25.1 Rapid Automated Differential Protein Expression Profiling with IEF Rotofor and SDS-PAGE Chip System

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Biomarker research relies on identification of differentially expressed proteins in a sample of interest and a suitable control. The two major experimental platforms include 2D gels followed by spot excision, tryptic digest and MS identification or alternatively automated multidimensional chromatography of trypsin digested biological samples followed by mass spec identification such as MUDPIT or iCAT. However, both approaches are very time consuming and often valuable MS time is wasted by analyzing either already known proteins or proteins with the same expression levels in both biological samples.

Here we are presenting an approach to minimize MS time by sample fractionation and fast identification of the fraction with the differentially expressed protein(s). The high resolving power of 2D gels is based on the combination of two independent separation mechanisms: first protein separation based on charge differences and in the second dimension on size differences. The same principle is preserved in the approach discussed here by using the charged-based Rotofor as the first dimension to obtain in a 3-hour experiment up to 20 fractions differing in their pI. Each of these fractions is then subjected to a SDS-PAGE based protein sizing analysis on a microfluidic platform. This analysis is automated, highly reproducible and quantitative and can be completed in less than one hour for all 20 Rotofor fractions. We demonstrating this approach using heat-shocked and normal E. coli samples and identify fractions with differentially expressed protein in one working day for further MS identification.

25.2 Primarily Study on Cucumber Fruit Proteins with 2-DE

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Fruit development is a forward topic of biological researches, however, its proteomics has not been studied thoroughly. Cucumis sativus is one of the most important vegetables, and play certain roles in vegetable supply and national income. So cucumber fruit proteins were primarily studied with an economical and handy 2-DE method in this experiment. The result showed that proteins of pollinated ovaria were more than those before anthesis. It's identical to SDS-PAGE, so our 2-DE method was feasible. This can laid foundation of studying cucumber fruit proteomics deeply, and gives reference for analyzing proteins of other fruits in ordinary laboratory. Procedures of the method were summarized as follows: 1. Total proteins of cucumber fruits were separated as described by R. F. He (2000), and dissolved in USB buffer (9.5 mmol/L urea, 4% NP-40, 0.5% ampholyte pH 3–9.5, 0.5% pH 4–6, 0.8% pH 6–9, 5% mercaptoethanol). 2. Glass pipes made from pipets of 1.5 mm bore were soaked 1 h with 2 mol/L NaOH, 2 mol/L HCl, double distilled H2O and ethanol respectively. Then isoelectric focusing gel (0.5015 g urea, 187.5 µL double distilled H2O, 10% NP-40 187.5 µL, 12.5 µL pH 3–9.5, 12.5 µL pH 4–6, 20 µL pH 6–9, 28.3% acrylamide-1.62% methylene bisacrylamide 122.5 µL, 10% ammonium persulfate 2.5 µL, 0.75 µL TEMED) was poured into the pipes that have been baked and with one end sealed by parafilm. The negative electrode buffer was 50 mmol/L NaOH while the positive was 25 mmol/L H3PO4. Condition of electrophoresis was 200 V/11003 15 min, 300 V/11003 30 min, 400 V/11003 18 h, 1000 V for 1 h. 3. After isoelectric focus electrophoresis have finished, one gel was cut into 1.5 cm long sections, then soaked overnight into 1.3 ml double distilled H2O in cleaned penicillin bottles to gauge the pH; and other gels which have loaded samples were immobilized 30 min in 12% trichloroacetic acid, then balanced twice with balanced buffer (2.3% SDS, 62.5 mmol/L Tris-HCl (pH 6.8), 10% glycerol, 5% mercaptoethanol, 0.1% bromophenol blue). The first balance was 20 min, and the second was 25 min. 4. The second dimensional electrophoresis was conducted under condition of 30 mA steady current after the balanced gel was fixed on the top of 12% separation gel with 1% agar. Finally gels were stained with coomassie brilliant blue.
25.3
Subcellular Comparative Proteomics Analysis Using the AACT Technique
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Due to the complexity of total proteomes, especially of eukaryotic proteomes, it is evident that there is an increasing need for standardized but versatile sample preparation methods in order to maximize the coverage of the proteome and to increase the chance to visualize low abundance proteins and make them accessible for subsequent analysis. Here we describe an extraction method that enables simple fractionation of proteins in their native state according to their subcellular localization, yielding three subproteomes enriched in (a) nuclear; (b) mitochondrial and (c) cytosolic proteins. At the same time, our strategy of amino acid-coded mass tagging (AACT) uses leucine (L) as an internal marker of the peptide, provides high specificity in MS and MS/MS signals. AACT strategy makes contributions to the validation and interpretation of MS/MS spectra, leading to more accurate and specific identification of protein that can significantly facilitate identity and quantity protein (Gu et al., Anal. Chem., 2002 and 2003). Here we report results from the combined AACT technique with subcellular enrichment method, to identify and quantify proteins from a double cell lines: human carcinoma cell lines QGY-7703 and normal human liver cell line QSG-7701.

25.4
High Throughput Proteomics with Magnetic Bead Based Technologies
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A significant challenge in proteomics today is overcoming the dynamic range of protein abundance. High throughput proteomics requires efficient methods to analyze complex protein mixtures. It is often necessary to reduce sample complexity for many proteomics strategies. The use of magnetic beads for sample preparation enables protocols to be automated and throughput to be increased. The kinetics of bead-based sample preparation is very efficient and washing can be done thoroughly. In addition, preparation is flexible regarding sample and buffer volumes. Consequently, low abundant proteins can be concentrated from large sample volumes.

We have developed protein purification methods based on the use of magnetic bead technology. These methods are used for the fractionation of complex protein samples, for isolation of proteins or peptides with post translational modifications and for isolation of recombinant proteins. These methods increase the resolution of 2D gels and MS analyses.

25.5
Impact of Water Quality on Bi-dimensional Electrophoresis
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Bi-dimensional electrophoresis is a basic technique in the study and the analysis of proteins. Although most parameters have been thoroughly evaluated to define protocols ensuring reproducibility and optimization of the technique, water has not been considered as a reagent so far, in spite of its large usage throughout the experiment. Hence, very few studies have reported on the impact of water quality on gel electrophoresis.

Data presented here highlight the effect of water quality on 2D gel protein electrophoresis. An experimental plan was designed to study the impact of water quality during the different steps of electrophoresis: sample preparation, rehydration of the strip, iso-electrofocalisation, migration in second dimension, and staining. Two types of water quality were tested: ultrapure water freshly delivered from a water purification system, and bottled water. A protein extract of E. coli was selected for the study, and electrophoreses were all run under similar experimental conditions. Gels were done in duplicate for each experiment, and staining was done both with silver staining and Coomassie blue. To show and quantify the effect of water on the gels quality, various analytical parameters were considered using the Phoretix 2D expression software.

Results highlighted a better gel quality with high purity water compared to bottled water. Differences due to water quality were also pronounced for Coomassie blue staining. MALDI-TOF-MS data then showed that contaminants that might be present in water used to run the gel electrophoresis also interfere with subsequent protein and peptide analysis.
25.6
Isoelectric Focusing of Peptides: A Ideal First Dimension Separation in Shotgun Proteomics

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An area of considerable interest in proteomic research is the development of multidimensional separation methodologies to resolve complex mixtures of peptides for mass spectrometric analysis. The most prevailing method employed for such investigations uses strong cation exchange chromatography and by reversed phase LC-MS/MS in the second in either an online or offline format. We have recently introduced a new multidimensional separation scheme, which relies on the use of isoelectric focusing of peptides using IPG strips in the first dimension, followed by reversed phase LC-MS/MS in the second. This approach has several distinct advantages that make it the ideal choice for a first dimension separation for shotgun proteomics. Samples to be analyzed by this technique are first digested using standard techniques. The digests are then loaded onto a conventional IPG-IEF strip as used for the first dimension of 2-D gels. Any pI range can be employed. Following the focusing step, the strip is cut into fractions (typically 43 for an 18 cm strip). The peptides are then extracted and analyzed by nano-reverse phase LC-MS/MS on a quadrupole ion trap mass spectrometer. The resultant data is then analyzed by SEQUEST and software written in-house (ID Sieve) that uses predicted pI and statistical models to minimize false positive and negative identifications. To date we have evaluated the utility of this technique in prokaryotic (E. coli) and eukaryotic (R. norvigicus) systems for comprehensive proteome analysis. The observed average pI of the fractions correlated strongly to the actual pI and the frequency distribution of peptide identifications as a function of pI matched well with theoretical calculations. A subsequent experiment in a eukaryotic system using a narrow range (3.5–4.5) strip to maximize dynamic range resulted in greater than 3000 protein identifications from 6000 peptides. A key to the use of pI as a data filtering criterion is the ability to predict peptide pI with a high degree of precision and accuracy. In order to examine this as a function of N-terminal amino acid position, both wide and narrow pI range strips were run with a variety of proteases. Using the E. coli soluble proteome, other work in progress is addressing the adaptation of the technique for analysis of the human serum proteome, a systematic study of the absolute sensitivity of the method and studies of peptide diffusion in the IPG strip as a function of time.

25.7
Comparative Proteome Analysis of Human B-cell, EBV-Transformed B-cells and Burkitt’s Lymphoma Cell Line

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Two-dimensional gel-electrophoresis (2-DE) in combination with mass spectrometry is a powerful approach to compare protein expression in cells. Analysis of protein in cells by high-resolution 2-DE offers promise in the identification of biomarkers that correlate with disease. In our experiment, we compared and analyzed protein expression level of human primary B cell, Epstein-Barr virus (EBV)-transformed B-cells and Burkitt’s lymphoma cell lines (RAJI, RAMOS). Protein analysis was performed using 2-DE with pH 3–10 immobilized pH gradient (IPG) strips followed by MALDI-TOF MS. We’ve selected about 120 spots on each gel and identified about 20 proteins by MALDI-TOF MS analysis and internet database (MS-Fit). Especially, the identified specific protein in EBV-B cell, RAJI and RAMOS cell was Heat Shock 70kD protein (HSP70) that was not identified in primary B cell. HSP70 is not typically expressed in all kind of cells, but it is expressed at high levels in stress conditions. Heat shock proteins play an important role in establishing of proper protein conformation, and prevention of inappropriate protein aggregation. Although the results are preliminary, the data has value in comparative proteomic studies.
25.8
High-throughput Genome-Scale Human Protein Production
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After human genome project, the complete human genome information has been made public. In addition, due to the public nature of the project, various DNA materials, such as YAC, BAC and cDNAs clones are also accessible. However, only a very small fraction of the gene products and antibodies to these gene products, which are essential for the functional analysis and practical applications, are readily available. We have obtained 13,247 full-length cDNA clones from the public Mammalian Gene Collection. 9,204 of these clones were identified as unique genes using LocusLink database. Of the 9,204 clones, we selected 3871 according to their functional annotations in the LocusLink database. We than sequenced and subcloned these genes into an expression vector and the proteins were expressed by CFS® wheat germ in vitro expression system. All proteins were GST tagged and were purified using glutathione affinity column. The sequence of 359 of the genes were found not matching to the sequences in GenBank database and were excluded from the study. A 50 µl in vitro expression reaction were tested for each genes. About 20 new proteins were successfully expressed each day with a yield about 5–10 µg per 50 µl reaction. To date, 1001 proteins were successfully produced and purified. The synthesized proteins were analyzed by SDS-PAGE and Western blot. Functional analysis of some of the kinase proteins showed that they retained many of the enzymatic activities. The overall successful rate for the protein expression system is about 70%. The major factors for the failure are the solubility of the proteins and the efficiency of column purification. Those genes that failed in the present standard protocol will require further experimentation. Our results showed that the genome-scale human protein production at high-throughput is achievable. The proteins that were successfully expressed are now publicly available and some of them have been selected for antibody production.

*CFS is abbreviated trade name of CellFree Sciences Co. Ltd., Japan.

25.9
Systems Investigation of Protein-Protein Interactions Networks/Pathways for Effective Therapeutic Interventions
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The emerging technologies of systems biology provide an opportunity for developing more effective strategies for therapeutic interventions in the progression of cancers or infectious diseases. Since 1999, we have introduced a mass spectrometric (MS)-based strategy of amino acid-specific mass tagging for protein molecules in the context of proteomics and functional genomics. Basically, this concept of the natural incorporation of stable isotope-enriched nucleotides or amino acids into biomolecules through in vivo/in vitro cell culturing represents a new type of sensitive and accurate molecular labeling other than radiological or chemical labeling, i.e., in addition to the mass-to-charge ratio (m/z) parameter in MS spectra, the use of these stable-isotope labels for tagging proteins in a sequence-specific way can enhance dramatically the specificity, accuracy, sensitivity, and throughput of MS-based technology for large-scale analyses. Our methodology has been proven to be more effective in comparative proteomics, e.g., characterizing changes in the expression of proteins corresponding to internal or external perturbations of cells or systems. The successful applications of this tagging technology will be presented, i.e., systems investigations of a broad range of the biomedical issues associated with 1) novel post-translational control by all-trans retinoic acid in treating acute promyelocytic leukemia cells, 2) p53-regulated apoptosis, 3) cellular signaling in response to radiation, 4) cellular responses to cadmium in Schizosaccharomyces Pombe, etc.

25.10
High Throughput Analysis of Proteome by Multidimensional Liquid Chromatography with Monolithic Column and MALDI Mass Spectrometry
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Due to the complexity of cellular proteomes, which contain thousands of proteins over a wide dynamic range of abundance, it is recommended to have a multidimensional type of analytical tools. A various fractionation and analysis columns can be used to extend the range and total number of proteins identified in the proteome analysis. In this study, a multidimensional LC separation with strong cation exchange and monolithic silica capillary columns has been followed by the off-line MALDI-MS analysis. Chromatographic performance and reproducibility of the monolithic silica capillary column were evaluated with the tryptic-digested peptides from model protein mixture. Compared to conventional capillary columns, high throughput and efficient separation of peptides was obtained using monolithic silica capillary column. And the quality of collision induced dissociation (CID) mass spectrum was comparable to the mass spectrum from ESI-CID-MS using capillary C18 column. The human plasma proteome was analyzed by multidimensional separation using monolithic column combined with LC-MALDI-MS/MS and the results were compared to those from LC/ESI-MS/MS.
25.11
Proteome Profiles of Grain Filling and Maturation in Rice Seed by SELDI-TOF MS and Affinity Chromatography
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The process of starch biosynthesis in rice requires many metabolic enzymes, defense proteins, regulatory proteins and unknown factors. Although the display proteomics using two-dimensional gel electrophoresis combined with mass spectrometry (2-DE/MS) provides powerful tools to identify proteins expressed conditionally, some drawbacks such as gel-to-gel variation, solubility of limited proteins, labor-intensive and time-consuming works exist. The present study describes the deeper analysis of novel proteins involved in rice seed maturation using protein chip-based technology called surface-enhanced laser desorption/ionization-time of flight-mass spectrometry (SELDI-TOF MS). Total soluble proteins prepared from 1-week, 3-week and mature seeds were overlaid on SAX2 plates for identification by Time-of-Flight MS. Three out of 20 candidates expressed specifically at the specific stage. In particular, the mass fragment identified 20 individual polypeptides in the range of 4,411 to 75,376. ProteinChip analysis with MASCOT software showed that 20 individual polypeptides correspond to the same profiling pattern during seed maturation as observed as 2-DE/MS. Three out of 20 candidates polypeptides were further verified as same expression pattern by affinity chromatography purification and SDS-PAGE analysis. Analysis by SELDI-TOF MS provides a robust and quick profiling of proteome and in particular, helpful information of low molecular weight polypeptides hard to detect on 2-DE/MS.

25.12
Two-dimensional Electrophoretic Analysis Reveals That Lipid Rafts Are Intact at Physiological Temperature
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Different proteins are present in lipid rafts depending on the isolation method. For example, a significantly increased quantity of insulin receptors from HepG2 cells was found in raft fractions prepared by Brij 35 than Triton X-100. In order to assess the effect of detergent type and temperature on raft isolation, raft proteins from HepG2 cells were analyzed by two-dimensional electrophoresis. More raft protein spots appeared when rafts were isolated by Brij 35 at room temperature or 37°C than by Triton X-100 at 4°C, indicating that lipid rafts are not disrupted in the presence of detergent at physiological temperature (37°C). Indeed, lipid-modified proteins such as Src, and Fyn were found in raft fractions although detergent-resistant rafts were isolated at room or physiological temperature. The 2-D gel profile of raft proteins isolated by detergent-free (high pH/carbonate) method was considerably similar to that of detergent-resistant raft proteins but showed more raft proteins. Whereas many detergent-resistant raft proteins disappeared upon cellular exposure with methyl-β-cyclodextrin, high pH/carbonate-resistant raft proteins did not, suggesting that many proteins isolated by high pH/carbonate could be contaminants. Taken together all these data, we conclude that liquid-ordered state of detergent-resistant lipid rafts are not destroyed at physiological temperature.

25.13
Monolithic Capillary Columns for Enhanced Glycoprotein Identification in LC/ESI/MS and LC/MALDI/MS
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Most biological processes are regulated by post-translational modifications of proteins. In the past decade, instrumental progress in the field of mass spectrometry has enabled the identification and characterization of protein modifications such as glycosylation and phosphorylation. In glycomics new strategies that provide rapid and sensitive characterization of carbohydrates in biological systems must be developed. Here we report on the application of polymeric monolithic capillary columns for the separation of glycopeptides. Polymeric monolithic stationary phases offer an alternative to the classical microparticulate sorbents, bringing improved separation performance for e.g. peptides and proteins. In contrast to the traditional stationary phases, which consist of packed particles, the monolithic separation medium is made of a continuous, rigid polymeric rod with a porous structure.

Glycopeptides are separated on monolithic capillary columns and subsequently detected by on-line ESI/MS or fractionated onto MALDI target plates for identification by Time-of-Flight MS. LC/MS method development has been performed with model glycoproteins. Different approaches to locate and identify glycopeptides using both ESI and MALDI/MS will be discussed. The methodology has been used for the identification of glycoproteins in a cell extract from parasitic worms.
Holes Formation in Cell Plasmic Membranes by Saponins as a Rapid Preparation Method of Cytosolic Soluble Proteins Released into Culture Medium

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The antiproliferative effects on human normal and carcinoma cells of hederacolchisid A1, a newoleanolic acid monodesmoside isolated from Hedera colchica K. KOCH, an ivy species endemic in Georgia, has been recently demonstrated (IC50 values from 4.5 to 12 μM) [1]. SEM analysis of treated human melanoma revealed that holes ≤ 1 μm are formed at the cell surface when treated with other related saponins like α-hederin [2].

The cytoplasmic Lactate Dehydrogenase release is used as a test for the evaluation of chemically induced membrane permeability. LDH is released during the treatment of human melanoma MEL-5 cells with Hederacolchisid A1. This release can be monitored in function of time. Instead of monitoring only LDH, the analysis of all proteins released can be performed using the proteomic approach (2D gels, 2DLC MS/MS).

This analysis shows that the action of hole producing chemicals can be used to selectively fractionate the soluble cytosolic proteins in serum free culture media. We describe in the results a new rapid strategy to identify cytosolic proteins by chemically inducing hole formation in cell membranes and release of proteins.

A New Ruthenium Complex for Staining of Proteins in One and Two Dimensional Gel Electrophoresis

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Since its first use in 1979, silver staining has been the pre-eminent visualization method for detecting low nanogram levels of proteins after 2D gel electrophoresis. Colloidal Coomassie Brilliant Blue stain is also attractive for a number of reasons including its cost and its simplicity of use even if it presents a low sensitivity that limits the detection for poorly expressed proteins. Recently, some new fluorescent detection methods have been optimized and seem to present the same sensitivity threshold as silver stains: SYPRO Ruby and Deep PurpleTM. These fluorescent dyes show interesting potentials (sensitivity, accuracy) but remain very expensive. There is thus a need in the detection techniques for 1D and 2D gel electrophoresis. Considering all of it, we have tested a new ruthenium complex including an activated ester for the selective acylation of amino acid side chain amines. This new ruthenium complex was first tested on control proteins after 1D gel electrophoresis. It performed very well: a larger number of proteins was detected compared to silver nitrate or SYPRO Ruby detection. This new detection method is thus more sensitive than SYPRO Ruby and silver staining and gives a response to protein concentration over than a 10 000-fold dynamic range allowing to visualize individual proteins over three orders of magnitude Finally, this methodology is applied to proteins from human colon carcinoma cells lines HCT 116 separated on 2D gel. We will present here the new ruthenium complex and a comparison with all other common detection (Colloidal Coomassie Blue, silver nitrate and SYPRO Ruby), including the commercially new Deep PurpleTM reagent.

A Quantitative Proteomics Study of NDST-1 Deficient Mouse Brains Using DIGE and LC-based Methods

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Heparan sulfate proteoglycans (HSPGs) are posttranslationally modified proteins with glycosaminoglycan side chains attached to certain serine residues of the core protein. HSPGs are widely distributed on cell surfaces and basement membranes of mammals. One major biological property of HSPGs is their capability to specifically interact with a number of growth factors. Several genes coding for enzymes, which polymerize and modify HSPGs have been deleted in different mouse systems by homologous recombination in embryonic stem cells. These strains are useful tools for studying the biological function of this type of glycosylation and their core proteins.

The brains from one such strain lacking the biosynthetic enzyme N-deacetylase/N-sulfotransferase-1 (NDST-1) was studied. These mice die as neonates from lung defects and also display defects in the central nervous system. Brain proteins regulated as a consequence of defective heparin sulfate biosynthesis were studied using two complementary quantitative proteomic workflows. In the 2-D gel based workflow, relative quantification was performed using DIGE and DeCyder 2-DE. In the LC based workflow, relative quantification was performed using unlabeled samples and DeCyder MS. The data from the two workflows was analyzed using uni- and multivariate statistics such as t-test, ANOVA, PCA and cluster analysis. Brain proteins regulated as a consequence of defective heparin sulfate biosynthesis were studied and the information from the DIGE and LC based methods were compared.
**25.17 Novel Strategy of High Abundance Protein Removal Using Multi-dimensional Liquid Chromatography**

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In many cases low-abundant proteins are most promising drug targets or provide the key for understanding pathogenesis in a disease. In biological samples the low-abundant and high-abundant proteins differ in concentration over 5 orders of magnitude. Thus, high-abundant proteins can mask low-abundant proteins during separation and MS identification of proteome samples.

Recently, affinity chromatography acts as the main means to be used to reduce the complexity of the protein or peptide by selecting the specific target. However, the inherent limitations including extreme specificity of binding of known proteins and high-cost have limit its widely applications in proteomics.

In our lab, we put forward and implement a novel strategy, which removed high-abundant proteins by using normal multi-dimensional liquid chromatography system. A combination of strong cation exchange (SCX) and reversed-phase (RP) chromatography was applied. The fractions of different charges were eluted by salt solution of different concentration. Then the fractions were lyophilized. The dried fractions were dissolved and re-loaded onto the RP column. The fractions were separated further according to their hydrophobicity. Then the high-abundant and low-abundant proteins can be collected individually or grouped. The fractions contained the low-abundant proteins were lyophilized again, digested and loaded onto an online 2D separation and tandem mass spectrometry system. The peptide effluents were deposited on MALDI target plate and detected by MALDI-TOF/TOF/MS. The processing approach can be done in parallel for several times because of the good reproducibility of chromatography. As a result, low-abundant proteins were capable of being in detected level. The experimental results proved this strategy was effective. Low-abundant proteins were identified.

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**25.18 Nano-electrospray-LC/MS Using a Polymer Microfluidic Device for Protein Identification**

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Nano-electrospray-LC/MS is a demanding analytical technique, typically practiced in the hands of LC-mass spectrometry experts. The ESI tip, enrichment column, or analytical LC column can clog or leak, limiting overall performance and system throughput. In this paper we demonstrate the integration of these elements into a single microfluidic chip that eliminates leaks by using seamless internal microfluidic connections between elements. High pressure switching of flows is accomplished using a novel microfluidic to rotary valve interface with low dead volume connections. Such devices exhibit state of the art performance in nano-electrospray, reverse-phase HPLC, with fast on-chip pre-concentration. Fluidic sensors and chemical functionality are easily integrated within these devices by taking advantage of the layered microfluidic architecture. High precision, laser ablation of biocompatible polymer materials is used to rapidly fabricate device structures and create nano-ESI tips with excellent performance and reproducibility. High pressure, low dead volume, and positive shut-off fluid switching are facilitated by using a microfluidic to rotary valve interface. Performance was determined for the laser ablated HPLC-Chip devices using a combination of small molecule tests and protein digests in aqueous buffers on an IonTrap MS system. Amounts of BSA digest as low as 600 amol were identified by tandem MS in combination with a protein database search. Compared to standard fused silica capillary nano-LC columns, polymer-based HPLC-Chip devices have demonstrated improved sensitivity and better peptide sequence coverage while simplifying the nano HPLC-MS operation.

**25.19 Today’s 2-D Electrophoresis Technology**

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Two-dimensional gel electrophoresis (2DE) with immobilized pH gradients (IPGs) combined with protein identification by mass spectrometry (MS) is currently the work horse for proteomics. Mass spectrometry has emerged to a highly sophisticated technology, whereas immobilized pH gradients have overcome the former limitations of carrier ampholyte based 2DE (O’Farrell 1975) with respect to reproducibility, handling, resolution, and separation of very acidic and/or basic proteins (NEPHGE). Narrow-overlapping IPGs provide increased resolution (&lt;0.1; pI = 0.001) and, in combination with prefractionation methods, the detection of low abundance proteins. Moreover, the development of IPGs between pH 2.5–12 has facilitated the analysis of very acidic and very alkaline proteins and the construction of the corresponding databases (www.wzw.tum.de/proteomik). In addition, the protocol for the enrichment of low-abundance proteins by IEF in granulated gels (Görg et al. (2002) Proteomics 2, 1652–1657) has been improved by including multicolored, low molecular weight pI markers visualizing the slope of the pH gradient.

In spite of alternative technologies that have emerged (MudPIT, stable isotope labelling, arrays), 2DE is currently the only technique that can be routinely applied for parallel quantitative expression profiling of large sets of complex protein mixtures. Furthermore, it delivers a map of intact proteins, which reflects changes in protein expression level, isoforms or post-translational modifications. Last but not least, today’s 2DE technology with IPGs (Görg et al. (2000) Electrophoresis 21, 1037–1053) in combination with prefractionation techniques, DIGE and mass spectrometry has greatly improved the coverage of the total proteome of a cell.
Expanding the Proteome Using Medium-range IPG Strips to Resolve Proteins in the Extreme Basic Region

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2-D electrophoresis is one of the most powerful protein separation techniques in terms of resolution, a method by which several thousand proteins can be separated in a single experiment. In combination with mass spectrometry, 2-D is the method of choice for separation and identification of complex protein samples. However, when digging deeper into the proteome, trying to find the least abundant or most acidic/basic proteins, using one wide immobilized pH gradient (IPG) strip is often not enough. Narrower pH gradients are required to increase resolution and sample loads. To demonstrate the advantages obtained by dividing and expanding the pH range, four overlapping medium-range IPG strips ranging from pH 3 to pH 11 were run with micro-preparative mouse liver protein loads. Selected areas of the resulting 2-D gels were then compared to the corresponding area on the broad-range pH 3–11 NL IPG strip with regards to number of spots. The software analysis of the 2-D gels resulted in a significantly increased spot number in the expanded medium-range pH gradients. Furthermore, the basic pH 7–11 NL IPG strip resolved a number of basic proteins not clearly present in pH the 3–11 NL IPG strip. Some of the most basic proteins were also identified by MALDI-ToF analysis. The pH 7–11 NL IPG strip has been chemically modified to increase robustness allowing higher sample loads and longer focusing times with maintained reproducibility. The extension towards the basic end of the pH 3–11 NL IPG strip is also shown by comparison with different commercially available pH 3–10 IPG strips.

Differential Analysis of Protein Expression and Phosphorylation in Yeast by Stable Isotope Labeling and Mass Spectrometry

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Understanding complex biological systems requires the analysis of protein expression and dynamics. An important aspect of protein regulation are post-translational modifications and in particular protein phosphorylation. Recent advances in proteomic techniques make the examination of protein expression by mass spectrometry feasible. Likewise, several strategies have been developed for the identification of phosphorylated peptides. However, application of these methods to whole cells is still challenging due to the wide dynamic range of protein concentration. Our interest was therefore to develop an integrated approach for the analysis and quantification of phosphopeptides in complex sample mixtures.

Cells were labeled in vivo by growing them in the presence of lysine and arginine marked with the stable carbon isotope $^{13}$C (SILAC). Peptides were generated from whole cell lysates and separated by using a combination of ion exchange and reverse phase chromatography. The characterization of phosphopeptides was improved by specific enrichment with affinity chromatography employing either Fe-NTA or TiO$_2$. Analysis of individual fractions by LC-MS-MS was performed with a Q-TOF mass spectrometer. In order to quantify relative peptide abundance, a software package was developed and integrated into VEMS, a program for the interactive analysis of mass spectra.

Yeast was used as a model system for the quantification of protein expression and phosphorylation and results from differential expression experiments are presented.
25.24
Delving Deeper Into the Proteome: Strategies for Discovery Screening and Characterization of Complex Biological Protein Samples
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While the complete elucidation of the human genetic code ended previous speculation over the number of human genes, the number of expressed genes per cell or per organism is still unknown. Although the number of genes is now believed to be somewhere around 35,000, this does not mean the same number of expressed genes or proteins are to be expected. Splicing, post-translational modifications and other mechanisms push the potential number of proteins at 10s of magnitude higher. The dynamic range can cover as much as 11 logs. This scenario poses a tremendous challenge for today's technologies. As a result, strategies for 'divide and conquer' are proposed in this presentation which starts from the cellular level, includes sub-cellular fractionation and utilizes selective protein enrichment techniques prior to protein fractionation and identification. Flow cytometry, cell sorting and/or cell imaging are used to ascertain the phenotypic and functional status of the cells. Sub-cellular fractionation is accomplished by centrifugation whereby the sub-components of cells are isolated. Selective enrichment techniques such as protein depletion or affinity capture are proposed as precursors to protein fractionation using multi dimensional chromatography, which results in liquid fractions that can be subjected to “top down” or “bottom up” analysis.

25.25
A “Divide and Conquer” Strategy for Simplifying the Analysis of Proteomes
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Despite attempts to analyze entire proteomes, it is becoming clear that their complexity and the extreme range of protein concentration presents a significant challenge for today’s technologies. As a result, strategies for simplifying the sample prior to analysis are being developed. A so-called “divide and conquer” strategy is proposed in this presentation which starts at the cellular level, includes sub-cellular fractionation and utilizes selective protein enrichment techniques prior to protein fractionation and identification. Flow cytometry, cell sorting and/or cell imaging are used to ascertain the phenotypic and functional status of the cells. Sub-cellular fractionation is accomplished by centrifugation whereby the sub-components of cells are isolated. Selective enrichment techniques such as protein depletion or affinity capture are proposed as precursors to protein fractionation using multi dimensional chromatography, which results in liquid fractions that can be subjected to “top down” or “bottom up” analysis.

25.26
A Novel Method for Simultaneous Validation of Multiple Candidate Biomarkers in Ovarian Cancer Plasma Samples Using a Nanowestern Immunoblot Based on Inkjet Printing Technology
Michael Hsu, Melissa Thomas, Emma Richards, Femia Hopwood, Rebecca Harcourt, Sybille Hunt, Lucille Sebastian, Susanne Pedersen, Jenny Harry, Andrew Gooley, and Russell Ludowyke
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Ovarian cancer is the fifth most common cause of cancer death among women world-wide. The symptoms are ambiguous and around 75% of patients present when the disease is advanced. If the cancer is detected and treated early, the 5-year survival rate is over 90%. Hence, there exists a critical need for a diagnostic test that detects the early onset of ovarian cancer.

We are using a piezoelectric-based ink-jet printing system (a Chemical Immunoblot Printer, ChiP™) to validate candidate proteins that may be useful as biomarkers for the detection of ovarian cancer. Using the ChiP™ to print antibodies, multiple proteins can be analysed simultaneously on the same sample. This results in the need for significantly less sample and less primary antibody, compared with conventional immunoblotting methods.

We have identified candidate biomarkers by the comparison of plasma from apparently healthy women and women with ovarian cancer using ProteomIQ™. Initially, 2DE gel image analysis of plasma samples from a small cohort of subjects revealed a number of proteins that were statistically differentially expressed between the subject groups. Subsequently these proteins were identified using MALDI-TOF mass spectrometry and peptide mass fingerprinting. Notably, some of these proteins have not been associated previously with ovarian cancer but have postulated biologically significant roles.

The ChiP™ was then used to perform nanoWestern immunoblotting, with the aim of screening a larger number of subject samples for the expression of these proteins. 1D gels of plasma samples were blotted to membranes. Using the ChiP™, nanoliter amounts of specific antibody against these proteins were printed onto the relevant regions of the blot. These regions were washed, and then nanoliter amounts of secondary antibody were also printed over these regions. The presence of these proteins in each plasma sample was then detected using a chemiluminescent detection system. Results from this analysis and a comparison with conventional immunoblotting will be presented.
An Improved Method for In-Solution Digestion of Proteins After Gel Separation

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An improved method for in-solution digestion of gel-separated proteins for mass spectrometer (MS) analysis was developed. The method involves electroblotting of the gel-separated proteins to a poly vinylidene difluoride (PVDF) membrane, extracting proteins from the membrane using a solution of 1% trifluoroacetic acid in 70% acetonitrile, digesting the proteins in-solution and identifying proteins by matrix-assisted laser desorption/ionization (MALDI) MS. Compared to the standard in-gel digestion procedure, the developed method significantly increased sequence coverage because it avoided the presence of SDS which is a major problem for the in-gel digestion method. In addition, proteins electro-blotted into PVDF membrane can be used for other purposes such as the immuno-detection of posttranslational modifications. Moreover, 200 mM ammonium bicarbonate was used to neutralize the extraction buffer instead of, as described before, lyophilizing the extraction buffer then reconstitution in digestion buffer. This resulted in about 15% increase in the protein recovery. In-gel and in-solution digestion methods were separately applied to identify 16 proteins in the Chinese Hamster Ovary (CHO) Cells. The results showed that proteins with high molecular weight (MW) had slightly higher scores for in-gel digestion than that of in-solution digestion; while proteins with relatively low MW (<40 KDa) had higher scores for in-solution digestion than that in-gel digestion.

25.28
Protein Expression Analysis and Biomarker Identification and Quantification Using Multiplexed Isobaric Tagging Technology—iTRAQ Reagents
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A multiplexed peptide tagging chemistry—iTRAQ reagent technology—has been developed for quantitative proteomic analysis. This technology uses a 4-plex set of amine-reactive isobaric tags for peptide derivatization following digestion. In MS spectra, peptides labeled with any of the tags are indistinguishable. Upon fragmentation, signature ions are produced that give quantitative protein expression information. Significantly, ion currents for the sequence informative ions are additive (no splitting), such that the peptide backbone fragments represent the cumulative ion current from up to four samples. The reagents derivatize the N-termini therefore all peptides in a digest mixture are labeled. Derivatization also occurs at lysine residues. The N-terminal tag contains a basic group, which enhances the abundance of b and y ions to yield greatly simplified MS/MS spectra as well as promoting ionization of lysine containing peptides. The principle features of this technology are demonstrated in the measurement of protein expression differences between control and knock-out strains of yeast (two different knockout mutants compared in the same experiment). The advantages over mass difference labeling approaches are illustrated, as well as the absolute quantitation of target proteins using derivatized synthetic peptide standards. Due to the advantages of multiplexing, this technology was then used to study effects of drug inhibition on a tyrosine kinase in a time-dependent manner over 4 time points, and in another study to quantitate and identify novel native peptides biomarkers from human saliva, also over 4 time points.

25.29
A Novel Technological and Data Analysis Tool for Proteomic Exploration of Plasma Membrane Proteins in Living Cells
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Despite the well-known intricacy involved in the elucidation of plasma membrane proteome, a full characterization of the plasma membrane surface proteins in healthy and diseased cells is vital for diagnostic and therapeutic purposes. We develop an integrated tool for comprehensive analysis of the surface proteins. The first step incorporates the identification and examination of the outer membrane proteins using our PROCEED (PROteome of Cell Exposed Extracellular Domains) protocol. Briefly, the living cells undergo biochemical treatment in order to collect the extracellular domains of the surface proteins. The process allows the cells to remain viable and no plasma membrane damage or cell disruption occurs, thus the sample contamination is absolved. Furthermore, the method allows the membrane topology determination, in addition to protein identification. The PROCEED protocol was applied on several mammalian cell lines. We illustrate the potential of PROCEED to determine protein topology in cases that prediction methods fail. Our tool further improves the identification and analysis of the membrane proteins by using an original computational approach to mass spectrometry (MS) data analysis. In addition to using a specialized prediction and/or annotation based membrane-focused sequence database, our tool implements a novel computational approach that exploits the isotope distribution profile of the MS spectra in order to enhance the surface protein discovery. Our combined tool aims to greatly facilitate the membrane protein exploration and investigation by bringing together two innovative approaches in both experimental and computational areas.
25.30
Polymeric Monolithic Capillary Columns in On-Line 2D-LC ESI-MS/MS Proteomic Analysis
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2-D LC has become a widely used method alternative to 2-D PAGE in global protein analysis. This work explores the use of polymeric monolithic capillary columns for the analysis of complex proteomic samples with 2D-LC ESI MS and the advantages of the monoliths over capillary columns packed with beads. Monolithic capillary columns were prepared in silanized fused-silica capillaries of 75–250 μm I.D. by thermally-induced in situ polymerization. The method has a potential for proteomics applications with wide dynamic range and high complexity of a sample. The method showed high efficiency in the pressure-driven elution mode for the second dimension (over 100,000 pL/m); ease of manufacture in comparison to particulate columns of similar dimensions; good column lifetime (up to 1000 injections per column) and reproducibility; ease of a column cleanup under harsh elution conditions helping to avoid cross-contamination between different samples injected. The value of the approach was shown in salt plug injection 2D-LC ESI MS analyses of 1) *S. pombe* cell lysate and 2) low-molecular-weight (LMW) human plasma protein digests. On-line monolithic 2D-LC ESI MS was applied for differential proteomic analysis of metabolically N14/N15 labeled chemically treated or genetically diverse *S. pombe* cell strains. The relative abundance of proteins was calculated from 14N/15N peptide current ratios. Upon MMS treatment we were able to observe the induction of DNA repair proteins indicating biologically relevant results. Another differential proteomic experiment involved chemical isotopic labeling of LMW human protein plasma digests by CD3OH/HCl and CH3OH/HCl esterification followed by 2D-LC ESI MS. Further development of 2D-LC ESI MS with the use of polymeric monoliths and coupling of these separation techniques to more sensitive and fast mass spectrometers is planned.

25.31
Global Validation of False Positive Results by 1DE-based Molecular Weight Correlation in Proteome Analysis of Human Serum Using Multidimensional LC-MS/MS
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Proteomics in general deals with the large-scale determination of gene and cellular function directly at the protein level. Cell, tissues and specially serum or plasma, which is generally considered the most informative proteome from a medical viewpoint, contain thousands of proteins spanning a wide range of abundance. A various fractionation and analysis method has to be used to extend the range and total number of proteins identified with high confidence. In this study, we have performed proteome analysis of human serum by multidimensional analysis at the level of proteins and peptides. We propose the 1D gel-based filtering method to remove the false positive proteins from searched ones. First, the six most abundant serum proteins were removed by immunaffinity chromatography, followed by 1D gel separation. The proteins separated by molecular weight were fractionated into gel bands and in-gel digested with trypsin. Resulting peptides were analyzed by MS/MS with 2D-LC/ESI and LC-MALDI. In 2D-LC/ESI/MS/MS analysis, a two-dimensional LC column packed with strong cation exchange support and reversed-phase material in series is used for the separation of peptides. Total 650 proteins from serum were identified by Sequest based on the results from LC-ESI/MS/MS analysis. After protein filtering with molecular weight, highly confident data was obtained and compared with those from LC-MALDI/MS/MS analysis. The proposed approach based on 1D gel filtration and 2D-LC/ESI-MS/MS analysis was proved to be a confident method for complex proteome analysis such as human serum.
Destreak Rehydration Buffer and Prefractionation Improve Resolution of Proteins in 2D Gels

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In the present study, the results of the investigations into the new commercial rehydration buffer Destreak and prefractionation of the proteins using Zoom IEF Fractionator are reported. The samples were either in the standard rehydration buffer or in Destreak (Amersham). Isoelectrofocusing was done using IPGphor, total vhrs-80,000. Equilibrated with SDS, reduced, and alkylated strips were subjected to SDS-PAGE. Use of Destreak visibly improved the results of 2D SDS-PAGE (higher number of spots detected, better spot shapes). 16 spots were excised from Destreak gel, digested with trypsin, and analyzed by LC-MS/MS. 12 proteins were identified (significant scores, correspondence to the molecular weights and pI values).

3D SDS-PAGEs were performed using Zoom IEF Fractionator (prefractionation); Zoom IPG Runner (IEF); and Zoom SureLock (SDS-PAGE). Every step was done by the manufacturer’s (Invitrogen) protocols. IEF Fractionator efficiently separated the proteins into 5 fractions. Each of them was taken for IEF on IPG DryStrips with overlapping pH ranges. Consecutively, every strip was transferred for SDS-PAGE followed by acidic silver staining. Total number of spots detected by 3D procedure was about 2.5 fold higher than in control (no prefractionation, IEF and SDS-PAGE as above). Spot shapes were also much better after prefractionation. Improved resolution was especially visible in the basic region. 10 spots/sample were excised, digested, analyzed by LC-MS/MS. 9 proteins were identified (significant scores, correspondence to molecular weights and pI values).

Protein Profiling of Mouse Oocyte and Sperm for Functional Analysis of the Mechanism of Fertilization

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Mouse is a model mammal and the recent completion of its genome sequence has supplied abundant information about functions involved in the development of this organism. However, comprehensive protein expression profiling is necessary to discover key new molecules with unique functional characteristics and to probe the mechanisms regulating expression from the genome to the transcriptome and proteome of mouse tissues. For functional analysis of the mechanism of fertilization, the protein expression profile of mouse oocytes and sperm was developed using proteome analysis techniques. The oocytes and sperm were collected from mouse, and total proteins were extracted with lysis buffer and separated by two-dimensional polyacrylamide gel electrophoresis. More than 80 protein spots in oocytes and 200 protein spots in sperm were visualized, and the relative abundances of the identified proteins were calculated through image analysis of the profile. Amino acid sequences of identified proteins were determined using a protein sequencer, MALDI-TOF/MS, and Q-TOF. Assignment of proteins identified in both oocytes and sperm to functional categories showed that most of these proteins are involved in metabolism (glucose-6-phosphoate dehydrogenase, lactate dehydrogenase, etc.) or calcium-related functions (calreticulin, actin, tubulin, heat shock protein, etc.). On the other hand, an oocyte-specific protein (zona pellucida glycoprotein) and a sperm-specific protein (acrosomal protein SP-10) were also identified. Furthermore, proteins of unknown function and novel proteins were identified. These data indicate that the information obtained using this proteomic approach will be helpful in predicting the function of the unknown proteins and analyzing the mechanism of fertilization.
25.34
An Approach to Quantitative Proteome Analysis by Labeling Tryptophan Residues

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This poster presentation describes a method for quantification and sequence identification of individual proteins in complex mixtures. The method is based on labeling with the chemical reagent 2-nitrobenzenesulfenyl chloride (NBSCI) in conjunction with tandem mass spectrometry.

In this method, selective introduction of the 2-nitrobenzenesulfenyl-(NBS) moiety onto tryptophan residues is achieved, and a 6 Da mass differential is generated using 13C6-labeled NBSCI (NBSCI-13C6) and 12C6-labeled NBSCI (NBSCI-12C6). The 6 Da mass differential between the NBS-12C6-labeled and the NBS-13C6-labeled peptides assigns a mass signature to all tryptophan-containing peptides in any pool of proteolytic digests for protein identification through peptide mass mapping. Using this approach, we compared the protein expression in rat sera using a normal (control) rat (Crj-Wister) and a hyperglycemic rat (GK/Crj). The stable isotope dilution techniques used in this method provide highly accurate relative quantification. The NBS approach offers a widely applicable means of analyzing protein mixtures derived from biological samples, and the method described here presents an effective and simplified approach to proteome analysis.

25.35
Top-Down Separation Technologies Targeting Proteome Dynamic Range

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This presentation aims to demonstrate a multidimensional protein separation platform, capable of providing selective analyte enrichment and extremely high resolving power for handling complex protein mixtures prior to mass spectrometry detection. Such selective enhancement toward low abundance proteins can drastically reduce the range of relative protein abundances in complex proteomes such as human plasma/serum, and greatly improve proteome coverage using the combined electrospray/liquid chromatographic separation platform. This top-down proteome separation technology is highly automated and offers robust and high throughput resolution of whole proteins while avoiding analyte dilution and loss in an integrated platform. By coupling with a nanoscale trypsin membrane reactor, the ultrafast proteolytic digestion of proteins resolved and eluted from the separations enables the combined top-down/bottom-up characterization of post-translational modifications in complex proteomes using mass spectrometry detection. The bioanalytical capabilities of this top-down proteome separation technology are illustrated for the identification and quantification of thousands of proteins in a single analysis. Proteomic studies of steroid-activated programmed cell death during development of the fruit fly Drosophila melanogaster serve as the model system for the demonstration of the technology.

25.36
Novel High-Affinity Concentrating Surfaces for MALDI Substrates

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The capture of biomolecules from complex mixtures for direct MS analysis represents a significant challenge for proteomics researchers. We have developed unique biochip surfaces to enable high-affinity binding and subsequent concentration of analytes for MALDI-MS. The platform technology employs virtual wells comprised of discrete zones of increasing wettability to initially confine, then concentrate liquids. Specific analytes are captured in a 3 mm diameter affinity zone. The analyte is released by addition of MALDI matrix, and surface forces concentrate both sample and matrix into a 0.6 mm analysis zone at the center of the site. The affinity zone is prepared with active chemistries (We might want to keep the idea of NHS for sale/development,), which are readily linked to specificity-conferring molecules, while the analysis zone is engineered to resist both covalent reactions and non-specific binding. Streptavidin was covalently linked to the analysis zone, and has been shown to specifically capture biotinylated target molecules. The surface density of biotin binding sites was measured both by MALDI-MS and by an orthogonal method (enzymatic assay of bound biotinylated horseradish peroxidase). The streptavidin surface provides a convenient entry into systems where either the capturing molecule (such as an antibody) or the analyte can be labeled with biotin. In another example, antibodies were directly immobilized on the active surface. An antibody directed toward human ACTH specifically captured spiked ACTH fragment 18–39 directly from rabbit serum. Post-translational modifications may be identified in cases where the antibody captures both modified and unmodified target molecules. In addition, surfaces may be designed to specifically capture species containing modifications such as phosphorylation and glycosylation both in native proteins and proteolytic digests.
Spatially Addressable Peptoid Microarray—Construction and Application in Proteomics
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Protein detecting microarrays are capable of testing thousands of proteins in parallel, thus promising to be a powerful tool for global analysis of protein expression, function, and post-translational modification at the proteomic level. To construct such a protein detecting microarray requires high-affinity and high-specificity ligands for every protein of interest and generating these ligands is by far the biggest technological barrier. Unlike DNA microarrays, where Watson-Crick base pairing rules allow the facile design of capture agents, there is no general way to design a ligand against a protein and most ligands are discovered by screen libraries. Therefore, a high-throughput approach for the ligand discovery is critical for development of protein detecting microarrays.

Here, we describe a new photolithographic chemistry to construct spatially-addressable peptoid libraries, an analog of peptide, on a glass slide platform. This strategy is similar to the technology that first developed by Affymetrix to synthesize oligonucleotide microarrays. However, our approach has been revolutionized by introduction of digital optical chemistry, a technology that uses computer-controllable micromirror chip to generate “virtual” masks. These high-density peptoid microarrays have broad application in the era of proteomics, such as facilitating ligand discovery process, profiling proteins from different tissues, etc. This new peptoidic tool will eventually lead to protein detecting microarrays that provide accurate, fast and cheap diagnostic tools for various diseases.

A Software Pipeline for Quantitative, LC-MS/MS Based Proteomics
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High throughput LC-MS/MS is capable of simultaneously identifying and quantifying thousands of proteins in a complex sample. The consistent and objective analysis of the obtained large amounts of data is challenging and time-consuming. Over the past three years, we developed software tools that facilitate and standardize such analysis. These include: (1) mzXML, an XML common file format that represents LC-MS/MS data from all types of mass spectrometers; (2) Pep3D, a tool that visualizes LC-MS/MS data in a graphic plot and is useful for optimizing the performance of an LC-MS/MS system; (3) ProbID and ProbIDtree, two new database searching engines. The latter is also capable of identifying multiple peptides from a co-fragmented MS/MS spectrum; (4) INTERACT, a sorting tool that allows users to easily organize and inspect database search results on MS/MS spectra; (5) PeptideProphet, a tool that evaluates the likelihood of peptide identifications being correct and provides an easy, accurate and standard criterion on peptide validation; (6) ProteinProphet, a tool that infers protein identifications from identified peptides; (7) XPRESS and ASAPRatio, tools that evaluate protein relative abundance in isotopically labeled samples. The latter also assesses the confidence level of protein ratios and the likelihood of proteins being up or down regulated; (8) Sisyphus, a relational data management system that automatically appends relevant biological information to LC-MS/MS identified and quantified proteins; and (9) SBEAMS, a relational database that stores LC-MS/MS results and allows users to pool information from multiple experiments. We have integrated these tools into a unique pipeline for the consistent and objective analysis of LC-MS/MS data. All the software tools are available under an open source software license at www.proteomecenter.org.

Differential Proteome Analysis of CCR6 Signaling Complexes in Lipid Rafts
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Proteomics-based technology is a powerful tool for analysis of differential protein expression. We have applied a gel-free proteomics-based technology, a lysozyme-specific isotopic labeling technique, to compare the molecules involved in CCR6 signaling in lipid raft domains. Lipid raft domains have been implicated to serve as scaffolds to facilitate the association of signaling complexes. The lipid rafts from CCR6-overexpressing Jurkat T cells either stimulated or un-stimulated with CCL20/MIP-3α were isolated by fractionation of detergent-resistant membrane followed by sucrose density centrifugation. The isolated lipid raft proteins were sequentially digested with CNBr and trypsin. The digests from un-stimulated and stimulated lipid rafts were isotopically labeled with light and heavy 2-thiomethyl-2-imidazoline at lysine residues, separately. The labeled peptides from un-stimulated and stimulated lipid rafts were mixed, fractionated by strong cation exchange, and subsequently subjected to reverse phase nanoLC/MS/MS. No isotope shifts were found in LC separation for isotopically labeled peptide mixture. The differentially labeled peptides were mass analyzed and quantified. Since no constraints on molecular weights and isoelectric points were posted as in traditional 2-dimensional electrophoresis, an unbiased proteome analysis for lipid rafts proteome can be realized. By this unique proteomics-based technology, we have identified about 500 proteins, of which 50 proteins were found significantly changed in lipid rafts from cells stimulated with CCL20/MIP-3α. These proteins can be organized into molecules involved in mediation of cytoskeleton rearrangement, in activation of small G protein, in generation of lipid mediators, and in regulation of protein tyrosine phosphorylation.

1D-ZOOMER: The Technique for Reconstitution of Proteins Profiles from the Molecular Scan of SDS-PAGE
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SDS-PAGE protein separation could be the method of choice for the profiling the membrane-bound proteins. However, the severe limitations prevent this separation technique from the wide-spread application. These limitations elucidate from the poor separation capacity of the method, i.e. several proteins can reside within one and the same band. Eventually that leads to the ambiguousness in protein identification and to the inability to quantify the expression level of the individual protein. To resolve the indicated shortcomings we propose to perform the molecular scanning of the SDS-PAGE and then to decompose the series of MS-spectra into the principal components. The decomposition enables the extraction of signals, relevant to the individual protein and that significantly increases the confidence of identification. In addition, the protein profiles can be reconstituted from sequential MS-data, thus addressing the problem of quantitative comparison between different samples. The proof-of-principal study of 1D-ZOOMER performed on human liver microsomes is reported. Trial version of the software is available from cpd.ibmms.ru/other/zoomer/index.shtml.
Automated 2D-LC Array/MALDI-MS/MS System for High Throughput Proteome Profiling of the Human Liver

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The 2D-LC-ESI/MS has proven to be a powerful tool for comprehensive proteome analysis. With the introduction of the MALDI technique, the coupling of 2D-LC with MALDI/MS became a promising option due to its advantages, including allowing multiple analyses for spots collected and no time constraints for the MS and MS/MS analysis. With the conventional 2D-LC system, however, multiple LC/EL elution cycles for the complex whole proteome profiling must be run one-by-one. This is time consuming, ultimately, limits proteomics analysis throughput. In the study, a new 2D-LC/MALDI-MS/MS system was developed based on the previously described 2D-LC system and the significant improvement on throughput, automation and robustness was demonstrated. The system, in which multiple capillary RPLC columns were employed as the second separation dimension and one SCX column as the first separation dimension, is fully automated and enables SCX fractions transferring to RPLC columns sequentially and the simultaneous RP chromatographic separations of the multiple fractions. With the novel system, high throughput 2D-LC separation of peptides mixture generated by proteins proteolysis was achieved in 2–3 h instead of 20–30 h for common 2D-LC system. In addition, the system allows effluents from multiple RPLC columns on-line mixing with matrix in parallel during the elusion of the RPLC array, and the analyte/matrix mixtures can be automatically deposited into discrete spots for subsequent rapid MALDI/MS analysis. The liver is the largest and one of most important organ in human body. The establishment of the comprehensive proteins expression profiling of the liver will help for the development of new therapeutic approaches for liver diseases. The application of the system to the protein mapping of the human liver was discussed.

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MSDViewer: A Toolbox for High-Throughput LC-MS Proteomics Data Visualization

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The classical LC-MS technique aims at giving an overview of proteins present (detected as set of peptides) in a sample, which could be e.g. serum, cell lysates, or cell membranes. By comparing proteomics-oriented pictures of samples in different status (e.g. disease versus healthy), LC-MS-based differential proteomics is expected to bring some prospects for effective and reliable disease diagnosis and prognosis.

Considering that a single LC-MS experiment can produce more than tens of thousands detections of peptide ions, recording data in a 3-dimensional coordinates (time, mass and intensity), a high demand has been raised to develop correspondingly powerful bioinformatics tools to handle and mine information from these high-throughput data. Among many steps of data processing, graphical exploration is often very helpful as a starting point of data mining, and is usually effective in guiding the design of following data analyses. However, the development of data visualization tools runs much behind the rapid pace of data generation.

We present here a toolbox (called Mass Spec Data Viewer, abbreviated as MSDViewer) with a simple-to-use GUI interface, written and run in MATLAB environment, which can be used for LC-MS proteomics data visualization, pre-processing, and data quality/repeatability analyses.

MSDViewer is designed to process accurate mass data that are a priori transformed from mass-to-charge ratios by other existing software like Mascot Distiller. The tools in MSDViewer can be put into three categories: data preprocessing, single data exploration, and pair-wise data comparison.

Data denosing and peak centroiding belong to the preprocessing module, which aims to compensate variations caused by random factors during experiment, and make data as clean as possible within a tolerable range of signal-to-noise ratio. For each single LC-MS data, MSDViewer provides four means of visualization: (1) two-dimensional projection plot on a time-mass plane with discrete color-coded intensity information, (2) three-dimensional plot, (3) a chromatograph provided with the interactive ability so that a chosen time point can be expanded to view a scan of masses, and (4) a overview of all masses with the interactive ability so that an ion intensity along time for a given mass can be monitored. We also developed a set of tools for pair-wise data visual comparison, including mirror plots of total chromatograms and total mass scans, two-dimension LC-MS projections overlay (similar to the concept in handling 2-D Gel data), data repeatability assessment by time-versus-time and intensity-versus-intensity plot, and data similarity matrix plot. For most of the functionalities, a capability of submitting a batch of data is implemented, if no parameters needs to be changed from data to data.

There is certainly a large space for improving and supplementing functions in MSDViewer toolbox. Our future development plans include: to test accurate mass data transformed by other software, to add a module for group data visualization (more than a pair of data), and to include some pattern recognition (e.g. classification/clustering) methods, etc.
A De Novo Sequencing Method for Identifying Novel Amino Acid Mutations and Post-translational Modifications in Human Serum Proteome

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Population proteomics is a potentially powerful tool for identifying biomarkers (e.g. mutations or post-translational modifications (PTM) in proteins) unique to different human sub-populations. However, most mutations and PTMs are not represented in the publicly available human genome database, which is the basis for protein identification following traditional mass spectrometry (MS) analysis. To circumvent this problem, a de novo sequencing method was developed.

Peptide derivatization by 2-methoxy-4,5–1H-imidazole (imidazole hereafter) prior to MS analysis has been shown to enhance protein identification and simplify MS/MS spectra. To test imidazole derivatization in de novo sequencing, several proteins and gel spots were digested and labeled with imidazole and analyzed by MALDI-MS/MS. Two or more peptides from each protein were directly sequenced from MS/MS spectra without database searching, resulting in unambiguous protein identification. An unanticipated point mutation in ORM2 was also identified by this approach. The reaction and mass spectrometry conditions were optimized to obtain maximum length of peptide sequence tags from MS/MS spectra. Derivatized peptide mixtures were also resolved by RP-HPLC prior to MS analysis, with the result of increasing protein sequence coverage.

This de novo sequencing method was applied to human serum samples to identify possible mutations and PTMs. Human serum samples were immunodepleted of the six most abundant proteins, then the remaining proteins resolved using a novel high recovery RP-HPLC approach, into 50 fractions. Selected fractions were analyzed following digestion and derivatization, by microscale RP-HPLC separation and MALDI-MS/MS. Several point mutations and PTMs were thus identified for lower abundance proteins. This method can be easily automated for large-scale studies, such as population proteomics.