30.1

Result Driven Strategies for Protein Identification and Quantitation

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As an alternative to a 2D PAGE separation of proteins followed by MALDI TOF measurement of the peptide mass fingerprints (PMF), one or multi-dimensional LC ESI MS/MS approaches have gained significant interest in quantifying and identifying proteins from complex biological samples. With the recent progress on MALDI based MS/MS technology, the off-line coupling of LC with MS quantitation and MS/MS identification methods allows the storage and consolidation of results from several acquisitions, processing and identifications steps in a relational database and to link them back to the sample based on the x and y coordinates and barcode(s) of one or several MALDI plates. Therefore MS and MSMS runs, quantitation and identification can be iterated with modified parameters or new runs are added until enough confidence in the results is gained. In the case of an expression dependent workflow, quantification can be decoupled from identification and only relevant expression ratios can be selected for further MSMS analyses. On the other hand, additional MSMS runs might confirm tentative PMF hits in a search result dependent workflow. This level of sophistication requires the development of new algorithms for expression, MS data and search result dependent MSMS analyses and also the definition of a quality metrics for MSMS spectra to support a higher degree of automation.

30.2

Monolithic Capillary Columns for High-speed Protein/Peptide Separations

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LC Packings Dionex Company, Amsterdam, The Netherlands

Capillary and Nano LC columns with inner diameters ranging from 50 to 300 μm are typically packed with 3 to 5 μm particle size stationary phases. Although these column are robust and yield reasonable efficient separations there are a few limitations: the slow mass transfer in the stationary phase limits the separation efficiency and the packed bed of this granular phases results in high back pressures. In contrast to conventional stationary phases, monolithic structures are highly porous, characterized by macro and meso pores which results in better mass transfer properties and in reduced back pressure.

In this poster we report on the chromatographic performance of 200 μm i.d. polystyrene/divinylbenzene monolithic capillary columns. The improved mass transfer in the stationary phase will be illustrated by H/u curve. Efficiencies up to a 1/4 million plates per meter are obtained routinely. To achieve these high efficiencies special attention must be given to the chromatographic system. The response time and sampling rate of the data system must be adapted accordingly. Other advantages using monolithic bed structures are the high mechanical stability of the column bed (no voiding) resulting in a superior lifetime.

Ultra-fast separation of proteins and peptides including protein digests will be shown to illustrate the performance of these monolithic capillary columns. For proteins and peptides peak width at half heights of 1 to 3 seconds are achieved routinely. Peak capacities with up to 100 peaks in less than 15 minutes in gradient mode are now possible

30.3

ECL *Advance*™: A New Chemiluminescent Substrate for Western Blotting

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As we move into the post-genomic era there is a greater emphasis on the analysis of proteins and their roles in disease and diagnosis. Of the many methods available to study proteins, Western blotting is a well-established technique, which can be used to identify the presence of specific protein entities in complex mixtures.

Western blotting describes the transfer of proteins from a gel onto a stable support such as a nitrocellulose membrane. Proteins are then detected on this blot through the use of specific labelled antibodies. Methods of detecting proteins on Western blots using labelled antibodies have advanced over the years from colorimetric techniques to chemiluminescent detection, which is most widely used today. Many current chemiluminescence detection reagents on the market work through luminol based chemistries whereby a Horseradish peroxidase (HRP) molecule on the antibody reacts with the detection reagent to produce a readily detectable light signal.

Here we introduce ECL *Advance* (Amersham Biosciences), a new generation chemiluminescent detection reagent for use with Western blotting, highlighting some of its features and benefits. The sensitive nature of this new reagent allows the detection of low amounts of target protein. Weaker primary and secondary antibody concentrations can also be employed. This offers a benefit where protein target or antibody supply is scarce. The intense light signal produced by these reagents is readily detectable by film or CCD imaging. Accompanying the reagents is a new blocking agent, which minimises background noise caused by non-specific binding.

30.4

High Throughput Dialysis of Small Volume Protein Samples

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As the field of Proteomics develops, there will be inevitable pressure to produce larger numbers of protein samples for analysis. Current methodologies are well adapted to the production of limited numbers of samples, but handling larger numbers—tens, hundreds or thousands—presents a logistical problem. One of the common steps in many protein purification protocols is dialysis, yet reliable, efficient and cost effective devices designed to handle large sample numbers are scarce. One such device is the BDTM Multiwell Dialysis System, which is designed to process multiple small volume protein samples simultaneously. This study examines the performance of the system in several standard dialysis procedures. The properties studied here include the rate of ion removal, effect of sample volume on dialysis rate and protein retention as a function of molecular weight.

30.5

CALI—An Innovative Approach for the Identification and Validation of Novel Drug Targets

Susanne Rubenwolf

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Chromophore-assisted laser inactivation (CALI) is a protein knockout technology that can specifically inactivate protein function in its physiological context. The combination of CALI with scFv antibody phage display and mass spectrometry has generated a powerful technology platform for the systematic identification and functional validation of proteins in cell-based assays. Starting point of our approach is a complex source of proteins such as the extracellular proteome of a diseased cell, against which a suite of specific scFv antibodies is selected. These antibodies are then systematically tested for their ability to mediate inactivation of a disease relevant biological function in a CALI experiment. Once a functional hit is generated, the bound target protein is isolated by immunoprecipitation and its identity is determined by subsequent mass spectrometry analysis.

30.6

High-speed Protein Digestion and Sample Preparation for MALDI-MS on a Microfluidic CD

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Limitations to efficient protein identification by MALDI-MS include manual sample preparation steps and low proteolytic digestion rates, particularly with dilute protein samples. A CD microlaboratory, Gyrolab MALDI SP1, has been shown to increase sensitivity and reproducibility of MS analysis by improving the sample preparation process. Protein digests are concentrated, desalted, crystallized and analyzed by MALDI-MS directly on a CD. We have now integrated an upstream protein digestion step. This microfluidic approach to miniaturization and integration enables low micromolar to nanomolar concentrations of protein to be digested and prepared for MS analysis within minutes. We demonstrate that digestion efficiency and subsequent protein identification can be improved compared to conventional techniques and that identification of proteins from dilute solutions can be facilitated.

30.7

Protein Quantification in Microfluidic Protein Arrays Using Affibody Affinity Binders in a CD Microlaboratory

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This work describes a novel assay format for quantitative determination of specific proteins in crude samples. Affinity binders, Affibodies, were covalently coupled as capturing reagents to particles packed within application-specific microstructures of a CD microlaboratory, developed using Gyros technology platform. Samples were processed under controlled flow conditions to ensure efficient capture of analytes to the capture bed. Bound analytes were detected by adding fluorescently-labelled secondary binders and quantified by scanning the laser-induced fluorescence on the columns. Discrete 100 nanoliter samples of undiluted cell supernatants were processed in parallel, using HSA-secreting cells as a model system. HSA was successfully quantified in supernatants containing 10% FCS. We believe that this format is suitable for use with other crude samples such as serum, plasma, urine and, possibly, tissue extracts.

30.8

Preparing Samples in Parallel in a CD Microlaboratory Improves Sensitivity of MALDI Analysis to Facilitate Successful Protein Identification

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In proteomics, the demand for a reliable sample preparation procedure prior to MALDI MS analysis is well recognized. Loss of sample and non-optimal sample preparation impair the performance of MS analysis, making it especially difficult to identify low-abundance proteins. To overcome these limitations we have miniaturized, optimized and integrated the sample preparation process into a CD microlaboratory, Gyrolab MALDI SP1. In comparison with traditional preparation techniques, we show improved sensitivity and high reproducibility. Together with the ability to process up to 96 samples in parallel, this microfluidic solution offers a significant step towards a higher success rate for protein identification and increased lab productivity.

30.9

UltraPlex™ Barcodes Molecules

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SmartBead has developed barcoded particle technology, UltraPlex™ that allows for a large number of protein assays to be performed simultaneously. UltraPlex™ combines the power of barcodes with microparticle arrays to unambiguously label molecules.

The UltraPlex™ system includes barcoded aluminium microparticles, attachment chemistry for binding analytes to the microparticles and an imaging based reader system with software for reading the barcodes.

The barcoded microparticles can be covalently bound to a range of analytes, such as antibodies, antigens and other peptides. Each analyte is therefore uniquely and permanently coded and can be combined together in one assay. The number of unique barcodes is unlimited, providing the flexibility to combine a few to hundreds of thousands of assays in one tube. The barcodes provide an unambiguous identification of each analyte in the assay, reducing the error rates often found in other coding systems used by competitive products.

UltraPlex™ can be combined with a range of standard detection methods, such as fluorescence.

Applications for UltraPlex™ include a range of protein assays, including multiplexed ELISAs, EIAs, protein:protein interaction and receptor-ligand screening.

30.10

eTag™ Reporter Assay System for Solution-phase Multiplexing of Gene Expression and Protein Analysis

Sharat Singh

Assay Technologies, ACLARA BioSciences, Inc.

The eTag™ Reporter system-a universal assay platform-enables solution-phase, multiplexed assays for the measurement of both gene and protein expression in biological samples. The system enables the concurrent measurement of intracellular, membrane and secreted proteins together with their post-transcriptional modifications. Multiplexing with eTag Reporters simplifies pharmacological profiling and deorphanization of receptor and enzyme families. The system offers rapid assay development, high level multiplexing, direct analysis of cell lysates with no sample preparation, one-step assays with no wash steps using standard laboratory instruments, and reliable assays with high sensitivity. Key application areas include analysis of signaling pathways, surrogate markers for cell function or toxic effects, or any useful panel of mRNAs and proteins.

30.11

Differential Protein Expression on Protein Arrays

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The detection of differences between biological samples is of fundamental importance in biology, whether comparing normal with diseased tissues or monitoring the effect of drug treatment. Classically, scientists have measured relative levels of gene expression by comparison of mRNA levels in differentially labelled samples on microarrays. However, a problem in examining gene expression at the mRNA level is that protein and mRNA levels do not necessarily correlate. More direct methods are required to compare the expression of protein products, in all their manifestations, in a highly parallel way. Until recently, the only practical way to examine a multiplicity of proteins simultaneously was by 2 dimensional fluorescence difference gel electrophoresis (2D DIGE), which allows direct and accurate analysis of protein differential expression by using matched dyes to pre-label samples prior to multiplexed separation. However, 2D electrophoresis is not ideally suited to rapid, large-scale protein expression screening applications. We are developing a high throughput, high sensitivity protein array for the differential analysis of multiple protein analytes in a highly parallel fashion. Preliminary data obtained from such protein arrays will be presented.

30.12

Detection and Identification by MALDI MS of Alzheimer's Brain Proteins in 2-D Gels Using a Novel, High Sensitivity Fluorophore

Mary F. Lopez[1], Simon Melov[2], David Bennett[3], Elena Chernokalskaya[4], Peter Jackson[5], Susan Kramer[6], Dan Sissors[6], Tom Erickson[6], and Joe Trometer[6]

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Protein expression was analyzed using Human brain tissue from older participants in the Religious Orders Study, a prospective longitudinal clinical-pathologic study of aging and Alzheimer's disease. All participants underwent detailed clinical evaluation within a year of death. Protein mixtures from brain samples were covalently pre-labelled with SuperBRIGHT™, a novel reactive fluorophore, after reduction using a rapid procedure. The labelled proteins were subsequently run on 2-D gels encased in low fluorescence glass cassettes. The 2-D image patterns were directly acquired through the glass cassettes without further gel handling, using a PerkinElmer Life Sciences ProXPRESS™ Proteomic Imaging System. Using this method, the protein detection sensitivity was on the order of 10pg indicating higher sensitivity than silver stain. Following image analysis, protein spots were identified by peptide mass mapping by excision from the gels and automated in-gel trypsin digestion using the MultiProbe®II Proteomics followed by MALDI Mass spectrometry.

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30.13

Applications of Magnetic Particles in Proteomics Using PickPen™ Technology with QuickPick™ Products

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Magnetic particle (MP) technology is based on the interaction between a ligand coated on paramagnetic particle and a target molecule. The MPs are mixed with a sample containing the target molecules, and during incubation the target molecules bind to the particles. After incubation, the complex formed between the target molecule and MP is extracted with PickPen™, a MP transfer device which supersedes traditional MP separation racks. The PickPen™ transfers the MPs themselves, not liquids, enabling simple, easy and fast separations.

QuickPick™ is a product family for robust, ready-to-use kits targeted to protein purification and fractionation using the PickPen™. The QuickPick™ system is optimized for small sample volumes, however, with samples of low protein concentration volumes up to 1 ml can be applied. The time needed for single preparation is below 10 min, which is of great importance when working with samples that need fast treatment due to e.g. stability problems.

The QuickPick™ protein product family at present contains three methods; weak cation exchange (CM), weak anion exchange (DEAE), and metal affinity (IMAC), but more methods are in development. The ion exchange applications focus on fractionation of complex proteomic samples before 2-D electrophoresis. Moreover, proteins expressed in low amounts can be concentrated by decreasing the volume of the elution buffer enabling more profound analysis of the sample. QuickPick™ IMAC MPs may be applied for direct purifications of recombinant his-tag proteins or for protein-protein interaction studies in a pull-down analysis by the capture of interacting "pray" proteins using his-tagged "bait" protein.

30.14

Phenotyping of a Diabetes Animal Model by Peptidomics

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Sedentary lifestyle and overly rich nutrition have resulted in rapid growth of the incidence of obesity and type II diabetes. Dysregulation of peptides such as insulin, glucagon, GLP-1 and leptin are implicated in the pathophysiology.

Peptides are the main focus of BioVisioN's Differential Peptide Display™ technology. Signals correlating with metabolic or pathologic events are identified by comparison of peptide patterns. Peptides with a molecular mass below 20 kDa are extracted from samples and analysed by a combination of liquid chromatography, mass spectrometry and specialised software. The technology has been successfully applied to various biological sources such as body fluids, biopsies, cell cultures and supernatants.

A disease area such as diabetes represents an ideal fit to our peptidomics discovery approach. Here we describe the phenotyping of a diabetic model by differential analysis of selected tissues of obese mice (ob/ob-strain) and normal mice. Since beta-cell failure is one hallmark of type II diabetes, we selected pancreatic tissue as a starting point. From several thousand signals, only a limited number of individual peptides strongly correlated with the ob/ob genotype.

In several cases, sequencing of peptides revealed a close functional relationship of the corresponding protein to diabetes.

This study will enhance the understanding of peptide networks in diabetes and obesity. It may contribute to novel approaches in the diagnosis or therapy of diabetes.

30.15

Automated Purification of Recombinant Proteins in a 96-well Format: No Centrifugation Required

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The complex problem of simultaneously isolating thousands of proteins each with unique physical and chemical properties is proving more difficult to solve than the problem of automated DNA preparation. Commonly employed fractionation protocols combine multiple steps of mechanical disruption, centrifugation or filtration, and finally capture and recovery. These time-consuming steps are necessary in order to separate cells from culture media, lyse the cells, reduce viscosity and purify the target protein from the cell extracts. New developments in robotic liquid handling platforms allow for the automation of the wide variety of tedious liquid handling steps involved in the purification process. However, sample processing is still encumbered by multiple mechanical extraction and separation procedures such as sonication and centrifugation. We have addressed these processing bottlenecks by combining several powerful purification tools including a detergent-based lysis reagent to lyse cells directly in their culture media, a combination of enzymes to digest cell debris and nucleic acids, and high capacity magnetic affinity capture resins to purify the target proteins all in a 96-well automation friendly format. Additionally, fusion proteins can be screened for soluble expression directly from the crude extracts after extraction using an ultrasensitive fluorescent assay. Data will be presented to demonstrate automated protein purification and expression level solubility screening for a variety of protein targets employing these reagents and associated protocols on a robotic protein purification workstation.

30.16

Staccato™—Automation for High Throughput Proteomics

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A given biological sample could easily contain more than a hundred thousand different protein molecules. An extensive separation of the proteins in many fractions is necessary in order to improve the chance of identifying and characterizing all proteins present in such a sample. Highly automated and multiplexed operations are needed to achieve this goal within reasonable time limits.

The Staccato™ Proteomics System provides a key interface to high throughput proteomics processes automating in-gel protein digestion, all liquid handling steps as well as preparation and spotting of peptide samples for direct analysis by mass spectrometry.

At the heart of each system are Zymark's Sciclone™ Advanced Liquid Handler, Twister™ II Advanced Capability Microplate Handler, TurboVap™ 96 Concentration Workstation and the API 44 Automated Precision Incubator.

Each of these components is connected and managed by Zymark's revolutionary CLARA™ System Integration Software.

The Zymark Sciclone™ ALH sets the standard for accuracy in automated liquid handling. With a 20-position deck that accommodates high- and low-volume pipetting heads, it supports a range of discovery applications using flexible 8-, 96- and 384-channel pipetting tools. Any combination of standard or custom disposables can be used.

As an example the process of on-target sample preparation and high precision spotting of peptides onto Bruker's Anchorchip™ MALDI targets with the 384 disposable tip array is running fully automated on the SciClone™ ALH 500.

With the Staccato™ Proteomics System Zymark provides the optimum balance of throughput and robustness required to advance the entire field of proteomics.

30.17

Integrated Sample Preparation Platform for In-Gel Protein Digestion

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Proteomics research is rapidly scaling up in terms of the number of samples analyzed. Although there are several approaches to proteomic analysis, electrophoretic separation followed by mass spectrometry is currently most common. The classic process for identifying stained proteins in 1- or 2-D electrophoresis gels is labor intensive requiring multiple transfers and, from start to finish, takes about 24 hours. We report on the design of a 96 well plate system that integrates all sample manipulations into a single unit ($ZipPlate_{C18}$). Each well has a conical bottom with a capillary drain that contains 300 nl of immobilized C18 resin. A feature is also incorporated that prevents the gel plug from clogging the outlet. Using vacuum as the driving force to drain the wells, 1.5 mm diameter gel plugs can then be immersed in as little as 15 μl of trypsin solution. With incubation, the digestion time can be reduced to 3 hours. Once digested, the peptides are desalted and concentrated on the C18. The peptides are then desorbed with a few microliters of 50% acetonitrile/0.1% TFA using vacuum or centrifugation into a 96 well conical bottom plate, then spotted. The entire sample preparation process takes about 7 hours for 96 excised gel spots. Acquisition and analysis with search results and report generation could be completed in less than 4 hours with confident identification results from a complex sample, resulting in completion of an entire set of 96 samples in significantly less time.

30.18

Uniform Labeling of Proteins Using Universal Linkage System ULS®

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The Universal Linkage System ULS® is a platinum-based labeling technology that allows labeling of biomolecules like RNA, DNA and proteins with any kind of label and haptens, including many fluorochromes, biotin, dinitrophenol and horse radish peroxidase. The labeling reaction takes place under a wide range of conditions and produces a stable bond. ULS labels DNA, RNA and nucleotides by binding to the N7 position of bases. In proteins, in contrast to NHS labeling, ULS labels by binding to histidine, methionine and cysteine, whereas NHS labels Lysines only. The labeling of Methionine and Cysteine is pH independent and fast (T ½ = 15 min) and Histidine labeling is at pH > 7 and slower (T ½ 12 hr, at 37°C aminoacid: label ratio 1:1.1). The ULS-labels are very stable in aqueous solution. ULS can label proteins separately and in complex mixtures like serum or cell lysates. We have compared ULS- with NHS-labeling on a number of proteins and cell lysates. Taking into account the relative abundance of aminoacids in the proteome, ULS labeling can improve uniform labeling as three more target amino acids can be labeled compared to NHS labeling alone. The potential of using ULS in protein target labeling and in the field of proteomics is therefore high.

31.1

High-throughput Approaches for the Identification of Carbohydrate-Protein Interactions in Functional Glycomics

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Glycomics is an integrated way to study the role of carbohydrates in normal or pathologic events. However, the identification and evaluation of carbohydrate-protein interactions in the complex cellular environment is difficult. We describe here high-throughput techniques applied for identification of carbohydrate receptors on killer lymphocytes. They are based on the combination of glycoaffinity techniques with modern methods for analysis of complex protein mixtures and protein complexes by mass spectrometry (MALDI and uLC-MS/MS in an ion trap). Supported by Ministry of Education of Czech Republic (MSM113100001) and Volkswagen Foundation.

31.2

Fast Topological Analysis of a Supramolecular Protein Complex by MALDI Mass Spectrometry and Hydrogen/Deuterium Exchange

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Hydrogen/deuterium exchange of amide protons analyzed by mass spectrometry (HXMS) allows a fast structural characterization of proteins. It involves isotopic exchange at room temperature is followed by quenching (low pH and low temperature) and proteolytic cleavage. The number of exchanged deuterons is determined by mass spectrometry and compared with a non-exchanged reference. To study protein-protein interactions within supramolecular complexes, topological analysis by HXMS can be conducted first with intact subunits: H/D exchange performed on the whole complex under native conditions is followed by a separation and mass analysis of the disrupted subunits. One step further, proteolytic digestion is applied prior to mass spectrometric analysis, to determine the distribution of incorporated deuterons within a given subunit. We have developed a strategy that uses MALDI mass spectrometry, fast micro-chromatography on reversed phase supports, as well as digestion with immobilized pepsin. This approach has been applied to the topological study of yeast mitochondrial ATP synthase, a 600 kDa supramolecular complex composed of 13 different subunits. We have performed a detailed study of the topology of the epsilon subunit of ATP synthase, known to interact closely with the gamma and delta subunits. Topological characterization was achieved with an amount of protein in the 10 pmole range. The procedure greatly benefits from the fast analysis capability of MALDI-MS, associated to its high mass accuracy and ability to deal with digestion mixtures, and thus opens a promising way to investigate the topology of supramolecular protein complexes.