POSTER SESSION

(Alphabetical order by presenter)

(1) Profiling Kinase Inhibition in Cancer Cell Extracts Using Active-site Probes and Targeted High Resolution, Accurate Mass Spectrometry

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Protein kinases are enzymes that play key roles in signal transduction pathways which regulate cellular processes including growth, division, differentiation and metabolism. Kinase inhibitors are essential tools used by researchers to understand kinase function and are also therapeutic compounds used in treating cancer and other diseases. Active-site probes are one technology that can be used to determine the specificity and potency of kinase inhibitors. Desthiobiotin-ATP and -ADP are two nucleotide derivatives that have been shown to selectively label kinase active sites. Using these probes to enrich kinase active-site peptides, we identified over 150 kinases from K562 chronic myelogenous leukemia and A549 lung carcinoma cell extracts using high resolution, accurate mass (HR/AM) spectrometry. We also assessed specificity of kinase inhibitors including staurosporine and wortmannin by determining the relative quantitation of kinase active-site peptides after drug treatment. In addition, we validated kinase inhibitor targets using a parallel Western blot workflow. Overall, kinase inhibition measured using high resolution, accurate mass spectrometry had strong correlation with Western blot data and enabled global profiling of kinase inhibitor target and off-targets.

(2) Proteomic Analysis of Induced Pluripotent Stem Cells as a Mechanism to Study Non-Alcoholic Fatty Liver Disease (NAFLD)

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Our goal is to study the proteome of iPSC lines created from Non-Alcoholic Fatty Liver Disease (NAFLD) and matching control patients. NAFLD is a complex metabolic disease common among obese individuals. A significant fraction of affected individuals will progress to liver cirrhosis, and will require a transplant.

iPSC provide an opportunity to examine liver cell dysfunction in a human cell-based model. In our initial analyses of iPSC, we characterized the proteome of undifferentiated hiPSC and hESC lines using an Orbitrap Velos mass spectrometer. Only a few proteins were detected at significantly different levels within (1.9-3.7%) or between (2.7-5.3%) cell lines. Our iPSC2a and iPSK3 lines had significantly lower levels of the transcription factor, Lin28A (30% and 49%) compared with our H1ESC line. Based on this developed protocol, we will differentiate control and NAFLD-patient iPSC lines to hepatocyte-like cells to identify significant proteomic and functional differences between these cells after differentiation.

(3) Post-Mascot Validation Using Retention Time Deviation to Improve Peptide and Protein Identifications

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Shotgun proteomics commonly utilizes database search like Mascot to identify proteins from tandem MS/MS spectra. False discovery rate (FDR) is often used to assess the confidence of peptide identifications. However, a widely accepted FDR of 1% sacrifices the sensitivity of peptide identification while improving the accuracy. We here developed a machine learning approach combining retention time based support vector regressor (RT-SVR) with q value based statistical analysis to improve peptide and protein identifications with high sensitivity and accuracy. The use of confident peptide identifications as training examples and careful feature selection ensures high R values (>0.900) for all models. The application of RT-SVR model on Mascot results (p=0.10) increases the sensitivity of peptide identifications. q value, as a function of deviation between predicted and experimental RTs(ΔRT), is used to assess the significance of peptide identifications. We demonstrate that the peptide and protein identifications increase by up to 89.4% and 83.5%, respectively, for a specified q value of 0.01 when applying the method to proteomic analysis of the human natural killer leukemia cell line (NKL). The software can be freely downloaded via http://pages.cs.wisc.edu/~yadi/bioinfo/rtsvr/rtsvr.html.

(4) Quantitative Mitochondrial Phosphoproteomics Provides Mechanistic Insight into the Regulation of Ketogenesis in Type 2 Diabetes

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Type 2 Diabetes Mellitus (T2DM) results from insulin resistance coupled with insufficient insulin production. Mitochondrial dysfunction has been postulated as a unifying theme of T2DM, yet the specific mitochondrial alterations that accompany the progression of the disease have been poorly defined. To address this, we took advantage of two mouse strains with contrasting susceptibilities to T2DM. We labeled mitochondrial peptides with 8-plex iTRAQ reagents, allowing us to measure eight experimental conditions simultaneously in each LC-MS/MS run on a Velos Orbitrap mass spectrometer. We performed five biological replicates for both peptide and phosphopeptide analyses, revealing many statistically significant changes. In total, our experiments quantitated 615 liver mitochondrial proteins and 441 phosphorylation sites across our eight conditions. Among the most robust and noteworthy phosphorylation targets in our study was HMGCS2, the rate-limiting step in ketogenesis. We have demonstrated that phosphorylation site serine 456 is critical for the regulation of HMGCS2 catalytic activity, and may lead to the observed increase in ketogenesis in the diabetic state. These data strongly suggest that reversible phosphorylation is an essential regulatory mechanism for mitochondrial processes, and motivate our ongoing efforts to identify kinases and phosphatases responsible for directing these modifications.

(5) Top-Down Proteomics Identified Upregulated PKC Phosphorylation Sites of Cardiac Troponin I in Spontaneously Hypertensive Heart Failure Rat

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Post-translational modifications (PTMs), particularly phosphorylation, of cardiac troponin I (cTnI) play a critical role in modulating Ca^{2+} -mediated contraction and relaxation in the cardiac muscle. Top-down mass spectrometry (MS)-based proteomics is a powerful technique for comprehensive analysis of protein PTMs since it can universally detect all existing modifications in one spectrum simultaneously without *a priori* knowledge and map the PTM sites precisely. Electron capture dissociation (ECD), a unique non-ergodic fragmentation method, is especially useful for mapping labile PTMs such as phosphorylation. Our previous studies have systematically characterized PTMs of cTnI purified from healthy human and animal cardiac tissues using such a top-down MS approach. However, a comprehensive study on endogenous cTnI modification in diseased animal models is still lacking. In this study, we have utilized top-down MS combined with immunoaffinity purification to quantitatively determine the PTM changes in spontaneously hypertensive rats (SHR) heart failure models. We found phosphorylation of cTnI is up-regulated in SHR in comparison to age-matched Wistar-Kyoto rats (WKY) control rat. The top-down ECD MS unambiguously localized up-regulated phosphorylation sites to Ser22/23 and Ser42/44 in SHR. Ser42/44 is known to be substrates for PKC-$\alpha/\delta/\varepsilon$ whereas PKC-δ/ε also cross-phosphorylate at the PKA sites of Ser22/23. The Western blot analysis further confirmed that PKC-α and δ is up-regulated in SHR heart failure rats. This is the first direct evidence of PKC phosphorylation sites of cTnI from a diseased animal model, supporting the hypothesis that PKC phosphorylation in cTnI may be considered maladaptive and potentially associated with cardiac dysfunction.

(6) Mass Spectrometry-Based Analysis of GFAP Overexpressor Mice's Cerebrospinal Fluid for Proteome Biomarker Discovery in Alexander Disease

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Alexander disease is a fatal neurodegenerative human disorder caused by mutations in the astrocyte intermediate filament, GFAP. CSF samples from GFAP overexpressor mice are pooled to 100 µL and depleted of their 14 most abundant proteins using a custom IgY spin column. The flow-through fractions, containing the low and medium abundance proteins are analyzed via mass spectrometry on the amaZon ion trap using nanoLC on a reverse phase C18 column. Biological triplicates from both affected and control mice are analyzed in technical triplicates and normalized spectral abundance factor (NSAF) is performed to assess differently expressed proteins. Initial data identified 289 proteins with 106 proteins subjected to NSAF analysis and 13 proteins exhibiting significant changes in expression between control and affected mice CSF. This study represents the first attempt of MS profiling of the CSF proteome for Alexander disease biomarker discovery.

(7) Determination of detectability for reference proteotypic peptides for human proteins

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The determination of protein/peptide detectability has been a key problem for shotgun proteomics due to the dynamic range of protein abundance observations and complex proteomics analysis process. In this work, we aim to study the detectability of proteotypic peptides of major plasma proteins and other human proteins using a large number of runs on both high and low resolution instruments. We proposed the usage of peptide ion significance index (PISI) and a model of peptide detectability generated using peptide precursor information obtained in the LC-MS analysis to build the human spectral library.

PISI uses a number of components of the LC-MS data to measure the detectability of the identified peptide ions of individual human proteins. These parameters include average peptide identification per run, peptide relative occurrence, and median relative abundance of multiple runs. From our results, a list of the top ranking proteotypic peptides is produced for human proteins that were readily detectable even under different proteomic experimental conditions. Utilization of the spectral library in determining proteotypic peptide detectability provides a reliable reference for the crucial selection of peptides of major human proteins in targeted proteomic experiments.

(8) Glycoproteomic Characterization of the Cancer-Specific Marker Carcino Embryonic Antigen (CEA)

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Colorectal cancer (CRC) is the second leading cause of cancer related deaths in the United States. A critical component of CRC care is post-surgical monitoring for cancer recurrence. CEA is a tumor marker for the clinical management of CRC which has the specific utility of monitoring post-operative disease recurrence. After surgery to remove cancerous tissue, the level of CEA in blood can be periodically monitored using an immunoassay. If the levels begin to rise above 6.0 ng/mL there is a high correlation with recurrence of the cancer.

CEA, like many tumor markers is a glycoprotein and there is a significant body of work showing protein glycosylation is greatly affected by diseases such as cancer. We are investigating glycoforms of CEA as sensitive and specific biomarkers of CRC. This presentation outlines our methods and includes qualitative and quantitative CEA glycoform data.

(9) Detergent Assisted Lectin Affinity Chromatography for Membrane Proteomics

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Membrane proteins play vital roles in many fundamental physiological and pathophysiological processes, constituting 20-30% of the human genome but making up more than two-thirds of drug targets. These cell surface proteins are generally heavily decorated with carbohydrate chains. Traditional shotgun proteomics analyses underrepresent these membrane glycoproteins due to their hydrophobicity that results in poor solubilization and inefficient protease digestion. Here we introduce a novel lectin affinity chromatography strategy for the enrichment of membrane glycoproteins. Under certain concentrations, mildly denaturing detergents can minimize nonspecific binding and facilitate the binding and elution of hydrophobic glycoproteins. Through a systematic investigation of the binding efficiencies of three lectins in the presence of four nonionic and zwitterionic detergents, we have developed and optimized a detergent assisted lectin affinity chromatography strategy that significantly increases the recovery of plasma membrane and glycoproteins. This study represents the first global identification of the mouse brain glycoproteome by mass spectrometry.

(10) Elevated Levels of Cardiac Myosin Binding Protein-C in Acute Coronary Syndrome

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Acute coronary syndrome (ACS) is a life threatening complication afflicts approximately five million people in U.S. every year. Evaluation of patients who come to the hospital with acute undifferentiated chest pain and signs suggestive of ACS are often a clinical challenge. Cardiac myosin binding protein-C (cMyBP-C) is a thick filament assembly protein that stabilizes sarcomeric structure and cardiac function. Recent studies from our laboratory show that elevated plasma cMyBP-C levels could be used as a biomarker to determine post-myocardial infarction (MI). However, it is unknown whether the level of cMyBP-C is increased in patients with ACS at the early stage. In the present study, we hypothesized that the level of plasma cMyBP-C in patients with ACS is increased significantly, compared to healthy

controls. To test the hypothesis, we utilized 132 plasma samples from patients with ACS that collected at the time of hospitalized and 23 normal healthy controls samples. To determine the level of cMyBP-C in the plasma, we used sandwich ELISA utilizing antibodies against cMyBP-C in mouse and rabbit species. Results show that plasma levels are significantly elevated in patients with ACS, compared to normal control ($P<0.0001$). In conclusion, the elevated level of cMyBP-C in the circulatory has a great potential as biomarker in field of cardiovascular diseases. These data demonstrate that cMyBP-C is a powerful marker for ACS and may be a useful tool in assessing risk and directing appropriate therapy that improves clinical outcome.

(11) Development and Validation of 8-Plex N,N-Dimethyl Leucines as Novel Tandem Mass Tags for Quantitative Proteomics and Peptidomics

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The mass spectrometric determination of relative protein or endogenous peptide expression levels in different biological states is important. Numerous MS-based quantitation approaches like mass-difference labeling and isobaric labeling exist. Tandem mass tags (TMTs), an isobaric labeling approach, provide identification and relative quantitation during MS/MS experiments. One popular but costly type, iTRAQ, utilizes four or eight isobaric labels with reporter ions one Da apart upon MS/MS fragmentation. Our group has developed a more cost-effective set of novel 4-plex isobaric TMT reagents, DiLeu, that are easily synthesized with comparable performance to 4-plex iTRAQ. The development of 8-plex DiLeu tags, described within this poster, could increase the number of biological replicates or states compared in one experiment while remaining more cost effective than iTRAQ.

(12) Investigation of the Impact of Phosphorylation on Top-down Electron Capture Dissociation Mass Spectrometry of Large Proteins

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Electron capture dissociation (ECD) is a non-ergodic tandem mass spectrometry (MS/MS) technique, which well preserves the labile phosphate group during the fragmentation process. Top-down ECD has become a powerful tool for mapping labile phosphorylation of large proteins. However, the potential impact of phosphorylation on ECD of large proteins is still unclear. Herein, we have systematically investigated the effect of phosphorylation on the ECD of large proteins using model phosphoproteins such as α-casein and cardiac troponin I (cTnI).

The commercially available "dephosphorylated" α-casein (De-α-cas) presents as un-, mono-, and bis-phosphorylated forms. ECD fragmentation unambiguously determined that Ser88 and Ser115 are the two phosphorylation sites in mono-phosphorylated (mono-p) De-α-cas (positional isomers). For cTnI, top-down ECD identified Ser22 as the phosphorylation site for mono-phosphorylated swine cTnI whereas Ser23 was phosphorylated in mono-phosphorylated primate cTnI.

We have demonstrated that the phosphorylation sites have a slight deleterious global effect on the total number of fragmentation ions of ECD. Moreover, the phosphorylation sites do not have a significant local effect on the fragmentation of amino acid residue adjacent to phosphorylation sites. In contrast, a previous study by Cooper and co-workers reported the deleterious effect of phosphorylation on ECD sequence coverage of small peptide and less ECD fragments adjacent to the phosphorylation sites compared to unmodified peptides. Our results support the hypothesis that for a given modifications in primary structure, large proteins may be less affected and change their physiochemical properties less than small peptides. This is the first investigation of the impact of phosphorylation on top-down ECD MS of large phosphoproteins (>24 kDa).

(13) Effect of Vascular Endothelial Growth Factor on Bone Marrow Endothelial Progenitor Cells

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Bone marrow derived endothelial progenitor cells (BM-EPCs) are stimulated by vascular endothelial growth factor (VEGF) and other potent pro-angiogenic factors. During physiological angiogenesis, an increase in VEGF expression stimulates BM-EPCs to induce endothelial tube formation and increase microvessel density. The goal of the present study was to use a proteomic strategy to examine the VEGF signaling network within rat BM-EPCs, while exploring unique methodology to directly identify interacting peptides and map downstream pathway components. BM-EPCs were isolated from Sprague Dawley (SD) rat hind limbs and signaling complexes were studied using a combination of cross-linkers and VEGF coupled to magnetic DynaBeads M-450 Epoxy resin. Both thiol-reducible and collision inducible dissociation cleavable cross-linkers were utilized. Signaling network analysis by orbitrap electrospray ionization mass spectrometry revealed proteins directly and indirectly related to the VEGF signaling pathway, calcium signaling pathway induced by VEGF, the related eNOS pathway, and related solute transport pathways.

(14) Discovery and functional study of a novel crustacean tachykinin via a multi-faceted MS approach

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Tachykinins constitute a large and structurally diverse neuropeptide family in the animal kingdom, including both vertebrates and invertebrates. In invertebrates these peptides are called Tachykinin Related Peptides (TRPs) and have the conserved C-terminal pentapeptide FX_1GX_2Ramide. With their presence in the central and peripheral nervous system, tachykinins mediate various physiological and pathological effects, making them very important in drug discovery. Here, we have discovered a novel TRP, in the blue crab *Callinectes sapidus*, which we named CalsTRP, via a multi-faceted MS based platform. We also examined the spatial distribution of CalsTRP in the *C. sapidus* brain using imaging mass spectrometry (IMS), and we explored quantitative CalsTRP changes in the *C. sapidus* brain and neuronal ganglia under different behavioral conditions.

Novel aspect:A novel tachykinin neuropeptide was discovered; its role in feeding and its physiological effects on crustacean nervous system were demonstrated.

(15) Quantitation Study of Biogenic Amines and Crustacean Hyperglycemic Hormone (CHH)-family Peptides in Crustacean Nervous System by Novel DiLeu Labeling Technique

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Crustacean hyperglycemic hormone (CHH) family neuropeptides play central roles in energy homeostasis, which is mediated by biogenic amines from eyestalk. Monitoring the variation of CHH-family peptides and biogenic amines provides insights into the underlying hormonal mechanism of energy homeostasis. In this study, we applied *N,N*-Dimethyl Leucines (DiLeu) tagging technique to simultaneously quantify the biogenic amines and CHH-family peptides for studying the dynamic interplay between these important biological molecules. Two crab species, *Callinectes sapidus* and *Cancer borealis*, were utilized as model organisms for the study of interplay between biogenic amines and CHH-family peptides. Two neuroendocrine organs, pericardial organ (PO) and sinus gland (SG), which are major neurosecretory structures that can release these biological molecules, have been studied. Quantitation of CHH-family peptides is challenging because of high molecular weight (~9 kDa) and post-translational modifications (PTMs). Novel isobaric DiLeu reagent is a powerful quantitation technique for assessment of expression levels of neuropeptides in multiple samplpes. The tissue extract of SG organ of *C. sapidus* was digested by trypsin and labeled with 4-plex DiLeu reagents respectively, followed by nanoLC-ESI-MS/MS analysis. The labeled tryptic peptides were detected and the amount variation were calculated according to the intensity ratio of DiLeu reporter ions m/z 114, 115, 116 and 177, enabling quantitation for several CHH-family peptides, including CHH, molt-inhibiting hormone, and mandibular organ-inhibiting hormone. Serotonin and dopamine were detected in the two crab species. The DiLeu isobaric labeling technique was applied to profile the variation of biogenic amines in the crustacean nervous system. The coupling reaction by DiLeu was complete as evaluated by the serotonin and dopamine standards. 4-plex DiLeu reagents were then employed for labeling tissue samples, allowing the biogenic amines to be quantified for the first time in these neuronal tissue extracts.

(16) Mass Spectrometric Imaging of Neurotransmitters and Neuropeptides in the Brain of the Lobster *Homarus americanus*

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The American lobster Homarus americanus is a decapod crustacean with both high economic and scientific importance. As part of the central nervous system (CNS), brain is connected with the stomatogastric nervous system that produces rhythmic motor activities. Thus, the characterization of the identities and localization of signaling molecules in the brain is very important. Previous work used primarily immunocytochemical approaches to map the distribution of neurotransmitters in the CNS. Here, we employed a mass spectrometric imaging (MSI) approach to examine the distributions of small molecule neurotransmitters and neuropeptides in H. americanus. A pseudo selected reaction monitoring (SRM) methodology was developed to image the distribution of small molecule neurotrasmitters. Furthermore, neuropeptide profiles in the brain at multiple developing stages were compared and MSI of juvenile and adult stages were highlighted.

(17) A Combined Mass Spectrometry and Imaging Approach for the Study of Human Mediator Complexes

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The understanding of protein complex assembly and mapping of protein interactions has rapidly grown in recent years due to significant advances in mass spectrometry. As experiments turn toward characterization of the human proteome

and the complexity it presents, a need remains to efficiently capture complexes intact, particularly weak or transient interactors as well as large multiprotein complexes. Here we present a new technology based upon the use of a protein fusion tag, termed HaloTag, which allows for highly specific and covalent immobilization of proteins complexes, as well as the ability to do correlative cellular localization studies using fluorescent ligands. In studies presented here, this technology was used to further understanding of the multi-protein Mediator complex assemblies and their cellular function.

Mass spectrometry analysis of pull-down samples from multiple human Mediator (MED) subunits fused to HaloTag reveals the successful and specific capture of the Mediator complex, as well as interacting partners for all tested fusions. No Mediator subunits were observed in control samples expressing the HaloTag protein alone. These data strongly support proper *in vivo* incorporation of each MED-HaloTag fusion into Mediator complexes. Further in-depth mass spectrometry analysis of the various MED subunit pull-down data reveals subtle, yet important biological differences between the compositions of each complex. These differences are reproducibly observed in replicates samples and can be clustered together relative to the position of the MED subunit within the core Mediator structure, supporting its modular function. Complementary cellular imaging studies for the different MED subunits show also unique localization patterns. Taken together, the preliminary results suggest multiple distinct Mediator sub-complexes exist within the cell, likely to regulate specific transcription events, and in addition Mediator subunits may have functions in complexes independent of the Mediator complex. These studies also highlight this ability to use the HaloTag technology to efficiently study multi-protein complexes, discover new interactions, and characterize cellular localization for macromolecular complexes within the cell.

(18) Glycosylation characterization of Human IgA1 by UPLC-ESI QTOF MS through differential deglycosylation

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Differential deglycosylation was investigated as an effective technique to characterize glycosylation in glycoprotein containing both N-linked and O-linked glycans at both protein and peptide levels.

Human IgA1 was treated with an array of enzymes then reduced with DTT. The reverse phase UPLC was used for separation with 0.1% formic acid in water as mobile phase A and 0.1% formic acid in ACN as mobile phase B. ESI-TOF was connected on-line with UPLC. The above deglycoproteins and unmodified glycoprotein were further digested to peptides by trypsin. ESI-TOF was connected on-line with UPLC.

Data from deglycoproteins were used to estimate the number of N-glycan and O-glycan sites. Data from deglycopeptides and glycopeptides were used to locate glycopeptide and deglycopeptides. Site specific N-glycan mass information was obtained by calculating mass difference between glycopeptide and deglycopeptide

(19) Absolute Quantification and Identification of Intact Proteoforms: Separation by HILIC and Analysis by Native UV-Induced Fluorescence and Top-Down Mass Spectrometry

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The field of proteomics currently lacks a method to quantify intact proteoforms, defined as proteins that differ by post-translational modifications or small changes in sequence due to alternative splicing or codon substitutions. Today, proteoforms are generally characterized by enzymatic digestion and analysis of the resulting peptides. While this approach can identify individual modifications, information on the forms and amounts of the intact protein is lost.

To address this problem, we have constructed a device that allows us to measure native tryptophan fluorescence of protein samples in real-time prior to injection into a mass spectrometer. This combines the quantitative capabilities of fluorescence with the identification capabilities of mass spectrometry, allowing us to obtain information that is vitally important for understanding the function of biological systems. We are currently working to use this in combination with GELFrEE (Gel-Eluted Liquid Fraction Entrapment Electrophoresis) and pH-gradient HILIC (Hydrophilic Interaction Liquid Chromatography) to separate, identify, and quantify intact proteoforms from molecular machines such as proteasomes and ribosomes.

(20) Imaging by Liquid Extraction Surface Analyses of Brain Tissue Sections Using High Resolution nano-ESI-MS and nano-LC-MS

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Matrix-assisted laser desorption ionization (MALDI) is a well established technique for the analyses of lipids from complex biological matrices like brain tissue. Complementary to MALDI, other Surface-oriented MS methods like DESI, LDI, DART or LESA allow the localization of individual molecular species and therefore the investigation of the spatial distributions. Liquid Extraction Surface Analyses (LESA) can have distinguished advantages providing soft ionization of analytes without introducing additional sample preparation steps like matrix deposition. Although the spatial resolution

of LESA (200 um - 1mm) is lower than that of MALDI (10 -100um), LESA allows the sensitive detection of a wide range of lipids classes in combination with nano-ESI-MS and nano-LC-MS.
Frozen Mice brain tissue, horizontally sliced in 15 um thickness and placed onto a glass slide, was analyzed by liquid extraction surface analyses. 0.5uL extraction solvent was robotically placed onto the tissue to form a micro liquid junction between a capillary and the surface. The extract was then aspirated into the capillary and transferred automatically to the inlet of a nano-ESI chip for direct analyses in a shotgun lipidomics fashion using high resolution mass spectrometry detection. Alternatively the extract was loaded onto a nanobore column enabling the chromatography separation and localization of individual Lipid species in single MS analyses. In booth modes positive and negative Ion detection was performed to increase the coverage.

Summary & Discussion

- LESA LC combines Liquid Extraction Surface Analyses with nanoLC separation
- The TriVersa NanoMate was modified using a nano Port switch valve for automated LESALC operation
- Spatial resolution was improved to 200um using a fused silica probe (170um OD) compared to 1mm on standard LESA operation using pipette tips
- Additional separation helps to distinguish isomers with will overlap in other imaging techniques like MALDI
- More than 250 lipid species from 10 lipid classes from a 200um diameter sampling area could be identified
- LESA with LC is the only surface analyses technique employing chromatographic separation

(21) False Discovery Rate in High-throughput Top Down Proteomics Identifications

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With the advent of high throughput top down proteomics it has become necessary to consider the false discovery rate (FDR) in top down protein identifications. For this project 9,838 high throughput top down MS/MS experiments were searched using ProSightPC against both forward and scrambled protein databases. This data was used to generate an initial estimation of the distribution of identification scores against random sequences. The scores from the forward database were then matched with the inferred null distribution to generate instantaneous FDR values for the identifications.

(22) Development of a Multiplex MRM Method for Measuring Proteins in High Density Lipoprotein Particles

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High density lipoprotein particles (HDL) are composed of some 40 proteins along with phospholipids , cholesterol esters, and other cargo. HDL protein composition changes in vascular diseases as well as neurological diseases. Changes in HDL are thought to be a response to vascular inflammation and other stressors. Methods to measure multiple proteins in HDL are lacking. Thus, we developed a method to detect and quantify selected HDL proteins from human plasma.

Using Liposorb capture beads, lipoproteins were enriched from human plasma samples. Proteins were digested on the beads with trypsin to generate peptides. Peptides were extracted, dried and resuspended in solvent (10% acetonitrile, 0.1% formic acid) for analysis. Samples were run on a nanobore C18 column using an Agilent 1100 instrument coupled to a nanospray -ESI ion trap mass spectrometer (MS). Two product ion transitions were monitored for each parent peptide and synthetic peptide standards were used for calibration.

In MS-MS mode, peptides identifying apolipoproteins ApoF, ApoL1, the HDL-associated protein, serum paraoxonase1 (PON1), and others were detected. Transitions for targeted peptides were programmed into the MS based upon these results. We also used the SRM Atlas (www.mrmatlas.org) to find peptide candidates for ApoD and ApoM. Plasma samples were analyzed in MRM mode and the targeted peptides were detected with sensitivity comparable to an ELISA assay. Affinity capture combined with MRM mode mass spectrometry allows high sensitivity analysis of multiple HDL-associated proteins.

(23) Characterization and Comparative Analysis of Proteomic Profiles of Leukemic and Primary Human NK Cells

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Natural killer (NK) cells detect and lyse tumor and virus infected cells. The morphology of NK leukemia cell line NKL resembles that of primary NK cells, and NKL are used as “model cell lines” in NK cell research. NKL cells and primary NK cells differ, however, substantially in the expression of cell surface receptors and intracellular signal transduction proteins. To obtain useful knowledge on the functional relevance of these two cell types, we characterize and compared the proteome of NKL cells and primary NK cells isolated from healthy donors using mass spectrometry-based proteomics. In this study, the method for protein extraction and 2D LC separation were optimized. Proteins extracted from primary NK cells and NKL cells were analyzed by Bruker amaZon ETD ion trap. Quantitative analysis will be conducted via spectral counting.

(24) A New High Throughput Microchromatography System for Protein Sample Prep

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Sample prep for LC-MS of proteins from complex matrices is a significant challenge. Generally, the protein(s) of interest must be selectively purified, proteolytic digestion performed and the resulting peptides concentrated and desalted. It has been a major challenge to develop a microliter-scale, multiplexed chromatography system which is simultaneously flexible, quantitative and high throughput.

This work describes the development of a high throughput micro-chromatography platform based on cartridges with a 5 µL bed packed with any 15 – 100 µm particles, including chromatography resins, immobilized antibodies and enzymes. The cartridges operate on a 96-channel liquid handler (Agilent Technologies) which incorporates 96 syringes that connect to the cartridges, providing positive-displacement flow control in either direction.

In addition to conventional resins, large particle, non-porous beads derivatized with either adsorptive surfaces (such as reversed-phase) or specific binding proteins (such as antibodies, streptavidin or protein A) were used. These cartridges have a binding capacity of only a few µg of target protein, which matches the sensitivity of LC-MS, but minimizes non-specific adsorption. Both binding and elution are extremely rapid and efficient.

Integrated automated workflows are being developed that include immunoaffinity isolation of trace target proteins, tryptic digestion and reversed-phase peptide cleanup.

(25) Towards an Understanding of Rbm20's Post-translational Modification State

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Rbm20 is a recently discovered splicing factor that affects the alternative splicing of the myofibrillar protein titin. Mutations in the protein have been linked to dilated cardiomyopathy in humans (Brauch et al J Am Coll Cardiol 54:530, 2009). Rbm20 contains an RNA recognition motif, an RS domain (rich in arginine-serine repeats), and two zinc finger-like domains. Many SR and SR-related proteins are post-translationally modified, most commonly through phosphorylation of serines in the RS domain. Our initial objective was to isolate a peptide or peptides from the 130 kDa Rbm20 protein that contained the full RS domain. Rbm20 expressed in E. coli was purified, digested with Glu-C, and analyzed using an LTQ/FT mass spectrometer. A peptide including the full RS domain was identified, and 68.4% coverage was obtained for the whole sequence. Work is in progress to analyze potential post-translational modifications in Rbm20 isolated from the heart.

(26) A High Yield Method for the Removal of Detergents from Low Concentration Protein or Peptide Samples for MS Analysis

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While mass spectrometry (MS) occupies a central role in high-throughput proteomic analysis, sample preparation ahead of MS analysis is pivotal to successful protein characterization. Sample complexity, interfering substances and large dynamic range of protein and peptide concentrations are major hurdles in the analysis of low abundance proteins. Detergents or surfactants play a significant role in protein chemistry, principally to solubilize and stabilize proteins and to disaggregate protein complexes. However, detergents are notoriously incompatible with MS analysis. Successful removal of detergents with good protein or peptide recovery especially for low abundant proteins is very critical for good MS analysis. We describe a simple, efficient and rapid method for the removal of detergents from samples with very low protein/peptide concentrations (0.1 mg/ml or less) with excellent protein recovery. The successfully removed >95% of nonionic, ionic, and zwitterionic detergents from 0.5-1% solutions with >90% recovery of protein or peptide. Tandem mass spectrometric analysis of 5-100 µg of BSA tryptic digests at 5-100 µg/ml prepared in the presence of detergents and processed to remove detergent revealed sequence coverage and MASCOT scores as good as control digested BSA samples processed without detergent. Additional data for other commonly used detergents and after detergent removal from Protein Discovery Gelfree 8100 fractions and cell lysate peptides is similarly promising. The method significantly removes detergents from samples containing low concentrations of proteins and peptides and eliminates the interference by detergents with the peptide mass spectral profile. The advantage of this method is the speed (less than 15 minutes) and >95% detergent removal from 5-100 µg protein or peptide samples at concentrations of >5 µg/ml samples before MS analysis.

(27) The Protein Partners of GTP Cyclohydrolase I in Rat Organs

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GTP cyclohydrolase I (GCH1) is the first and rate-limiting enzyme in tetrahydrobiopterin (BH4) biosynthesis. The normal BH4 biosynthesis is required for the degradation of phenylalanine, the biosynthesis of catecholamines, and the balance of nitric oxide and superoxide generation. GCH1 has been shown to be a promising therapeutic target in ischemic heart disease, hypertension, atherosclerosis and diabetes. However, the understanding of molecular mechanisms mediating the protective functions of GCH1 is limited and the endogenous GCH1-interacting partners have not yet been identified. In this study, we aimed to determine the endogenous GCH1-interacting proteins in rat and characterize the interaction in different rat organs by proteomics and western blot analysis. We demonstrated that GCH1 interacts with at least 17 proteins including GCH1 regulatory protein (GFRP) in rat liver by affinity purification followed by proteomics and validated 6 protein partners in liver, brain, heart and kidney by immunoblot. GCH1 interacts with GFRP and VLCAD in the liver, TB22A in the liver and brain, DNJA1 and ALDH in the liver, heart and kidney and EIF3I in all tissues. Our results show that GCH1 might be involved in fatty acid metabolism and protein biosynthesis, indicating that it has broader functions beyond BH4 biosynthesis. Additionally our results suggest that the regulation of GCH1 by its partners might be organ-dependant and EIF3I might be a general regulator of GCH1.

(28) Comparative Secreteome Proteomics of an ALS Cellular Model Using Human ESC-derived Astrocytes

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Amyotrophic lateral sclerosis (ALS) is a debilitating disease affecting motoneurons in the brain and spinal cord that control muscle movement. Astrocyte cells are critical contributors to the progression of this disease and may serve as potential targets for therapeutics. We have created a transgenic cellular model with human embryonic stem cell (hESC) derived astrocytes that express mutant (A4V, G85R) and wild-type (WT) superoxide dismutase 1 (SOD1). These astrocyte cells exhibit hallmarks of ALS cells including the mutants' ability to induce a selective toxic effect upon motoneurons in coculture and incubation with astrocyte conditioned medium (ACM). Here, we present results of a label free proteomics study of proteins secreted from mutant and WT hESC derived astrocytes to determine possible disease factors.

(29) Defining the Proteome by Deep sequencing Ribosome Protected mRNA Fragments Using Polysomes Isolated by Size Exclusion Chromatography.

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Gene expression profiling using RNA abundance measured either by micro arrays or by high-throughput sequencing reflects protein levels for transcripts that are not subjected to translation control. Isolation of polysomes and analysis of the associated mRNAs provide a better measure of translation rates to estimate the copies of the synthesized protein. Current polysome isolation methods rely on ultracentrifugation using either a sucrose gradient or a cushion. While the sucrose cushion avoids the need for more specialized gradient fractionation step, it still requires access to an ultracentrifuge and several hours of centrifugation. We have investigated size exclusion chromatography as an alternate to ultracentrifugation to isolate polysomes. As reported in the literature, we found polysomes in the void volume of commonly used size exclusion resins. The size-exclusion method is simpler and rapid and because of polysome exclusion it does not require any special equipment. We are investigating the use of size-excluded polysomes for ribosome profiling using the method of Ingolia et al [Science 324, 218-223 (2009)] to monitor the protein production in cells and define the proteome.

(30) Advancing Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry Imaging (MALDI-MSI) for Capillary Electrophoresis (CE) Analysis of Peptides

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Compared to on-line CE-MS, off-line coupling of CE with MALDI-MS offers an alternative with increased flexibility for the independent optimization of CE and MS experiments, and makes CE fractions available for further reanalysis. However, the most widely used CE-MALDI-MS interface -- discrete spotting method, sacrifices chromatographic resolution caused by the time intervals between fractions. Herein, we describe a continuous deposition approach by direct contact of a capillary tip with a MALDI target. MALDI-MS imaging (MALDI-MSI) was then conducted along the deposited CE track, and electropherogram can be generated with high temporal resolution dependent on the step size of imaging. This novel combination of CE and MSI thereby holds great promise for effective and automatic separation and quantitation of peptides.

(31) Mass spectrometric imaging of brain proteins following neonatal exposure to an NMDA antagonist and ethanol

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Compounds which inhibit glutamate N-methyl-D-asparate receptors (NMDA) or activate ·-aminobutyric acid ($GABA_A$) receptors and cause excitation/inhibition imbalance in the brain, can induce widespread neuroapoptosis, impair neurogenesis in the developing rodent brain and cause impairment of learning and memory. Ethanol acts by these mechanisms and is therefore neurotoxic for the developing brain. In this study we used imaging mass spectrometry (IMS) to study changes in peptide expression in the infant and adolescent rat brain following neonatal exposure to the NMDA antagonist MK801 or the combined NMDA antagonist and $GABA_A$ agonist ethanol. Rats were injected with MK801, 1mg/kg, or ethanol, 3g/kg, on P2 and P4 and were euthanized on P10 or P21. Analysis of rat brains treated in infancy with the NMDA antagonist MK801 or ethanol and comparison of MALDI images reveals differential distribution of several putative peptide signals. The findings indicate that a brief neonatal exposure to compounds that alter excitatory/inhibitory neurotransmission in the brain has long term impact on neuropeptide patterns in the brain.

(32) *De Novo* Sequencing of Swine Cardiac Troponin T (>35 kDa) by Top-Down Mass Spectrometry

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Swine hearts are highly valuable for cardiovascular research because of their similarity to human hearts. The post-translational modifications (PTMs) and alternative splicing of cardiac troponin T (cTnT) may represent important regulatory mechanisms of cardiac contractility. However, the sequence of swine cTnT is not available in public databases. Due to its large size (>35 kDa) and complex modifications, *de novo* sequencing of swine cTnT is extremely challenging. We have explored the ability of top-down high-resolution MS/MS in *de novo* sequencing of swine cTnT. The largest peptide has been sequenced by top-down MS/MS is 13.6 kDa to date. Electron capture dissociation (ECD), a unique fragmentation method, generates fragment-rich tandem mass spectrometry (MS/MS) data and preserves labile PTMs benefiting from its non-ergodic nature. Therefore, ECD is especially useful for *de novo* sequencing of proteins/peptides with complex PTMs in addition to other fragmentation techniques such as collisionally activated dissociation (CAD). Herein, based on investigations of the accurate mass, ECD and CAD fragmentation, in conjunction with homology alignment, more than 92% of the amino acid sequence of swine cTnT was identified. Modifications such as acetylation and phosphorylation site were located to Ser1.

(33) Monolithic Based Immobilized-pH Gradient Capillary Isoelectric Focusing and Monolithic Liquid Chromatography for Enhanced Neuropeptide Analysis

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The study of neuropeptides with mass spectrometry has been attractive but challenging due to the chemical complexity and low abundance of these signaling molecules. A number of microseparation techniques such as LC and CE have been employed and coupled to MS for improved analytical coverage. The monolithic materials have attracted increased attention since their integration into typical column format as novel separation media for protein and peptide analysis. Here we develop monolithic material based columns for improved separation efficiency with reduced time before mass spectrometry analysis. Acrylate-based monolithic immobilized-pH gradient capillary isoelectric focusing (IPG-CIEF) and monolithic LC column were fabricated and coupled with mass spectrometers for the analysis of tryptic digest peptides and neuropeptides extracted from a crustacean neuroendocrine organ.

(34) Mass Spectrometric Analysis of DNA-Bound Proteins in the UAS/$_{GAL}$ Region Using Novel GENECAPP Technology.

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Numerous proteins mediate DNA stability, control its activity, and regulate transcription of the encoded genetic information. To overcome this challenge, the Wisconsin CEGS developed **G**lobal **E**xo**N**uclease-based **E**nrichment of **C**hromatin-**A**ssociated **P**roteins for **P**roteomics (GENECAPP). While Chromatin Immunoprecipitation (ChIP) characterizes DNA interacting with individual known proteins, GENECAPP, utilizes oligonucleotide capture to isolate targeted DNA regions in a sequence-specific manner and analyzes captured DNA-associated proteins using mass spectrometry.

We utilize GENECAPP in *S. cerevisiae* to study the Gal upstream activator sequence (UAS/$_{GAL}$). Nuclear extracts were cross-linked and captured by hybridization after exonuclease digestion. After trypsin digestion, peptides characteristic of bound proteins, were identified by tandem mass spectrometry using an Orbitrap Velos instrument. We have been able to detect specific DNA-associated proteins from less than 1 fmol of captured and eluted DNA; thus showcasing the power and capability of high resolution mass spectrometry to identify DNA-associated proteins from a complex matrix.