

Proteomics: from Gel Based to Gel Free

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Since our last review in 2002, there have been significant changes in the field of proteomics. The hype surrounding this field has diminished as it has matured. The initial and overly simplistic view that studying a proteome would provide high-throughput discovery commonly overlooked issues such as the quality and validation of the results. The maturation of proteomics has led to the realization that it does not provide black and white answers, but is best employed as part of an overall discovery or validation effort. Proteomics is without question recognized as a highly valuable scientific area that can provide unique insight into many areas of biology. Fortunately, the application of mass spectrometry (MS)-based proteomics has shifted from reporting lists of proteins present in cells (often equated to building a large

stamp collection) to a differential expression approach based on the difference between two or multiple biological samples, akin to valuable stamps.

On the positive side, reports detailing proteomic applications have roughly doubled from 2002 to 2004 (from 1333 publications with the words “proteome” or “proteomics” in 2002, to 2555 in 2004). Functional proteomics has grown tremendously with numerous reports on protein–protein interaction mapping in different organisms. Additionally, a strong swing toward the application and discovery of biomarkers was driven by industrial needs for preclinical and clinical biomarkers. In regard to technology, an emphasis on increasing the dynamic range through fractionation and depletion has been observed, as well as the introduction of a plethora of approaches for differential peptide quantitation by MS.

2D gel electrophoresis (2DE) coupled to MS is a now a mature and well-established technique. This is illustrated by many reports on 2DE-MS over the last two years being mainly of an application nature, with a dwindling number of technical papers reporting incremental improvements. In fact, most developmental efforts over the last two years have been focused on alternative approaches to 2DE, such as “gel-free” proteomics and protein arrays. Although these novel approaches were initially pitched as replacements for 2DE-MS, it is likely that they will turn out to be complementary with their own limitations, some of which have become clearly apparent. 2DE-MS is now routinely used, while “gel-free” proteomic experiments are not and, conversely, are increasing in complexity.

In this review, we will discuss the major technical developments and applications that were published in proteomics in 2003–2004. This review does not attempt to be exhaustive; rather, it focuses on 200 publications that illustrate the breadth of development and diversity in application of proteomics over the past two years and provides comments on the future challenges in this field.

PROTEIN INTERACTION MAPPING

The mapping of protein–protein interactions is arguably the most significant development in proteomics to date. The two main approaches for mapping protein–protein interactions are the two-hybrid approach, as well as immunoprecipitation (IP) coupled to MS. The last two years have seen many reports of large-scale interaction data sets; the bulk of which were obtained by two-hybrid methodology. Bioinformatic developments for the analysis and integration of interaction data sets to build networks and pathways have also increased over the last two years.

Protein Interaction Data Sets. Protein interaction reports fall under three categories: low-throughput experiments that often focus on a single protein, medium-throughput experiments that focus on specific pathway components, and high-throughput

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experiments that focus on large interaction maps and networks. The majority of recent literature has reported low-throughput experiments and is too extensive to adequately review in this forum. Rather, we have decided to focus on reviewing the medium- and high-throughput experiments that have been recently reported.

Pathway Specific. Immunopurification coupled to MS was performed by Bouwmeester et al. (1) specifically on the TNF- α /NF- κ B pathway components. They identified 221 molecular interactions as well as 80 novel interactors. Two-hybrid screening was used by Colland et al. (2) to map the Smad signaling system; their work led to a network of 755 interactions involving 591 proteins, 179 of which had limited or no annotation. Tewari et al. (3) performed yeast two-hybrid experiments of known *Candida elegans* DAF-7/TGF- β pathway components and generated 71 interactions from 59 proteins. Their results from the screen were validated by coimmunopurification and RNA interference (RNAi).

Global Mapping. A high-throughput, two-hybrid approach was also used to map the interactions of *Drosophila* centered on cell-cycle regulators (1–4). They detected 1814 reproducible interactions from 488 proteins. In a similar approach, Formstecher et al. (5) selected 102 baits from *Drosophila* that were orthologous to human proteins linked to cancer. The yeast two-hybrid screen delivered 710 high-confidence interactions. The same technique was used by Goehler et al. (6) to identify potential interactors for 35 bait proteins related to Huntington's disease. They obtained 186 possible interactions from 35 bait and 51 prey proteins. Landgraf et al. (7) synthesized a series of peptides that matched the relaxed consensus binding sequence for eight yeast SH3 domains. These domains were screened against a high-density nitrocellulose membrane and identified a series of potential binding sequences. Li et al. (8) obtained over 4000 protein interactions using yeast two-hybrid on a set of genes from *C. elegans*. Giot et al. (9) also used the two-hybrid method to map protein interactions for 10 623 predicted transcripts from *Drosophila melanogaster* leading to a draft map of 20 405 interactions from 7048 proteins. They subsequently refined these data to produce a higher confidence map of 4780 interactions from 4679 proteins.

Tong et al. (10) built a genetic interaction network for ~1000 genes leading to over 4000 genetic interactions from ~4700 viable yeast gene deletion mutants. It is important to note that genetic interactions do not equate to protein interactions but implicate a potential association between two genes; the nature of this association is often unknown. Furthermore, genetic interaction networks are commonly hampered by significant levels of false positive results.

The wealth of protein interactions that have been elucidated in different species has facilitated the construction of theoretical protein interaction maps in humans. Lehner and Fraser (11) compiled a network of over 70 000 predicted physical interactions for ~6200 human proteins using the protein interaction maps from lower eukaryotic species. To date, this extrapolative technique has been used to produce the majority of the worldwide interaction data for humans.

It is also important to note that, to date, a relatively small amount of research has been focused on mapping the different

interactions between the basal and activated states of proteins in high-throughput proteome mapping experiments.

Bioinformatics and Protein Interactions. The number of publications reporting protein interactions has drastically increased over the years. Unfortunately, the data in these reports have not been systematically deposited in public databases compared to, for example, the standard practice with gene sequence data. The lack of a standardized procedure for reporting protein interaction data makes subsequent systematic reporting difficult. Current proteomic curation efforts are commonly hampered by data that have to be manually evaluated and extracted from the literature; this is a very expensive way of building public databases. Recently, a community standard data model for the representation and exchange of protein interaction data was reported (12). For this model to work the standard will have to be universally adopted; publishers will have to require that protein interaction data be submitted to a database in order to be accepted and published. Furthermore, evaluating the quality of the information that is reported is another significant issue. Protein interaction mapping experiments are being performed using different approaches and variable degrees of repetition, leading to different levels of confidence in the data. Most of the time, the experimental confidence levels are not reported, which may lead to significant levels of error propagation once the results are integrated into public databases.

CHEMIPROTEOMICS

The study of interactions between proteins and small molecules is an emerging field. Chemiproteomics, or chemical proteomics, aims at using small molecules as bait to isolate interacting proteins and thus identify possible novel drug targets (13). Gold et al. (14) developed a method to identify the target of SB 203580, a p38 kinase inhibitor. Immobilization of an SB 203580 analogue enabled affinity purification to be performed and yielded a concentrated sample of potential SB 203 580 targets. Using their novel method, they determined that many proteins other than p38 were inhibited by SB 203580 causing a reevaluation of the mode of action of SB 203580 in vivo. A recent publication by Saghatelian et al. (15) demonstrated that the matrix metalloproteinase inhibitor GM6001 (ilomastat) inhibited the neprilysin, aminopeptidase, and dipeptidylpeptidase clans of metalloproteases, in addition to its intended target despite the lack of sequence homology between the protein classes, clearly demonstrating the need for thorough laboratory testing. The study used a combination of sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE), and micro liquid chromatography (LC)–tandem mass spectrometry (MS/MS) to identify the proteins targeted by ilomastat, which had been purified using a trifunctional HxBP probe (containing ilomastat). Wissing et al. (16) used MS and an immobilized pyrido-[2,3-*d*]pyrimidine ligand (a class of tyrosine kinase inhibitor) as an affinity probe to simultaneously purify and identify potential protein targets. They discovered many nontyrosine kinase proteins that interacted with the ligand, exposing the lack of selectivity of these tyrosine kinase inhibitors.

Adam et al. (17) developed a gel-free method to map enzyme active sites with activity-based chemical probes. The method relies on the covalent cross-linking of chemical probes containing a sulfonate ester to the active site of a protein, affinity purification

and sequencing by LC/MS/MS; the method can be successfully used to analyze proteins of interest within complex mixtures. Although chemoproteomics in its current form does not provide final answers and follow-up validation experiments are commonly required, it is a promising area of research for discovering compounds that can be effectively tethered without changing their activity or specificity.

BIOMARKER DISCOVERY

Biomarker discovery studies have become a trendy area of research in proteomics due to an increasing need for preclinical and clinical biomarker targets. A biomarker may be defined as a characteristic, or a combination of characteristics, that can be objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or a pharmacological response to a therapeutic intervention. In the last two years, biomarker research has evolved at an accelerated pace. Although most reports claim that they have discovered biomarkers, the reality is that they have discovered candidate biomarkers. These candidate biomarkers must then go through a validation process that is linked to its potential usage. For example, the validation of a candidate biomarker for preclinical usage is significantly less demanding than its validation as a clinical surrogated end point or as an approved diagnostic marker.

Surface-Enhanced Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (SELDI-TOF MS). The combination of SELDI with conventional TOF or orthogonal TOF instruments has been widely applied for the discovery of candidate biomarkers. However, it is worth mentioning that this technique is not without controversy from a technical as well as a clinical point of view (see refs 18–21). It is still far from clear if the data generated by this technique can truly identify novel biomarkers. Furthermore, it is often not obvious how one would validate the peaks obtained from this approach. In general, it is important to be aware of a growing scepticism toward this approach.

Ovarian Cancer. The identification of novel biomarkers is, in some instances, limited by the amount of material present in the samples. Metha et al. (22) have examined the effect of carrier proteins in plasma (i.e., albumin) on the analysis of a particular biomarker of ovarian cancer (mass-to-charge ratio (m/z) = 6631.7043) by SELDI-TOF. The carrier protein seems to accumulate low molecular weight biomarkers significantly increasing their measurable concentrations. Additionally, they demonstrated that the concentration of the biomarker was predominantly affected by the clearance rate of the carrier protein rather than the clearance rate of the biomarker itself.

Johann et al. (23) implemented a novel method for analyzing MS data that enabled the detection of biomarkers in the low-amplitude region of mass spectra upon healthy and ovarian cancer samples. Their approach was based on data reduction by culling via peak intensity rather than by peak location. The new method enabled a better visualization of the signals since all of the diagnostic information was contained in the lowest amplitude region of the mass spectra.

Colorectal Cancer. Potential novel biomarkers for colorectal cancer were identified and tested by Chen et al. (24) by analyzing serum samples from colorectal cancer patients and healthy controls using SELDI-TOF. Artificial neural network with a back-

propagation algorithm was used to identify four peaks of unknown origin; they were selected to create a potential fingerprint of colorectal cancer.

Renal Cancer. Tolson et al. (25) used SELDI-TOF for the detection of three biomarkers (haptoglobin 1 a, SAA-1, and an unknown marker of mass 10.84 kDa.) in serum samples from renal cell carcinoma patients; the serum samples were compared to healthy controls.

Pluripotent Embryonic Carcinoma Cell Line. Hayman et al. (26) used SELDI-TOF to identify biomarkers produced during the differentiation of pluripotent stem cells. They used established human stem cell lines treated with retinoic acid as a model of differentiation and analyzed the cell lysate for biomarkers. Using this approach, they were able to identify proteins expressed exclusively in undifferentiated or differentiated cells.

Prostate Cancer. Ornstein et al. (27) used SELDI-TOF to analyze serum from patients with elevated prostate-specific antigen to determine the need for prostate biopsies. Using a weak cation exchange (WCX) protein array, a hybrid quadrupole (Q)-TOF mass spectrometer, and a pattern recognition bioinformatics tool, a discriminatory model was put forward. The proposed model had a sensitivity of 91% and specificity of 67% when tested on samples from 91 men.

Pancreatic Cancer. Bhattacharyya et al. (28) analyzed serum from 49 patients with pancreatic cancer and 54 healthy controls using SELDI-TOF. The resulting spectra were analyzed using classification and regression trees and logistic regression to develop a classification model that would distinguish between cancerous and normal samples. Using classification and regression trees and logistic regression, models with specificities of 93.5 and 100%, respectively, were developed for pancreatic cancer.

Endometrial Carcinoma. Yang et al. (29) analyzed samples from normal and malignant endometrial tissue using SELDI-TOF. Using strong cation exchange (SCX) SELDI-TOF, the level of chaperonin 10 was observed to be significantly enhanced. The increased level of chaperonin 10 was confirmed by western blotting and immunohistochemistry analyses.

Gastric Cancer. Ebert et al. (30) used SELDI-TOF and a pattern-matching algorithm to analyze sera from patients with and without gastric cancer. A classifier ensemble was created, which consisted of 50 decision trees that enabled the classification of a patient as cancerous or noncancerous.

Liver Disease. Cadieux et al. (31) analyzed urine from patients with urolithiasis by SELDI-TOF and determined that the relative intensity of two proteins p67 and p24 differed from control (healthy) patients. The ratio of p67/p24 was found to be below 1.0 for all healthy patients but to be above 1.0 for 72% (18/25) of the patients suffering from urolithiasis. Using SELDI-TOF, Paradis et al. (32) analyzed sera from hepatocellular carcinoma (HCC) patients with cirrhosis and healthy controls and determined which protein peaks were indicative of HCC discrimination by receiver operative characteristic curves. The authors identified the highest discrimination peak to be a 8900-Da fragment of vitronectin and proposed its use as a biomarker of HCC in patients with chronic liver disease.

Improvements in SELDI-TOF. Tibshirani et al. (33) developed a novel algorithm to identify biomarkers in SELDI-TOF and MALDI-TOF spectra. The “peak probability contrast” or PCC

algorithm first produces a list of common peaks among two different groups of spectra. It then analyzes them to define statistical significance and relative importance enabling class predictions (e.g., healthy versus cancerous) to be made. The PCC algorithm was compared to previously published algorithms for the analysis of an ovarian cancer data set; it matched or exceeded the performance of the previous algorithms.

Xu et al. (34) developed a three-step method for the classification of sera from rats with different types of liver cirrhosis. The samples were first analyzed using SELDI-TOF. A machine learning algorithm was then used to find statistically significant biomarkers, and the markers were identified by peptide sequencing. The sensitivity and specificity of this technique led to classifications higher than 92% being achieved.

A simple sample preparation strategy for the analysis of peptides and small proteins in serum was developed by Chertov et al. (35) to improve the discovery of biomarkers. The addition of acetonitrile and 0.1% trifluoroacetic acid (TFA) enabled the precipitation of large abundant proteins which improved the subsequent SELDI-TOF analysis.

LC/MS and Capillary Electrophoresis (CE)-MS. Different forms of separation techniques have been coupled with MS for the discovery of candidate biomarkers. For some biological samples, such as plasma, the dynamic range of proteins in the sample surpasses the dynamic range of the analytical techniques. Thus, researchers have been attempting to increase the analytical dynamic range by combining different separation approaches (multidimensional separation). Although multidimensional separation approaches improve the number of observed peptides, they add a serious level of complexity to the overall procedure. This can have a negative effect on reproducibility for well-designed biomarker studies that require multiple biological and analytical repeats.

Biomarkers in Urine. The presence of protein in urine has been associated with many diseases and conditions. Furthermore, the complexity of the urine proteome fits well within the current analytical capability of proteomic approaches. Pieper et al. (36) have done a comprehensive analysis of urine proteome using 2DE enabling 1400 distinct spots to be analyzed by matrix-assisted laser desorption/ionization (MALDI)-TOF. To improve the protein recovery, the sample proteins were first separated into two fractions (<30 and >30 kDa) prior to 2DE and prepared under two distinct protocols. Samples from renal cell carcinoma patients were analyzed before and after nephrectomy and showed a significant decrease in the level of kininogen after surgery. Mischak et al. (37) analyzed up to 2000 polypeptides in urine samples from patients with type II diabetes versus control patient samples by CE-MS. Comparison of the polypeptide profiles led to a distinctive pattern indicative of renal disease. Kaiser et al. (38) analyzed urine and serum samples derived from patients with renal diseases and discovered polypeptide patterns indicative of the diseases. Interestingly, no polypeptides were common to all individuals affected by a specific disease. A similar study was performed by Weissinger et al. (39) in which urine samples from patients with different renal diseases (minimal change disease, membranous glomerulonephritis, or focal segmental glomerulosclerosis) were analyzed by CE-MS. Characteristic profiles were obtained for each disease.

Kiernan et al. (40) reported the use of a mass spectrometric immunoassay (MSIA) for the qualitative analysis of β -2-microglobulin (β 2m), transthyretin, cystatin C, urine protein 1, retinol binding protein, albumin, transferrin, and human neutrophil defensin peptides in human urine samples. The method relied on the parallel purification of the proteins of interest with an MSIA-tip followed by MALDI and TOF analysis. The main advantage of this approach over conventional immunoassay methods is the ability to distinguish between mass-shifted variants of the proteins under investigation.

Serum. Villanueva et al. (41) described an automated platform that couples peptide concentration, purification, and analysis by MALDI-TOF. Peptide capture and concentration was done with reversed-phase (RP) media (1D) or RP and SCX media (2D) in a magnetic particle-based format, automated with a liquid handler robot. The robot also deposited the purified peptides on 384-well MALDI plates. A pilot study analyzing sera from brain tumor patients and healthy controls led to a biomarker profile that correctly predicted 96.4% of the samples (normal or disease).

Roy et al. (42) developed a method based on LC-MS for the determination of proteomic and metabolomic profiles of patients suffering from rheumatoid arthritis that distinguish them from unaffected individuals. Barnidge et al. (43) developed LC/MS/MS method for the quantification of prostate-specific antigen (PSA) in serum using protein cleavage-isotope dilution mass spectrometry (PC-IDMS) with an isotope-labeled analogue of PSA as an internal standard. The method, while not as sensitive as an immunoassay, showed a potential for biomarker analysis in biological fluids. Sen et al. (44) developed an on-line immunoaffinity (IA)-LC-MS method with in-line filters and an on-line preclear column for the analysis of serum amyloid P component, amyloid β -peptide, and β 2-microglobulin in serum.

Other Biological Fluids. Gronborg et al. (45) described the use of a combination of one-dimensional gel electrophoresis and LC/MS/MS. Pancreatic juice was first fractionated using one-dimensional gel electrophoresis and subsequently analyzed by LC/MS/MS. Using this technique, 170 unique proteins were identified including known cancer biomarkers (CEA, MUC1). Proteins not previously known to be in pancreatic juice were also detected (pg96, azurocidin, and PAP-2). Celis et al. (46) used an analogous protocol, 2DE and MALDI-TOF, to analyze interstitial fluid perfusing from breast tumors, enabling 267 proteins to be identified. Alexander et al. (47) analyzed breast nipple aspirated fluid using 2DE and MALDI-TOF to identify novel biomarkers associated with breast cancer. A total of 41 proteins were identified (25 of which were known to be secreted). In particular, the expression of gross cystic disease fluid protein-15 and α 1-acid glycoprotein was observed to correlate with different stages of breast cancer. Wenner et al. (48) studied ventricular cerebrospinal fluid by 2D-LC/MS/MS and identified 249 proteins.

Technology Development. Li and Cohen (49) developed a method for the quantitation of analytes by LC-MS in the presence of a high background using a surrogate analyte approach. A method was developed for the analysis of α -ketoisocaproic acid (KIC) by LC-MS using deuterium-labeled KIC (d_3) as a surrogate analyte. They reported a linear range of 10-5000 ng/mL and an error in their experimental accuracy below 10%.

Wang et al. (50) developed novel software, MassView, for the quantitation of proteomic and metabolomic profiles by electrospray ionization (ESI) and LC–MS based on the linearity of signal versus concentration and the reproducibility of sample processing. It is to be noted that this method does not rely on isotopic labeling or the use of a standard material. A protein standard spiked in human serum was measured from 50 fmol to 100 pmol using MassView.

Wang et al. (51) used a two-dimensional liquid-phase separation combined with isoelectric focusing and nonporous reversed-phase high-performance liquid chromatography (HPLC) for the separation of proteins obtained from whole cell lysate of cancer cells. Intact proteins were analyzed by ESI-TOF and 2-D images of the isoelectric points (pI) and molecular weights (M_r) of the observed proteins were created. Proteins exhibiting expression changes between normal and cancerous cells were collected from the HPLC eluent, digested, and analyzed by MALDI TOF MS and MALDI TOF MS/MS. The expression profile for over 300 proteins from 8 pI regions were obtained for two ovarian adenocarcinoma cell lines and one ovarian surface epithelial cell line.

Pitarch et al. (52) detected ~85 immunoreactive proteins by 2DE followed by western blotting of material derived from patients infected by *Candida albicans*, or systemic candidiasis. They then proceeded to identify the proteins by a combination of peptide mass fingerprinting (PMF) by MALDI-TOF, de novo sequencing by nano-LC/MS/MS, and genomic database searches. They observed that housekeeping proteins from *C. albicans* could generate an immune response and that the use of anti-*Candida* antibodies could be an appropriate therapeutic approach for these infections.

Two-Dimensional Gel Electrophoresis. Using 2DE with in-gel proteolytic digestion, peptide mapping, MS/MS analysis, and immunodepletion assays, Melle et al. (53) identified two significant proteins (calgranulin A and B) that were present in head and neck tumors.

Juan et al. (54) studied the plasma proteomes of nude mice previously injected with different cancer cell lines (SC-M1, HONE-1, CC-M1, OECM1, GBM 8401) by 2DE-MS; the over-expression of many acute phase proteins (APP) were reported. In particular, plasma from mice injected with SC-M1 cells contained serum amyloid A, a protein not previously observed in xenotransplantation experiments. The authors concluded that APP might be used as a nonspecific tumor associated serum marker.

Directional Surface Plasmon-Coupled Emission. Matveeva et al. (55) demonstrated the utility of directional surface plasmon-coupled emission in immunoassays of biomarkers. They developed an immunoassay for myoglobin, a cardiac marker, on a thin silver mirror surface using surface plasmon-coupled emission. They observed a reduced background noise, which was explained by the enhanced fluorescence emitted at a specific angle (72°) following binding of a labeled anti-myoglobin antibody.

Near-Infrared Fluorescence Detection. A competitive immunoassay with near-infrared (NIR) fluorescence detection was developed by Zhao et al. (56) for the analysis of 1 μ L of biological material (serum). The method relies on the competitive binding between NIR fluorescent-labeled antigen and unlabeled analyte for antibodies immobilized on paramagnetic beads. The limit of

detection was reported as 400 pg/mL when 1 μ L of sample was used.

PLANT PROTEOMICS

The incidence of proteomic studies of plants has increased over the past years, due, in part, to the availability and growth of proteomic technologies. Furthermore, new proteomic strategies have helped facilitate the study of hydrophobic and membrane proteins. Many new reports on differential expression, as well as global and organellar proteomic elucidation have been put forth. Model organisms and cash crops (e.g., rice and wheat) continue to be dominant in the plant proteomic literature.

Calikowski et al. (57) reported a proteomic characterization of the RNA-dependent nuclear matrix of *Arabidopsis* suspension-cultured cells. 2D-PAGE revealed ~300 spots; however, the identification of only 36 proteins was reported using 1D gel separation followed by digestion and ESI-MS/MS. Borderies et al. (58) reported a proteomic technique to identify cell wall proteins on living *Arabidopsis thaliana* cells in culture. Specific and sequential extraction procedures allowed for cell wall proteins to be separated from those of the cytoplasm. In all, 50 proteins were identified from the cell wall, 13 of which had not been previously localized to this region. Ferro et al. (59) reported a novel method of preparing highly purified envelope membranes from *Arabidopsis* chloroplasts; from this, proteins were extracted using several techniques. Proteomic analysis was achieved by LC/MS/MS and resulted in the identification of 100 proteins, 80% of which were known (or were likely) to associate with chloroplasts. A nearly identical study was carried out by Froehlich et al. (60), utilizing a novel method of purifying chloroplast envelope membranes from *Arabidopsis* and extracting the proteins in several ways. Proteomic analysis was carried out in two different ways including off-line multidimensional protein identification technology (MudPIT) analysis and 1D gel separation followed by digestion and LC/MS/MS; this proteomic study successfully identified 392 nonredundant proteins. In a similar manner, Brugiere et al. (61) used the method developed by Ferro et al. (59) to analyze the proteome of *Arabidopsis* mitochondrial membranes; LC/MS/MS analysis led to the identification of more than 110 proteins.

Ndimba et al. (62) characterized the proteomic response of *A. thaliana* to two different fungal pathogens, chitosan and extracts of *Fusarium moniliforme*. Proteomic analysis by MALDI-TOF revealed an increase in the levels of two classical cell wall proteins, as well as two novel proteins following elicitation. Furthermore, using a phosphotyrosine antibody (Ab), the differential phosphorylation of two cell wall proteins was determined, implicating the existence of an extracellular phosphorylation network which could be involved in intercellular communication.

Kerim et al. (63) studied the proteomic dynamics of developing rice anthers. Anther materials representing six discrete pollen developmental stages were collected; their proteins were extracted and separated by 2D-PAGE to produce proteome maps. A total of 150 protein spots were observed to be differentially expressed; generation of PMF by MALDI-TOF yielded the identification of 40 proteins, 33 of which were unique gene products. Rakwal et al. (64) studied the proteomic response of rice seedlings to exposure of the air pollutant SO_2 through SDS–PAGE, immuno-

blotting, N-terminal amino acid sequencing, northern blot analysis, and MS. Twenty-seven different proteins were reported to have variable expression levels following treatment of the pollutant. Similarly, Shen et al. (65) reported the proteomic response of the rice leaf sheath to physical cutting at 4 different time points; 10 proteins were observed to be upregulated, while 19 were downregulated. A large study by Komatsu et al. (66) extracted and characterized proteins from rice under various kinds of stress. A total of 10 589 proteins were visualized using 2D-PAGE; 272 were N-terminally sequenced, and a further 633 were sequenced using a peptide sequencer or MALDI-TOF following enzymatic digestion.

Gallardo et al. (67) studied the seed development of the legume *Medicago truncatula* using the same techniques as Kerim et al. (63). A total of 120 proteins were observed to be differentially expressed, 84 of which were identified by their PMF. A more comprehensive proteomic investigation on the same model organism was performed by Watson et al. (68). In this study, the proteomes of the leaves, stems, roots, flowers, seed pods, and cell suspension cultures were characterized; 551 proteins were separated and visualized, 304 of which were identified by their PMF using MALDI-TOF. Furthermore, expressed sequence tag (EST) counting was used as an indication of transcript abundance; they estimated that ~50% of the identified proteins appeared to correlate with their corresponding messenger RNA (mRNA) levels.

The proteomics of wheat has also been recently explored. Two reports by Islam et al. (69, 70) compared the proteomes of diploid, tetraploid, and hexaploid wheat and also studied the relationship between chromosome deletion and protein expression. Both studies utilized 2D-PAGE and imaging software for differential expression analysis. The chromosome deletion experiments employed the isotope coded affinity tag (ICAT) strategy and ESI-MS/MS to quantitatively determine the relative protein concentrations before and after the deletion.

BACTERIAL PROTEOMICS

Many different aspects of bacterial proteomics have been studied in the past two years using a variety of approaches. Hynes et al. (71) used WCX2 SELDI-TOF (ProteinChip) technology to analyze surface extracts from *Helicobacter pylori*, *Helicobacter bilis*, *Helicobacter pullorum*, and "*Helicobacter sp. flexispira*" and characterize cell surface changes following bile stress. Two distinct response patterns to bile stress on the cell surface of *Helicobacter spp.* were observed, as indicated by relative abundance changes in the SELDI spectra; the proteins that gave rise to these peaks were not identified. Govorun et al. (72) created and compared four different proteome maps of *H. pylori* clinical isolates from two different regions in Russia using a combination of 2D-PAGE and MALDI-TOF. Approximately 500 spots were visualized; 6 proteins were identified as being differentially expressed and 6 other proteins migrated to different isoelectric points in the 2DE, suggesting the presence of posttranslational modifications.

Guina et al. (73) reported a large-scale qualitative and quantitative study on the proteome of *Pseudomonas aeruginosa* under normal and magnesium-limited growth conditions. ICAT was used for quantitative purposes and to simplify the peptide mixture. Following ICAT labeling, peptides were separated by 2D-LC and

analyzed by MS/MS on an ion trap mass spectrometer. A total of 1331 proteins were identified, and of these, 145 were observed to be differentially expressed upon limitation of Mg. Furthermore, they reported a comparison between the whole cell and membrane-only fractions of both cell populations using the above techniques as well as 2D-PAGE analysis; low Mg content was shown to alter the subcellular compartmentalization of some large protein complexes.

Giometti et al. (74) developed a nondenaturing 2D electrophoresis method and separated proteins and protein complexes from the bacterium *Shewanella oneidensis*. Spots were excised, digested, and analyzed by LC/MS/MS; 23 proteins were identified from the *S. oneidensis* proteome. Vanrobaeys et al. (75) monitored the proteomic dynamics of *S. oneidensis* grown on different electron-accepting media: fumarate and ferric oxide. Twenty differentially expressed proteins were visualized using 2D-PAGE and were subsequently identified using LC/MS/MS and MALDI-TOF.

Chen et al. (76) reported a differential proteomic investigation of the effects of long-chain *N*-acyl homoserine lactone exposure on the bacterium *Sinorhizobium meliloti*. Prolonged exposure induced the differential expression of over 100 polypeptides, identifying 56 different proteins. A time-dependent exposure study of variants of the lactone molecule on *S. meliloti* was presented and discussed in detail.

Encarnacion et al. (77) characterized the aerobic and fermentative metabolism of *Rhizobium elii* using proteome analysis. Differential analysis was conducted by 2D-PAGE and several pattern recognition software packages; protein identification was achieved using N-terminal sequencing and MALDI-TOF.

Lai et al. (78) conducted a comparative proteomic analysis of the spore coats of *Bacillus subtilis* and *Bacillus anthracis*. A total of 38 *B. subtilis* spore proteins were identified, 12 of which were known coat proteins; 11 *B. anthracis* spore proteins were identified, 6 of which were likely coat proteins. Overall the spore coat proteomes between the two species were determined to be quite similar. Bernhardt et al. (79) conducted a temporal proteomic analysis of *B. subtilis* cells as they grew and encountered increasing degrees of glucose starvation. A sequential series of 2D-PAGE gels were constructed at different time points leading up to complete glucose exhaustion in the growth medium. The time-resolved starvation experiments revealed 150 proteins that were produced de novo as starvation ensued, accompanied by the cessation of nearly 400 proteins; many were identified by MALDI-TOF. A similar study was conducted by Beyer et al. (80) using 2D-PAGE to analyze proteome expression patterns under purine nucleotide starvation in *Lactococcus lactis* subsp. *cremoris*. Nineteen differentially expressed proteins were successfully identified by MALDI-TOF.

Vollmer et al. (81) used 2D-LC/MS/MS to analyze the proteome of *Escherichia coli* grown on either glucose or lactose media. Hundreds of proteins were identified, several of which were observed in the lactose grown cells only. A similar study was conducted by Yoon et al. (82) whereby 2D-PAGE and DNA microarrays were used to visualize the proteomic and transcriptome dynamics, respectively, of *E. coli* during the high cell density cultivation. Fountoulakis and Gasser (83) used 2D-PAGE and MALDI-TOF to elucidate 394 different gene products within the

membrane of *E. coli*. Mihoub et al. (84) observed a total of 26 proteins that were differentially expressed in two different strains of *E. coli* in the process of cold adaptation using 2D-PAGE and MALDI-TOF.

Schmidt et al. (85) compared the proteomes of two different strains of *Mycobacterium tuberculosis* both qualitatively and quantitatively using two different and complimentary MS-based approaches. The first approach involved 2D-PAGE followed by MALDI-TOF and identification through PMF; low molecular weight proteins (as well as proteins with no cysteine residues) were easily identified using this method. The second approach involved ICAT labeling of cysteine residues followed by LC/MS/MS; this method showed a bias toward higher molecular weight proteins and did not identify proteins that did not contain a cysteine residue. Together the two approaches effectively characterized the proteome of the two strains of *M. tuberculosis*, revealing a data set of 60 000 peptide MS/MS spectra and 1800 gel spots; from this information, 280 proteins were identified and 108 proteins were quantified. Mattow et al. (86) compared the proteomes of *M. tuberculosis* and attenuated *Mycobacterium bovis* using a combination of 2D-PAGE, MS, and N-terminal sequencing. A total of 39 different spots representing 27 different proteins were observed to be differentially expressed between the two species, including 5 proteins encoded by open reading frames absent from *M. bovis*.

Riedel et al. (87) reported a study that compared the proteomes of wild type *Burkholderia cepacia* and a mutant form that lacked the *N*-acylhomoserine lactone gene. The intracellular, extracellular, and surface protein fractions were compared using 2D-PAGE. A total of 55 proteins out of the 985 detected spots were differentially expressed, 19 of which were identified using N-terminal sequencing.

Liao et al. (88) created proteome maps of the intracellular and extracellular matrixes of *Shigella flexneri* using 2D-PAGE and MALDI-TOF. A total of 488 protein spots were processed, identifying 388 proteins from 169 different genes.

Len et al. (89) characterized the cellular and extracellular proteome of the oral pathogen *Streptococcus mutans* using 2D-PAGE, MALDI-TOF, and ESI-MS/MS. A total of 421 spots were analyzed and matched to the translation products of 200 open reading frames (ORF) deduced from the contigs of the *S. mutans* genome. Of these, 172 were identified as cellular proteins, and 28 were localized to the extracellular matrix.

Krayl et al. (90) monitored the proteomic dynamics of *Pseudomonas putida* upon exposure to methyl *tert*-butyl ether to help determine its degree of toxicity. 2D-PAGE, western blotting, and N-terminal sequencing revealed that several oxidative stress proteins were amplified in the presence of methyl *tert*-butyl ether, indicating how the chemical affects the physiology of the bacterium.

Kolker et al. (91) utilized 2D-PAGE with MALDI-TOF to characterize the proteomic differences between wild type *Staphylococcus aureus* and a mutant that was deficient in heme. Comparison of the 2D-PAGE patterns revealed major differences, consistent with the rationale that the electron transport chain of this mutant is disrupted. A total of 15 proteins were identified and reported as being differentially regulated between the wild type and mutant organisms.

Kohler et al. (91) used LC/MS/MS to rapidly characterize the entire proteome of *Haemophilus influenzae* and identified 25% of all predicted ORFs. They discovered that 15 genes originally annotated as conserved hypothetical encoded expressed proteins.

FUNGAL PROTEOMICS

Grinyer et al. (93) reported a proteomic approach to map the proteins of the biological control strain of *Trichoderma harzianum*. Proteins were extracted in acidic conditions and separated on 2D-PAGE gels; hundreds of spots were visualized. A total of 25 of these spots were successfully identified by a combination of MALDI-TOF with PMF, as well as LC/MS/MS and de novo peptide sequencing. In the same issue of that publication and using the same methods, Grinyer et al. (94) also reported a proteomic investigation of the mitochondrial proteins of the biological control strain of *T. harzianum*. Hundreds of spots were visualized, 31 of which were identified. Of those identified, 25 had not been previously characterized in existing *T. harzianum* databases.

Bruneau et al. (95) reported a proteomic study on the fungus *C. albicans* whereby they investigated the impact of four different antifungals commonly used to treat infection and noted the differential proteomic patterns that were induced. Expression responses were visualized using 2D-PAGE analysis; dose-dependent studies were then also investigated in the same manner. It was revealed that the induced proteomic expression changes of the antifungals were similar.

EUKARYOTIC PROTEOMICS

Forler et al. (96) reported strategy that combines the tandem affinity purification tag approach with double-stranded RNAi to avoid competition from corresponding endogenous proteins, allowing the determination of the functionality of the tagged protein and increasing the specificity and efficacy of the purification.

YEAST PROTEOMICS

Ghaemmaghami et al. (97) reported the creation of a fusion library of ORFs that had been tagged with high-affinity epitopes within the organism *Saccharomyces cerevisiae*. Each ORF was then expressed from its natural chromosomal location, and the tags were immunodetected to obtain a census of proteins expressed during log-phase growth. They reported that ~80% of the proteome was expressed during normal growth conditions and that the abundance of proteins ranged from fewer than 50 to more than 1 million copies per cell.

Bro et al. (98) used a microarray approach to study the global protein and gene expression profiles of *S. cerevisiae* grown in galactose in different time intervals after addition of lithium. Their results showed that 664 ORFs were downregulated while 725 ORFs were upregulated. mRNA levels were in agreement with 26% of the downregulated and 48% of the upregulated proteins.

Ohlmeier et al. (99) presented a parallel analysis of the yeast mitochondrial proteome with an analysis of the cellular transcriptome when the organism switched from fermentative to respirative growth. Overall, the concentrations of 17 proteins were observed to increase while only 1 decreased. Transcriptome analysis revealed that the genes encoding mitochondrial proteins were heterogeneously up- and downregulated, contradictory to the proteomic results.

TECHNOLOGIES

Proteome Fractionation Procedures. Arnold et al. (100) proposed a protein fractionation method that utilizes acid-labile surfactant for protein denaturation. Their method was compared to conventional tryptic digestion in urea for the analysis of homogenized liver and was found to produce similar protein identification patterns. The major advantage of their method was that it reduced the need for washing steps, which improved the ability to detect low-abundance proteins. Du et al. (101) used preparative gel electrophoresis with an acid-labile surfactant coupled to nano LC–Fourier transform (FT)-MS to perform a “top down” analysis of 37 proteins with molecular masses between 6 and 30 kDa.

Zhao et al. (102) proposed a novel method for the analysis of integral plasma membrane proteins known as biotin-directed affinity purification (BDAP). The method relies on biotinylation of cell surface membrane proteins in viable cells, followed by affinity purification with streptavidin beads, and removal of plasma membrane-associated cytosolic proteins with high-salt and high-pH washes. The purified integral plasma membrane proteins are then separated by SDS–PAGE gel and subsequently analyzed by HPLC–MS/MS. They identified 898 different proteins derived from human lung cancer cells using BDAP, of which 781 were noted for their plasma membrane location. Similarly, Blonder et al. (103) used iodoacetyl-PEO biotin to tag cysteinyl-containing proteins and were able to detect 786 proteins (42% of which had a least one transmembrane domain) from a *P. aeruginosa* membrane fraction.

A different approach, used by Zhang et al. (104) for the characterization of plasma membrane proteins, was based on solubilization using a surfactant, digestion in the presence of SDS, and auto-offline LC/MALDI MS/MS. The method was compared with gel electrophoresis/MS and 2D-LC/MS for the analysis of the proteome of HT29 cell lines and was found to provide a complementary view of the HT29 proteome.

Gevaert et al. (105) developed a diagonal method to isolate N-terminal peptides. Kuhn et al. (106) refined the technique by implementing an effective sample preparation strategy and called it protein sequence tag (PST). Prinz et al. (107) showed that the use of PST with an efficient membrane preparation protocol enabled the detection of 148 proteins from *S. cerevisiae*, 84% of which were known membrane proteins.

Marko-Varga et al. (108) used laser capture microdissection to isolate airway epithelial cells in combination with 2DE, MALDI-TOF, and LC/MS/MS to study changes in protein expression. Laser capture microdissection on normal ductal epithelium and metastatic ductal carcinoma cells was combined with expression proteomics by Zang et al. (109) using $^{16}\text{O}/^{18}\text{O}$ labeling. Liao et al. (110) studied the amyloid plaque proteome in human brain, a pathological feature of Alzheimer's disease, by laser capture microdissection combined with mass spectrometry. The purified amyloid plaque proteins (<5 μg) were analyzed by nano LC/MS/MS and 488 proteins were identified. A total of 26 were enriched in amyloid plaques compared to the surrounding tissue.

Rahbar et al. (111) modified the cationic colloidal silica technique to improve plasma membrane isolation. Western blots showed that 50% of proteins isolated from human multiple

myeloma and breast cancer cells were from the plasma membrane. They showed that 30 proteins previously thought to be hypothetical proteins are in fact present in the plasma membrane.

Improvement in “Figures of Merit”. Improvements in the figures of merit of proteomic methodologies are driven by the need to study and characterize samples that are increasingly diluted and complex. Shen et al. (112) published an ultrasensitive nanoscale proteomics approach for the characterization of proteins in complex mixtures from less than 50 ng of sample. Their method utilized micro-solid-phase extraction and nanoLC coupled to an ESI-FTMS. Analysis of 2.5ng sample from the microorganism *Deinococcus radiodurans* provided 14% coverage of all open reading frames of the microorganism with an estimated detection dynamic range of 10^5 – 10^6 .

Liu et al. (113) developed a new quantitative cysteinyl-peptide enrichment technique coupled to LC/MS that provides improved dynamic range and throughput. They combined ^{18}O labeling of tryptic peptides, enrichment of cysteine-containing peptides, LC/MS, accurate mass, and a time tag. They identified and measured 603 proteins from in vitro differentiated human mammary epithelial cells.

Raman Spectroscopy. The first demonstration of non-enhanced Raman spectroscopy coupled to LC was published by Zhang et al. (114). The acquisition of high-quality spectra from 1 fmol of protein was made possible by the drop coating deposition Raman (DCDR) technique. DCDR consists of microdepositing a solution of interest on compatible substrate, followed by solvent evaporation and backscattering detection. DCDR followed by MALDI-TOF detected spectral differences of isobaric glycan isomers.

Drachev et al. (115) developed a protein sensor based on nanostructured adaptive silver films for soft protein adsorption and detection with surface-enhanced Raman scattering. The novel protein sensor was used to analyze two insulin isomers that differed by one amino acid inversion. The sensor was able to differentiate the two insulin isomers at 80 fmol/ mm^2 , with only 25 amol in the probed area.

Improved Sample Digestion. Zhong et al. (116) published a novel protein sequencing method for small proteins based on polypeptide ladders analyzed by MALDI-TOF obtained from acid hydrolysis assisted with microwave irradiation. The method relied on the hydrolysis of the proteins in 3 M HCl with fast (<2 min) microwave irradiation to create polypeptide ladders. The protein sequence and the presence of posttranslational modifications could be directly read from the mass spectra acquired through this method.

Nomura et al. (117) presented an improved in-gel tryptic digestion strategy by adding acid-labile surfactant. They demonstrated better sequence coverage for eight proteins excised from an SDS gel and analyzed by MALDI-TOF.

Posttranslational Modifications (PTM). Ge et al. (118) have developed a technique capable of detecting glycosylation, phosphorylation, and total protein expression in a single gel using three different fluorescent dyes. They applied this approach to the 2DE differential analysis of membrane proteins from susceptible and resistant mammary epithelial cells.

Protein Arrays. Publications on protein arrays have been numerous both in fundamental development and in their applica-

tions. Improvements in designs, materials, and applications of protein arrays in the last year will be reviewed.

Kinoyaka et al. (119) introduced a novel semiwet peptide/protein array composed of supramolecular hydrogel based on glycosylated amino acids. This novel semiwet array shows promise for the prevention of protein denaturation by mimicking the normal aqueous environment of proteins.

Ramachandran et al. (120) simplified the preparation of protein array by developing a self-assembling protein array. This array is formed by printing complementary DNA sequences on a glass support, which is then transcribed and translated by a cell-free system. The epitope tag fused to the translated proteins allows their direct immobilization in situ requiring no additional purification methods. The self-assembling protein array was used to map protein–protein interactions of DNA replication initiation proteins, to observe the regulation of Cdt 1m, and to map its geminin-binding domain.

Torres et al. (121) presented an innovative calorimetric protein array referred to as enthalpy arrays. This array provides the unique ability of measuring different molecular interactions such as protein–ligand binding, enzymatic turnover, and mitochondrial respiration. Therefore, it represents a universal interaction measurement technique, and it does not require antibodies or fluorescent labels.

A noted trend in protein arrays has been continued miniaturization to accommodate minute sample volumes. Lynch et al. (122) created a protein nanoarray in which each spot measured 1–2 μm in diameter (compared to $\sim 55 \mu\text{m}$ for conventional arrays). This novel protein nanoarray could be analyzed by optical fluorescence microscopy or by atomic force microscopy. Ressine et al. (123) developed a novel and highly sensitive protein chip using a macroporous silicon substrate. The fabricated macroporous silicon network enabled small spots (100 pL/spot, 4400 spots/ cm^2) and reduced quantities of antibody (0.7 pmol/ cm^2) to be used.

Blank et al. (124) described the fabrication of a double-chip format protein array that consisted of a capture array with fluorescent labeled detection antibodies coupled to a reference array via DNA duplexes. This double-chip format protein array allowed cross-reacting antibodies to be used without the generation of false positives. Gilbert et al. (125) continued to develop the double-chip format to make use of single-chain Fv antibody fragments.

Lin et al. (126) developed a novel stamping system using a back-filling microstamp chip for the simultaneous and precise spotting of thousands of samples on dense 1-mm protein arrays, which greatly reduces the time required for generating multiple copies of an array.

Wegner et al. (127) reported the fabrication of an oriented His-tagged protein array using nitrilotriacetic acid (NTA) monolayer for surface plasmon resonance imaging of protein–protein and DNA–protein interaction. Koopmann et al. (128) developed a protein array based on poly(L-lysine) poly(ethylene glycol)-biotin polymer attachment to glass for the selective capture of biotin-tagged proteins. This approach is compatible with MALDI-MS analysis.

Kwon et al. (129) demonstrated the fabrication of protein arrays with immobilized antibodies in a single orientation, but with variable density, using the covalent interaction between cutinase

and its suicide substrate. The immobilization of antibodies required less than 5 min while preserving their reactivity and a low background. Cha et al. (130) described another method to prepare a protein array with a specific orientation using a high-density poly(ethylene glycol) (PEG)-coated Si (111) surface to which polyhistidine-tagged proteins were attached. This method removed the need for protein purification, diminished nonspecific binding, and ensured a controlled protein orientation. Camarero et al. (131) described the use of expressed protein ligation for the covalent attachment of proteins through their C-termini to a modified glass surface containing an N-terminal Cys PEG linker. The major advantage of this technique over traditional protein attachment methods is that the protein retains its folded state and biological activity.

Hall et al. (132) screened a yeast proteome microarray with DNA probes to identify novel DNA-binding proteins. More than 200 DNA-binding proteins (50% of which were novel) were identified by probing the array with single- and double-stranded DNA. A different method was presented by Kersten et al. (133) to probe protein arrays for DNA-binding proteins using ultraviolet (UV) light to cross-link the DNA–protein complexes.

Eppinger et al. (134, 135) described the use of protein arrays and fluorescent tagged affinity labels for studying the kinetics of papain. Striebel et al. (136) developed a label-free method for the analysis of protein–protein interactions on a protein array using the change in decay time of tryptophan and tyrosine intrinsic fluorescence.

POSTTRANSLATIONAL MODIFICATIONS

Glycosylation. Earlier studies were focused on global glycosylation to detect the different glycoforms. However, the focus has recently shifted toward site-specific glycosylation. Several techniques for the specific detection of glycosylation sites have recently been reported.

Krokhin et al. (137) developed a technique for the simultaneous detection and characterization of glycopeptides using an HPLC-MALDI-QqTOF. The differentiation of glycopeptides from peptides was based on their common fragmentation patterns in MALDI ionization; both the peptide and glycan components can be further analyzed using MS/MS.

Hägglund et al. (138) used hydrophilic interaction chromatography to capture glycopeptides and analyze them by ESI MS/MS. They cleaved the glycans using specific enzymes leaving one or two monosaccharide moieties, which allowed for more efficient MS/MS analysis. They used their techniques to identify 62 glycopeptides in 37 glycoproteins in human plasma.

Bunkenborg et al. (139) used successive lectin affinity separations to extract glycopeptides and analyzed them by MS following Lys-C digestion, glycan removal, and tryptic digestion. The sites of glycosylation were detected by the transformation of asparagine to aspartic acid. They identified 86 glycosylation sites from 77 glycoproteins present in human serum.

Kristiansen et al. (140) developed a technique to study the glycosylation of proteins in human bile. The technique consisted of glycoprotein purification using lectin chromatography followed by 1D-SDS–PAGE. The bands were tryptically digested, and glycans were detached using PNGase-F in the presence of H_2^{18}O . This led to the labeling of asparagine residues that were linked

to the glycan, and a characteristic shift of 3 Da was observed in the MS analysis. This approach reduced the number of false positives resulting from spontaneous deamination. The technique was demonstrated through the identification of 87 proteins and 33 glycosylation sites.

Phosphoproteomics. There has been significant progress surrounding the biochemical analysis of reversible protein phosphorylation in the past two years. New techniques have been developed to isolate, purify, and enrich phosphopeptides, and large-scale phosphoproteomic analyses have been conducted. Significantly, some of these analyses have monitored the phosphorylation dynamics of a system as a stimulus is added.

Many studies in tyrosine phosphorylation have been conducted, most notably due to the efficacy of phosphotyrosine IP strategies. Salomon et al. (141) monitored the temporal variation of tyrosine phosphorylation in the activation of human T cells, as well as the inhibition of the oncogenic BCR-ABL fusion product in chronic myelogenous leukemia cells in response to treatment with STI571 (Gleevec). In both cases, phosphopeptides were immunoprecipitated with anti-phosphotyrosine antibodies, enriched by methyl esterification and immobilized metal affinity chromatography (IMAC), and analyzed by LC/MS/MS. The experiments identified 64 different sites of tyrosine phosphorylation on 32 different proteins, half of which had been previously reported in the literature. In a similar study, Brill et al. (142) identified nearly 70 tyrosine-phosphorylated peptides from proteins in human T cells using a combination of phosphotyrosine immunoprecipitation, IMAC, LC, and MS/MS. Furthermore, they reported that methylation improved the selectivity of IMAC for phosphopeptides (eliminating the acidic bias of interfering peptides) and that desalting the methylated peptides prior to IMAC enrichment resulted in the assignment of ~3-fold more tyrosine phosphorylation sites. An impressive study was recently published by Blagoev et al. (143), combining many phosphoproteomic strategies to monitor the temporal variation of phosphotyrosine-dependent signaling networks in HeLa cells. The authors also presented an extension of the stable isotopic amino acids in cell culture (SILAC) strategy so that three isotopically labeled forms of arginine (therefore three different cell populations) may be differentially analyzed in a single MS experiment. HeLa cells were stimulated with epidermal growth factor (EGF) for three different amounts of time, each with a unique isotopic variant of arginine in the growth medium. The cells were mixed, immunoprecipitated with anti-phosphotyrosine antibodies, tryptically digested, and analyzed by LC/MS/MS. Proteins were quantified against the zero time point of stimulation. A total of 81 proteins were observed to be differentially tyrosine-phosphorylated through these experiments, 31 of which being novel effectors. Moreover, the time course of tyrosine phosphorylation was determined for each of these proteins upon EGF stimulation. Gembitsky et al. (134) described a prototype antibody microarray platform to track the temporal and positional phosphotyrosine changes across a cellular proteome. A rationally designed subset of the tyrosine phosphoproteome was spotted on a microarray chip, and bound proteins were probed with a fluorescently labeled p-Tyr-specific monoclonal Ab, PY-KD1. Bcr-Abl-expressing cells were probed before and after treatment with a specific inhibitor, Gleevec; and HeLa cells were probed before and after treatment with EGF using these methods.

Differentially expressed receptor targets were subsequently identified by MS.

Methods to analyze phosphoserine and phosphothreonine were also developed and tested over the past two years; several groups conducted large-scale phosphoproteomic investigations of all phosphorylated residues in a complex sample. Ibarrola et al. (144) used a two-sample SILAC approach to study temporal variations in the phosphoproteome of calyculin A-treated human embryonic kidney 293T cells. They did not use any enrichment strategies but analyzed all phosphoproteins pulled down with an anti-FLAG antibody by LC/MS/MS. Bonenfant et al. (145) developed a simple-isotope labeling strategy to measure quantitative changes in the phosphorylation of a protein in response to a stimulus. Their three-step strategy involved labeling two pools of differentially phosphorylated proteins by enzymatic digestion in H₂¹⁶O and H₂¹⁸O, IMAC enrichment, and dephosphorylation with alkaline phosphatase for quantitation purposes. Selected phosphopeptides were analyzed by MALDI-TOF. The strategy was successfully demonstrated on the yeast nitrogen permease reactivator protein kinase involved in the target of rapamycin signaling pathway; alteration in the extent of phosphorylation was easily assessed in wild-type and SIT4 phosphatase knockout cells. Ballif et al. (146) observed the phosphoproteome of the developing mouse brain. Embryonic mouse brain cell lysate was run on a 1D gel, cut into four equal pieces, and digested, and the peptides were separated and analyzed using offline SCX followed by RP LC/MS/MS on each SCX fraction. No phosphopeptide enrichment strategies were used; however, over 500 phosphopeptides in the developing brain were reported. Garcia et al. (147) characterized the phosphorylation sites on the major histone H1 isoforms using a combination of IMAC enrichment followed by LC/MS/MS; a total of 19 phosphorylation sites were identified. Ficarro et al. (148) identified over 60 phosphoproteins in capacitated human sperm using a combination of methyl esterification followed by IMAC enrichment and LC/MS/MS analysis. Nuhse et al. (149) utilized SCX prior to IMAC enrichment to decrease the sample complexity and increase the yield of monophosphorylated peptides in plasma membrane digests of *Arabidopsis*. LC/MS/MS of all IMAC-enriched SCX fractions yielded a total of nearly 300 phosphopeptides in the *Arabidopsis* membrane. Metodiev et al. (150) utilized a Qiagen phosphoprotein purification kit to enrich the phosphoproteins of human U937 cells. Isolated proteins were separated by electrophoresis and identified by direct de novo sequencing using MS/MS. The phosphoproteomes of the cells were monitored in the course of monocyte to macrophage differentiation; two cancer-related phosphoproteins were implicated in being fundamental in this process. Hegeman et al. (151) devised a method whereby phosphopeptides are modified by methyl esterification followed by phosphatase treatment in either deuterated or nondeuterated methyl alcohol. The site-specific phosphorylation stoichiometries of two samples (deuterated or nondeuterated) were derived from the comparison of their mass spectral isotopic patterns, as analyzed by LC/MS/MS. Ten phosphorylation sites of two standard phosphoproteins were mapped; 80% of these sites had discernible phosphorylation stoichiometries using this strategy. Haydon et al. (152) characterized 14 phosphorylation sites on *Xenopus laevis* His6-Aurora A and used this system as a model to test different IMAC strategies. The effect of methyl esterification

prior to IMAC enrichment was tested, and the strengths and limitations of different IMAC resins were studied and discussed using this phosphoproteomic system.

Different methods of chemical modification of phosphoproteins for facilitating analysis were also recently developed in addition to the isotopic methods mentioned above. McLaughlin and Chait (153) introduced an improved method of β -elimination-based affinity purification of phosphopeptides. The strategy used a thiol tag as the ligand for affinity purification via disulfide exchange with an activated thiol resin and was shown to be effective in simplifying complex peptide mixtures. The reaction did, however, produce a side reaction in which nonphosphorylated residues were converted to the affinity-tagged form; quantitation of this side reaction revealed a maximum of 1.7% of the unphosphorylated peptide was converted. Despite the side reaction, the strategy was shown to remain effective through the successful enrichment and analysis of *in vitro* phosphorylation of bovine synapsin I by Ca^{2+} /calmodulin-dependent kinase II. Klemm et al. (154) derivatized phosphorylated peptides with S- and N-nucleophiles through a β -elimination/Michael addition reaction scheme to increase the ionization efficacy of phosphopeptides in MALDI analysis. The nucleophiles were not identified; however, successful elimination/addition reactions led to the increased ionization efficiency of a sample of phosphopeptides and hence their identification by MS analysis. Knight et al. (155) used a novel method mapping phosphorylation sites based on chemical modification of phosphorylated residues followed by specific enzymatic cleavage. Phosphoserine and phosphothreonine residues were modified with aminoethylcysteine and β -methylaminoethylcysteine, respectively, and then cleaved with a lysine-specific protease to map sites of phosphorylation. Their strategy was effectively demonstrated on a mixture of four proteins. Qian et al. (156) presented a stable-isotope labeling method that uses a phosphoprotein isotope-coded solid-phase tag for isolating and measuring the relative abundances of phosphorylated peptides from complex peptide mixtures. Phosphorylated residues were derivatized by β -elimination followed by the Michael addition of 1,2-ethanedithiol. Modified peptides were captured and labeled in one step using solid-phase reagents that were also isotopically coded. Captured peptides were released by photocleavage and analyzed by LC/MS/MS. This method simultaneously enriches and quantitates phosphopeptides; it was successfully demonstrated on a sample of phosphoproteins from human breast cancer cells. Goodman et al. (157) reported the use of a new formulation of a small-molecule organic fluorophore stain that permits the selective detection of phosphoproteins on electroblotted membranes. The protocol for its use is relatively straightforward and can deliver results in ~ 1 h. The dye binds selectively and noncovalently to phosphate moieties and is thus compatible with MALDI-TOF analysis. Proteins are separated by electrophoresis, electroblotted, and stained with the Pro-Q Diamond dye. Schulenberg et al. (158) also reported the use of the Pro-Q Diamond dye to suitably detect phosphoserine, phosphothreonine, and phosphotyrosine residues. Phosphoprotein samples were separated by SDS-PAGE and stained directly. Its utility was demonstrated on NADH:ubiquinone oxidoreductase (complex I); exposure of mitochondria to cAMP-dependent protein kinase increased the incidence of phosphorylation of this protein.

Two different strategies for column-based phosphopeptide enrichment were also recently presented. Larsen et al. (159) compared the ability of RP columns and graphite powder columns to bind hydrophobic phosphopeptides. MS experiments revealed that the graphite columns had a greater ability to bind some phosphopeptides, compared to standard RP columns. Furthermore, of those that bound to the RP material, stronger signals could be achieved when using a graphite column. Their findings suggest that graphite powder has the ability to selectively retain phosphopeptides to a greater degree than the more common RP resins used and may be a packing material of choice for phosphoproteomic applications. Pinske et al. (160) reported the use of titanium dioxide as a stationary-phase material within a novel, two-dimensional column setup to enrich and analyze phosphopeptides. Phosphopeptides were trapped and separated from non-phosphopeptides by being trapped on the TiO_2 column; the latter were trapped on a downstream RP column and independently analyzed. Following the analysis the non-phosphopeptides, the phosphopeptides were bumped off of the TiO_2 column using a basic solution, trapped on the RP column, and analyzed by gradient elution LC/MS/MS. The viability of this approach was demonstrated on a synthetic peptide model, as well as on a digest of a large phosphoprotein. Its utility to phosphoproteomic studies in general was implicated and discussed. Kuroda et al. (161) also presented a method of using a TiO_2 precolumn for the selective enrichment of phosphopeptides in a complex mixture of peptides. Phosphopeptides were bound to the column in acidic medium and eluted with a phosphoric buffer. The behavior of the titania precolumns was optimized and observed to function similar to that of an IMAC column.

Different instrumental methods were also developed to enhance and facilitate phosphopeptide analysis. Chalmers et al. (162) demonstrated the utility of activated ion electron capture dissociation (ECD) using a micro-ESI-FT-ICR MS. They used the high resolution and accuracy (~ 0.7 ppm) FT-ICR instrument to unambiguously identify phosphopeptides of protein kinase A and structurally characterize the location of the phosphorylated residue within the peptide using MS^2 and MS^3 experiments. Furthermore, they confirmed their structural findings using ECD and infrared multiphoton dissociation (IRMPD) experiments. The ECD and IRMPD experiments produced c- and z-type ions while the MS^n experiments produced mainly b- and y-type ions; thus, the location of the phosphorylation sites was elucidated using different experiments that produced different types of data on one instrumental platform. Syka et al. (163) revealed a modified quadrupole linear ion trap that allowed ECD-like reactions to take place, predominantly producing c- and z-type ions. The application of this novel instrumentation was focused on the analysis of phosphopeptides, whose collisionally induced dissociation pathways often heavily favor the loss of the phosphate group. Using the novel instrumentation, ECD-like reactions allowed for PTM-independent fragmentation chemistry to take place along the peptide backbone, generating spectra of phosphopeptides that revealed a greater amount of sequence information. Le Blanc et al. (164) revealed how the recently introduced MDS Sciex QTRAP instrument may successfully and effectively analyze low-abundance phosphopeptides through the negative ion mode precursor ion scan (-79 Th), followed by rapid switching into positive ion mode

and MS/MS of the phosphopeptide. Alternatively, a positive ion mode neutral loss scan may identify phosphopeptides (loss of 49 Th) followed by rapid MS/MS.

Gerber et al. (165) presented a method for absolute quantification of proteins and their modification states through the use of synthetic internal standards within a sample. The synthetic peptides were manufactured to contain modifications identical to the PTM being observed to more closely mimic the behavior of the peptide(s) of interest. Quantitation was achieved through selected reaction monitoring experiments of the peptide of interest and the internal standard. This strategy quantitatively determined the cell cycle-dependent phosphorylation of Ser-1126 of human separase protein, as well as kinases capable of phosphorylating Ser-1501 of separase in an *in vitro* kinase assay. Finally, Diella et al. (166) reported a new database of all experimentally verified phosphorylation sites curated from literature called Phospho.ELM. Version 2.0 contains 1703 phosphorylation site instances for 556 phosphorylated proteins.

Lipidation. Another protein posttranslational modification that commonly occurs *in vivo* is lipidation, i.e., the covalent attachment of a lipid to a specific residue. Despite being a common PTM, very few reports of routine lipidation have appeared in the literature. Boisson and Meinel (167) developed a rapid and easy diagnostic method that both checked for effective N-myristoylation of any given protein and allowed it to be identified. The N-myristoylation reaction was coupled to pyruvate dehydrogenase, causing NADH to be continuously detected spectrophotometrically. Their method was optimized for and applied to *S. cerevisiae* and three derivatives of *A. thaliana* and was validated by comparison with a previously described discontinuous assay. Using their methods, they successfully determined the kinetics of N-myristoylation for a full-length polypeptide substrate and a small G protein of the RAB family. Kho et al. (168) developed a method for selectively enriching proteins containing lipid PTMs known as the tagging-via-substrate approach. The strategy was demonstrated on farnesylated proteins and involved metabolic incorporation of a synthetic azido-farnesyl analogue and chemoselective derivatization of azido-farnesyl-modified proteins using a biotinylated phosphine capture reagent. Modified peptides could be enriched using standard affinity purification methods, thereby enhancing their probability of detection. Application of the technique to a sample of COS-1 cell lysate successfully identified 18 farnesylated proteins.

BIOINFORMATICS FOR PROTEOMICS

There has been a significant development of bioinformatic tools for proteomics since our last review. Major developments have been driven by an increasing interest in the validation of peptide identifications, especially in the context of high-throughput experiments, and the increasing use of statistical methods to analyze results across multiple samples due to an increased emphasis on differential proteomics. Furthermore, the need to link mass spectrometric data to a multiplicity of genomic sequence entries in databases, and the development of common standards for the dissemination of proteomic results, have increased the global interest in proteomic bioinformatics.

Development of Common Standards for Data Dissemination. To date most proteomic publications have suffered from a

lack of access to essential information necessary to verify and utilize the results being reported. For example, it is a standard practice to report a list of identified proteins. However, rarely do researchers also report their list of peptides that led to the protein identifications or their search engine scores. Databases constructed from DNA sequencing efforts often have intrinsic errors associated with them; e.g., three-pass sequencing provides greater than 99% accuracy while one-pass sequencing provides less than 90% accuracy. Unfortunately, proteomics is far more complicated with many different types of experiments being performed on different platforms (see refs 169 and 170). The Proteomics Standards Initiative is aiming to define common standards for data representation, comparison, exchange, and verification in proteomics. They have already defined a common extensive markup language (XML) standard for molecular interactions as well as MS data (171) (<http://psidev.sf.net/>). An even more pressing issue is in regard to the different types of mass spectrometers currently being used in proteomics research, each having different analytical characteristics and data formats. Aebersold et al. (172) introduced the "mzXML" format for the generic XML representation of MS data obtained from different instruments. The expectation is that this common format will facilitate data management, interpretation, and dissemination in proteomics research. Additionally, the Proteome Experimental Data Repository (173) has developed another data model for the dissemination of proteomic information.

MS Information Matching and Validation. Beer et al. (174) introduced the idea of clustering MS/MS spectra to reduce the increasing amounts of data generated by proteomic experiments to a reasonable size (Pep-Miner). Their technique reduced the overall analysis time and improved the spectral quality leading to the identification of more peptides with higher confidence. For example, they used Pep-Miner to reduce 517 000 spectra to 20 900 clusters and identified 2518 peptides derived from 830 proteins. Bern et al. (175) also introduced approaches to minimize the number of spectra that are searched by SEQUEST. They presented a binary classification approach for MS/MS spectra, which predicts if SEQUEST will be able to make an identification, as well as a statistical regression approach, which provides a quality measurement involving the number of b- and y-ion peaks present in a spectrum. They were able to eliminate 75% of the unidentifiable spectra while losing only 10% of the identifiable spectra. Anderson et al. (176) recently described a learning algorithm, called the support vector machine, which can help distinguish false positive results from the SEQUEST search engine. Baczek et al. presented a method based on an artificial neural network to evaluate the results provided by SEQUEST searches (177). Lopez-Ferrer et al. (178) developed a statistical model of the SEQUEST results for high-throughput studies. The method calculated a probability score for each peptide assignment and provided an evaluation of the false discovery rate among all of the peptides identified. Cargile et al. (179) performed searches of 120 000 MS/MS spectra generated from an LCQ Deca XP Plus using SEQUEST and Mascot against a randomly generated protein database containing 40 000 entries. Even using stringent scoring cutoffs, over 500 and 1400 proteins were identified by Mascot and SEQUEST, respectively. Thus, the false positive rate in large-scale projects is significant and can lead to erroneous conclusions.

A comparison of the results obtained from multiple search engines was reported by two different groups. Boutilier et al. (180) performed a systematic analysis of four MS/MS search engines and concluded that with a low level of real samples the number of MS/MS matches significantly drops when two or more search engines are used for cross-validation. Furthermore, they indicated that strategies employing the summation of the results from two search engines can increase the number of matches but that the selection of search engines needs to be tested for individual proteomic platform. Chamrad et al. (181) also performed an evaluation of different search engines. They reported that SEQUEST was able to identify more proteins from MS/MS data than Mascot for a specific data sets generated on a postsource decay (PSD)-based platform. It is important to realize that on different instrumental platforms, the "best" search engines may differ; ideally, each should be evaluated before a final interpretation of MS/MS data is reported.

A new computational strategy, called Genome-based fingerprint scanning (GFS) matches PMF with unannotated genomic sequence and was introduced by Wisz et al. (182). Briefly, GFS searches experimentally derived PMF spectra against an unannotated genome sequence, revealing a list of the best matching regions of the genome (see <http://gfs.unc.edu>). Samuelsson et al. (183) also introduced a set of new algorithms and tools for PMF that performs peak extraction, peak filtering, and protein database matching. Demin and Walden (184) introduced a program, termed Sequit, for the de novo sequencing of MALDI-PSD mass spectra (available as a freeware for academic institution <http://www.sequit.org/>). Craig and Beavis (185) introduced TAMDEM, a set of software components, to search MS/MS spectra against protein/sequence databases. TANDEM source code is available under Artistic license at <http://www.proteome.ca/opensource.html>. The Open Mass Spectrometry Search Algorithm (OMSSA) is a probability-based matching search engine that was recently introduced (186). OMSSA is available at <http://pubchem.ncbi.nlm.nih.gov/omssa/>. Fernandez-de-Cossio et al. developed a software program called Matching to help with the interpretation of mass spectra obtained from approaches that rely on stable-isotope labeling (187) (see <http://bioinformatica.cigb.edu.cu/Matching>). Finally, Habermann et al. (188) used the MS-BLAST to perform sequence similarity searches using MS-derived peptide sequence information for the cross-species identification of proteins.

Platforms and Databases. Numerous proteomic experiments requiring vast amounts of data handling have led to the development of proteomic platforms that combine sample tracking capabilities with data handling. Radulovic et al. (189) reported a proteomic platform to handle and analyze proteomic data derived from gel free profiling and biomarker experiments. Similarly, Xirasagar et al. (190) developed the Chemical Effects in Biological Systems (CEBS) database by implementing a strategy known as the systems biology object model (SysBio-OM). This database was designed for the capture, storage, access, and exchange of annotated gene expression, proteomics, and metabolomics data sets (see <http://cebs.niehs.nih.gov/>). Linke et al. (191) reported the development of a platform called ProDB. ProDB provides automated data analysis and annotation of MS/MS spectra obtained from different mass spectrometers (see

http://www.cebitec.uni-bielefeld.de/groups/brf/software/prodb_info/index.html).

2DE-based proteomics databases and interfaces have also been developed. Babnigg and Giometti (192) reported the development of ProteomeWeb, a Web-based interface to display protein profiles obtained by 2DE; the interface provides access to protein identifications on the fly (<http://proteomeweb.anl.gov>). The same authors (193) also reported the development of GELBANK, a publicly available database of 2DE and annotation. A similar platform called PARIS (194), which is accessible through the Web and also downloadable was developed (See <http://www.inra.fr/bia/J/imaste/Projets/PARIS/index.html>). An XML standard schema was also proposed for the management, analysis, and dissemination of annotated 2DE (195).

Bandeira et al. (196) developed a novel technique for the de novo identification of proteins using pepsin. The nonspecific proteolysis of this enzyme increased the multiplicity of data and created overlapping peptides. They developed an algorithm that combined the overlapping MS/MS spectra to create a summed spectrum that contained intense b- and y-ions, greatly facilitating de novo interpretation.

Rejtar et al. (197) developed a multistep strategy to increase the quality of Mascot database searches using variable cluster areas and a wave denoising algorithm. They observed a 33% increase in unique peptides and a 22% increase in the number of protein identifications; the method also reported a slight increase in the number of false positives reported.

Yang et al. (198) developed an algorithm called DBParser capable of comparing database searches from multiple LC/MS/MS analyses of the same sample and identifying which proteins were consistent throughout all of the experiments.

CONCLUSIONS

Clearly, the quantity, quality, and diversity of proteomic research initiatives have increased across a broad spectrum of biological areas since our last report. Some proteomic approaches such as 2DE-MS and IP-MS are now routinely applied in many laboratories.

The dynamic range and low abundance of some biological samples still remain beyond current proteomic abilities. Solving these issues will require further improvements in our ability to fractionate biological samples as well as improvements in overall analytical sensitivity. Multidimensional gel-free methodologies have the potential to partially address the dynamic range issue. However, as analytical dynamic improve through the addition of further dimensions, overall systems become increasingly complex. The reproducibility of these approaches still needs to be studied.

We predict that the application of statistical methodologies to the design and implementation of proteomic strategies will become essential over the next few years; it is no longer reasonable to have a proteomic study report on a single biological sample. We also predict that reporting long lists of proteins will be less acceptable without additional follow-up validation experiments.

GLOSSARY

2DE	2D gel electrophoresis
Ab	antibody
APP	acute phase protein

BDAP	biotin-directed affinity purification
CE	capillary electrophoresis
DCDR	drop coating deposition Raman
ECD	electron capture dissociation
EGF	epidermal growth factor
ESI	electrospray ionization
EST	expressed sequence tag
FT	Fourier transform
GFS	genome-based fingerprint scanning
HCC	hepatocellular carcinoma
HPLC	high-performance liquid chromatography
IA	immunoaffinity
ICAT	isotope coded affinity tag
ICR	ion cyclotron resonance
IMAC	immobilized metal affinity chromatography
IP	immunoprecipitation
IRMPD	infrared multiphoton dissociation
KIC	α -ketoisocaproic acid
LC	liquid chromatography
MALDI	matrix-assisted laser desorption/ionization
M_r	molecular weight
mRNA	messenger RNA
MS	mass spectrometry
MSIA	mass spectrometric immunoassay
MS/MS	tandem mass spectrometry
MudPIT	multidimensional protein identification technology
m/z	mass-to-charge ratio
NIR	near-infrared
NTA	nitrotriacetic acid
OMSSA	open mass spectrometry search algorithm
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PC-IDMS	protein cleavage-isotope dilution mass spectrometry
PEG	poly(ethylene glycol)
pI	isoelectric point
PMF	peptide mass fingerprint
PSD	postsorce decay
PST	protein sequence tag
PTM	posttranslational modification
Q	quadrupole
RNAi	RNA interference
RP	reversed phase
SCX	strong cation exchange
SDS	sodium dodecyl sulfate
SELDI	surface-enhanced laser desorption/ionization
SILAC	stable isotopic amino acids in cell culture
SysBio-OM	systems biology object model
TFA	trifluoroacetic acid
TOF	time of flight
UV	ultraviolet

WCX	weak cation exchange
XML	extensive markup language

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