applications the two most

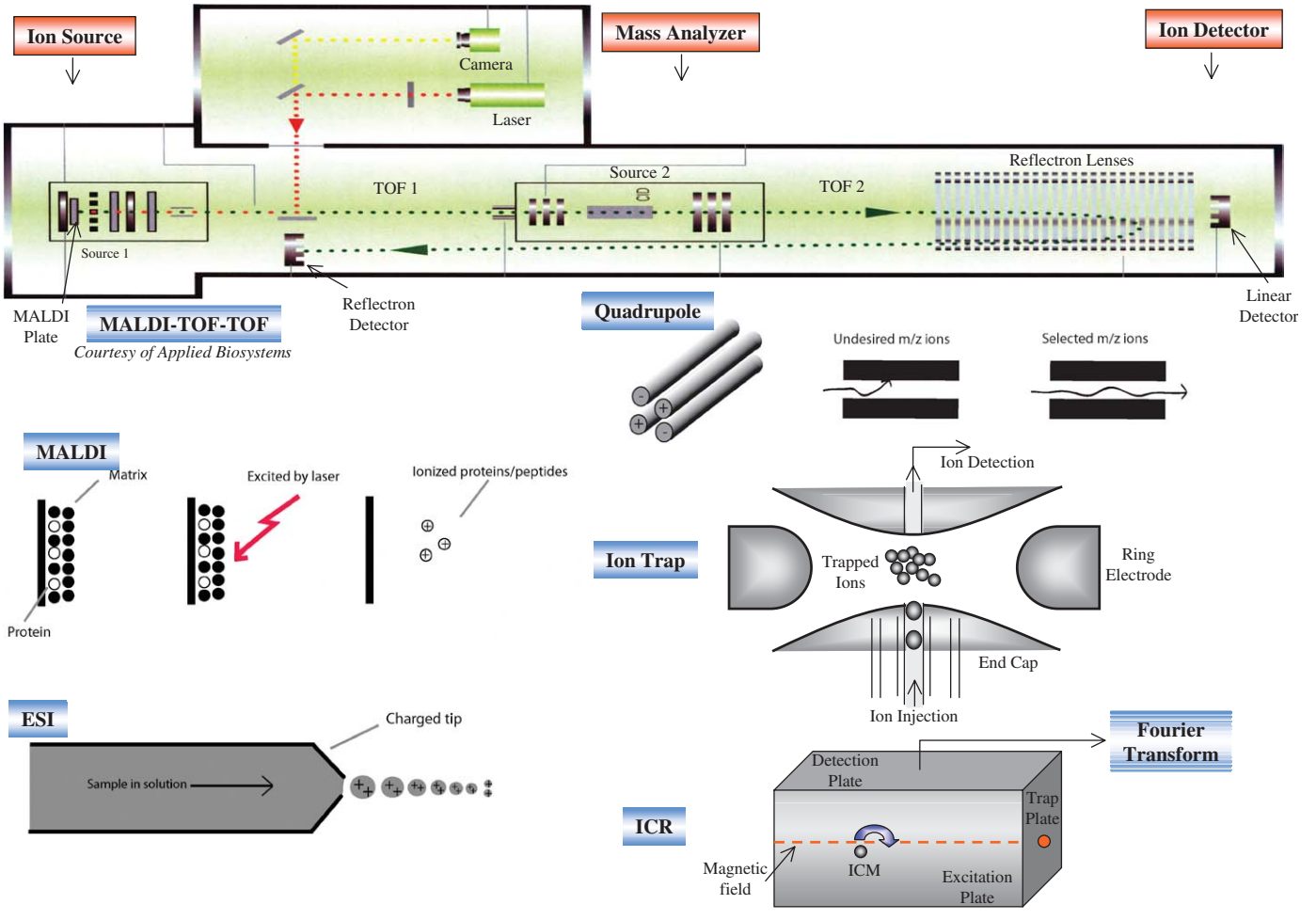

Fig. 5. Schematic representation of MS components (elaborate description in text). Adapted with kind permission from Springer Science and Business Media, and Applied Biosystems (Freeman and Hemby, 2004; Schrader and Klein, 2004; Biosystems, 2005).

commonly used matrices are α-cyano-4-hydroxy-cinnamic acid (CHCA) for peptides and small molecular-mass proteins (<10,000 Da), and sinapinic acid for high-mass proteins. Many techniques such as dried-drop, fast evaporation, sandwich matrix, spin-dry and seed-layer have been tested (solvents used: ACN, methanol, acetone, chloroform and propanol) to obtain a homogenous sample-matrix critical for obtaining good sample ion yield. A general theme for the formation of a homogenous sample-matrix, confirmed by Tannu et al. (2004b) among many studies undertaken so far, has been a hydrophobic MALDI plate surface. MALDI is capable of analyzing positive as well as negative ions. The MALDI mass spectra typically constitutes signals from singly protonated target molecules and their oligomeric ions ($[M+H]^+$, $[2M+H]^+$). The coupling of MALDI with quadrupole ion trap and FTICR instruments has also emerged, especially the MALDI-FTICR, contributing significantly to accurate mass measurements pre-requisite for assigning critical PTM to peptide residues.

ESI

ESI is an atmospheric pressure ionization (API) technique which can be performed in either positive or negative ionization mode to form $[M+nH]^{n+}$ and $[M-nH]^{n-}$-type ions, respectively. In ESI (Fig. 5) (and nanospray ionization), ions are produced in a liquid phase as three step process of droplet formation, droplet shrinkage, and gaseous ion formation (Wilm and Mann, 1996). The protein sample, in a solvent solution, is ejected as a mist of droplets from a charged capillary tip. As the solvent in the droplets evaporates the total charges of the proteins in the droplet remain but with a reduced surface area of the droplet. This continues to a point at which individual ions leave the droplet. Individual ions then pass on into the mass analyzer. The ESI has dominated bottom–up proteomics method to be coupled with the multi-dimensional chromatography. The wide popularity of interfacing ESI with HPLC and MS has been the continuous-flow function, acceptance of wide flow rates, tolerance to different types of solvents, and the ability to generate intact multiple-charged ions.

Mass analyzer

Which ever method of ionization is used, once the ions are created they must be separated before being detected in such a way as to provide information on the m/z ratio. Mass analyzers do not actually detect the ions or measure ion mass; they are only used to separate ions according to their m/z ratio. A number of mass analyzer types exist: time-of-flight (TOF), quadrupole, ion trap and FT-ICR. The coupling of HPLC with quadrupole and quadrupole ion trap (QIT) is widely practiced due to the high tolerance to low vacuum and absence of high potentials in the ion source. The coupling of LC with FTICR is known for high resolution and mass accuracy.

Time-of-flight

Time-of-flight (TOF) (Fig. 5) mass analyzers can be thought of as a tube. The ionized proteins/peptides enter the tube by passing through a high-voltage accelerator. The speed at which the ion travels is proportional to its mass (m). The TOF-MS is a velocity spectrometer separating the ions based on velocity differences. The principle of mass analysis is that after acceleration to constant kinetic energy (zV: where z = charge and V = accelerating potential) the ions travel at velocities, ν which is an inverse function of the square root of m/z values. The short pulse of ions is dispersed as isomass aggregates such that the ones with lower mass travel faster and reach the detector early than the heavier ions. The time of arrival (t) is used for mass analysis of the ions for a particular length of the flight (L) and is given by

$$t = L(m/2 \cdot zV)^{1/2}$$

The measurement of flight times for two known mass ions is used for converting the time spectrum into the mass spectrum, typically displayed as m/z (Dass, 2000). MALDI ion source most commonly coupled with TOF mass analyzer is many a times referred to as the workhorse of proteomics due to its capability of detecting proteins of >300 kDa with the detection sensitivity in attomole–femtomole range. The MALDI-TOF-TOF (Fig. 5) has

also found routine success in detection of peptide residues with PTM along with their locations.

Quadrupole

Quadrupole mass analyzer offers most of the desirable features necessary for MS viz. high scan speed, adequate mass range and resolution, high sensitivity and useful dynamic range. Also QIT is more sensitive and cheaper than quadrupole. Quadrupole mass analyzers (Fig. 5) also involve ions traveling down what can be thought of as a tube. In this case though, the tube consists of four parallel rods. The rods are two pairs of two that can be tuned to different currents and radio frequencies. The two pairs of rods have opposite currents and shifted radio frequencies allowing a form of tuning in which only ions of a particular m/z ratio pass though the tube. A range of m/z ratios can be scanned, generating an m/z profile of the sample. Quadrupole mass analyzers are often used with an ESI ion source.

Ion trap

Ion trap mass analyzers (Fig. 5) use the same principles as the quadrupole in that specific combinations of current and radio frequencies are used to select particular m/z ratios (Jonscher and Yates, 1997). The ion trap can be thought of as a small ball with one electrode around the equator and two more electrodes at the poles. Ions are introduced into the center of the ball and are kept in orbits within the trap. By changing current and radio frequency combinations particular m/z ratio ions are ejected from the ion trap through a port to the detector. By scanning through these voltages and radio frequencies a complete m/z profile can be made (Douglas et al., 2005).

FTICR

The major advantages of FTICR are high mass-resolution and -accuracy, and the ability to trap ions for extended periods of time along with performing multistage tandem MS. A cyclotron cell consists of three pairs of electrode plates assembled as a cube such that the front and end electrode act as trapping plates to trap the ions, the two excitation plates connected with a radio-frequency transmitter to excite the ions, and the detection plates detect the induced mirror current (Fig. 5). The basic principle of the ICR is to detect the ions in the cyclotron cell having a cyclotron motion (ICM) inside a uniform magnetic field produced by superconducting magnet with fixed field strength (4.7, 7, 9.4 and 12 T). A Fourier transform algorithm is used to digitize and process the transient signals of all the ions to give ion abundance for specific m/z (Marshall et al., 1998; Marshall et al., 2002; Schrader and Klein, 2004). The LC-FTICR (ESI ion source), LTQ FTICR and Qq FTICR are commercially available for proteomics applications.

Tandem mass spectrometry

A number of instrument designs strategies exist for MS/MS, each fulfilling special needs (Fig. 5). All of these were generally designed to increase the accuracy of m/z measurements and sensitivity to low abundance ions. Some of the instruments perform MS/MS in space i.e., the mass selection, fragmentation, and analysis is carried out in different regions of the tandem mass spectrometer (e.g., quadrupole and TOF instruments). In some of the instruments this is done tandem in the same region using temporal sequence (tandem-in-time: e.g., QIT and FTICR). Conceptually the operating principles of both designs MS/MS are practically similar. TOF analyzers can be placed in series (TOF/TOF) with a reflectron or collision cell (CID) between them (Fig. 5), quadrupoles and TOF can be placed in series (Q-TOF)(Morris et al., 1997) (Fig. 5), and extremely powerful magnets and Fourier transform algorithms (FT-ICR) (Bogdanov and Smith, 2005) can be used to determine the m/z ratios of all ions within an ion trap (Marshall et al., 1998) (Fig. 5). In the collision cell, an intermediate region of a tandem instrument is filled with neutral gas (helium and argon). The CID, a two-step process of collision activation and unimolecular dissociation, consists of selecting a product ion after dissociation of the precursor ions to be subjected for further activation and unimolecular dissociation (Fig. 5).

Most of the recently available tandem mass spectrometers are effective in identifying proteins in a complex mixture. Obtaining at least one MS/MS spectra by selecting couple of most-intense precursor ions from each constituent protein from a mixture can be used to confirm the identification of constituent proteins. With ions generated and separated, instruments like electron multipliers and scintillation counters detect the ions. Detectors change the kinetic energy of the ions into an electrical current that can be measured and passed along to a computer. While these detectors give information on abundance of ions, quantitation of protein abundance differences between samples by MS is limited unless samples are linked to isotopes (see ICAT™). Tandem mass spectrometry has enabled unprecedented sequence determination of large number of peptides. The various ion sources and mass analyzers which can be conceptually coupled to give a tandem mass spectrometer are schematized in Fig. 5. The most commonly used mass spectrometers today for quantitative analysis are the quadrupole ion trap (3D-IT), the triple quadrupole (QqQ) and the quadrupole TOF (QqTOF) (Chernushevich et al., 2001). The 3D-IT is relatively small and inexpensive instrument as compared to the standard triple quadrupole and had MS^n capability. Recently, the linear ion traps (LIT) combined with QTOF (QqLIT), having the uniqueness of Q3 to be run in two different modes, large ion storage capacity and higher trapping efficiency, permit very powerful scan combinations for information-dependent data acquisition (Hopfgartner et al., 2004, Douglas et al., 2005).

Protein identification

Peptide mass fingerprinting (PMF) and tandem mass spectrometry (MS/MS), are the main methods for determining protein identities (Rappsilber and Mann, 2002; Johnson et al., 2005). PMF was developed by a number of research groups and begins with digestion of a protein with sequence-specific endoproteinase, typically trypsin (Henzel et al., 1993; Mann et al., 1993; Pappin et al., 1993). PMF of spots from 2-DE gels is one very common application. Gel plugs are either excised by hand or robot and an in-gel-trypsin digestion performed. CBB, silver and negative staining are common post-electrophoresis methods available for the 2-D gel-based proteomics analysis. The gel plugs must often be destained, and some stains work better than others. The CBB stain remains the stain of choice for MS identification of proteins. Briefly, the gel plugs are washed for 20 min, twice in 50 mM ammonium bicarbonate/50% (v/v) methanol in water and once with 75% (v/v) acetonitrile in water. After drying, the gel plugs are incubated overnight at 37°C in 140 ng of sequencing grade trypsin (Promega, Madison, WI) resuspended in 20 mM ammonium bicarbonate. The peptide fragments are then extracted twice with 50% (v/v) acetonitrile, 0.1% (v/v) TFA in water and concentrated using a vacuum concentrator. The peptide fragments dissolved in 50% (v/v) acetonitrile, 0.1% (v/v) TFA in water are spotted on matrix-assisted laser desorption-ionization (MALDI) target plates, dried and mixed with a 50% saturated solution of CHCA in 50% (v/v) acetonitrile, 0.1% (v/v) TFA in water. Trypsin cleaves proteins at very specific locations (carboxy ends of arginine and lysine), resulting in a series of peptides. The experimentally obtained peptide masses are compared with the theoretical peptide masses of proteins stored in databases by means of search programs (Thiede et al., 2005); (1) Mascot: http://www.matrixscience.com/search_form_select.html, (2) ProFound: http://prowl.rockefeller.edu/profound_bin/WebProFound.exe, (3) MS-Fit: http://prospector.ucsf.edu/ucsfhtml4.0/msfit.htm and (4) Aldente: http://www.expasy.org/tools/aldente/ (Johnson et al., 2005).

A number of different strategies exist for MS/MS, in general the process entails the selection of one ion/protein generated during initial MS and then fragmenting this ion/protein into smaller pieces and measuring the mass of the resulting ions (Hernandez et al., 2005). These secondary ions can be decoded into protein sequence information which are searched against protein sequence databases to identify the protein (Perkins et al., 1999; Zhang and Chait, 2000). Almost all of the ionization and mass analyzer types can all be used in an automated mode for MS/MS as well

peptide fragmentation identification provided that the instrument is appropriately configured (Fenyo and Beavis, 2002). One MS/MS method that is particularly suited for proteome determination, and recently become amenable to quantitation, is MuD-PIT. In this method all the proteins in a sample are digested and loaded onto LC columns (see previous explanation). After fractionation of the peptides, the peptides are fed into a MS/MS instrument for protein identification (Hernandez et al., 2005). This method has identified thousands of proteins, can detect membrane proteins and is similar in concept to shotgun sequencing of DNA at the same time when coupled with iTRAQ is capable of quantitative analysis as well (Rappsilber and Mann, 2002).

Some of the more traditional methods for identifying proteins are still used for proteomic experiments. Edman protein sequencing can be performed on proteins or peptides extracted from gels or blotted from gels. This method is limited by low throughput and requires a comparatively large amount of protein. Another technique is the Far Western blot where a 2-DEG is blotted and the blot is probed with an antibody against a specific protein. This approach does not offer much progress over conventional immunoblotting.

Protein arrays

Because of some of the limitations of electrophoresis and MS methods, selected research groups are attempting to create proteomic chips/arrays. The basic approach is very similar to that of microarrays (Petricoin et al., 2002; Wilson and Nock, 2003; Lopez and Pluskal, 2003). Antibodies or other affinity reagents (e.g., aptamers and peptides) are spotted onto some sort of matrix. Hundreds to thousands of spots are on a single array. A labeled sample is then washed across the array and proteins bind to their specific antibody. The process can also be reversed whereby the protein samples of interest are spotted onto the matrix and then probed with different affinity reagents (Paweletz et al., 2001). While these array or chip approaches have potential for greatly increasing the throughput of proteomic experiments, the use of affinity reagents as the separation method is a severely limiting factor and cannot be ignored. A high-quality antibody is needed for each protein of interest and each modification of that protein. Generation of antibodies remains a laborious task which is almost as much art as science. Separate antibodies also have to be generated for different organisms. In order to generate quantitative data from antibody arrays, and because association kinetics between different antibodies and antigens can vary tremendously, relative concentrations of each antibody and antigen have to be optimized for each protein in order to have quantitative information (Haab et al., 2001). Lastly, it should not be forgotten that sequence/structure knowledge is needed of any protein to be analyzed by protein microarrays in order to generate the affinity reagent, limiting this approach to known protein sequences and modifications. Though there seem to be a number of pitfalls to proteomic chips/arrays as an open-screen technique they do hold promise for routine examination of a small group of proteins. Well-known pathways or gene families could be easily examined by such an approach.

Conclusion

The immense potential for neuroproteomic has increased its recognition over years. It becomes crucial for neuroscientist to address some preliminary questions; such as the completeness of the database for the animal model to be used for study, the amount of tissue available for experiment, the subcellular location showing most promise for scouting and the precedence of quantitative or functional evaluation. The long-term success will most certainly depend on defining the questions to be answered and thereafter integrating the advanced technology with sound and relevant experimental design. Ever-increasing neuroscientists have adopted proteomic approaches to their research; the true advances will come from those that use these new tools to create not just data but discoveries.

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