Mass Spectrometry Imaging for Anatomic Pathology and Oncology

*Nathalie Y. R. Agar, Ph.D.*

Mass spectrometry provides multiple options for the direct characterization of tissue to support surgical decision-making, and provides significant insight in the development of drugs targeting tumors of the central nervous system (CNS). Using an array of mass spectrometry (MS) applications, we rapidly analyze specific tumor markers ranging from small metabolites to proteins from surgical tissue for rapid diagnosis and surgical guidance. Using similar clinical protocols, we visualize drug and metabolites penetration in brain tumor tissue and correlate with tumor heterogeneity and response to support drug development.

Quantitative Proteomics for Epigenetic Cancer Mechanisms

*Benjamin A. Garcia, Ph.D.*

Mass spectrometry has become a powerful tool for characterization of thousands of proteins from any cellular source. Here in this presentation, I will highlight advances in mass spectrometry based methodology to detect post-translational modifications on histone proteins involved in epigenetic regulation of gene expression, especially during cancer pathogenesis. We will specifically describe high-throughput comparison of proteins from multiple cellular states (ii) mass spectrometry methods for quantitative tracking of combinatorial modifications and (iii) monitoring in vivo post-translational modification dynamics These studies in combination with biological experiments will help provide a systems biology outlook on gene expression that will lay down the basic scientific foundation to advance several applications in human health.

Expanding our View of the Surfaceome: New Bioinformatic Tools and Technologies for Mapping Glycoproteins from Small Sample Sizes and Human Primary Cells

*Rebekah L. Gundry, Ph.D.*

Cell surface proteins, glycoproteins, and glycans play critical roles in maintaining cellular structure and adhesion, and act as gatekeepers controlling how cells send and receive exogenous signals. Therefore, the collection of these molecules (i.e. surfaceome) is a rich source of accessible targets for developing new tools and strategies to identify, study, and manipulate specific cell types of interest, from immunophenotyping to immunotherapy. Classic Cell Surface Capture Technology (CSC) is a chemoproteomic approach that enables selective enrichment and identification of extracellular domains of cell surface N-glycoproteins. Since 2007, CSC has been applied to identify >3000 cell surface N-glycoproteins from >125 human and rodent cell types, providing unique surfaceome views in a cell-type specific manner to reveal new immunophenotyping markers and proteins involved in development and disease. However, while the classic CSC approach is highly specific for cell surface N-glycoproteins, the method
requires >80 million cells on average to produce high quality results, precluding its application to rare cell types. In this study, we developed new bioinformatic and technological approaches to address the need for surfaceome analyses of small numbers of cells, including human primary cells. Our new bioinformatic approach combines predictive and empirical evidence to efficiently prioritize the selection of cell type-specific proteins. Our new technological approach implements an automated liquid handling workstation for CSC sample processing, which minimizes human intervention and decreases processing time from 5 days to 50 hours. This new μCSC method successfully identifies >500 cell surface proteins from just 5-10 million cells with >90% specificity. We show the utility of these new bioinformatic and technological developments for discovering surface markers on primary isolated human cardiomyocytes and blood cell types.

Proteomics of Right Versus Left Colon Cancer

Amanda Hummon, Ph.D.

The use of short-term fasting is a potential cancer treatment that could be used in tandem with current cancer regimes to increase their potency. The Hummon lab has determined that simultaneous short-term glucose starvation and treatment with a common antimalarial drug, right and left colon cancer cells can be sensitized to chemotherapy treatment. We have demonstrated with colon cancer cells that tumor suppressor proteins are increased in their abundance after treatment with the FDA-approved malaria drug, chloroquine, and short-term glucose starvation for 72 hours. This treatment rendered the cancer cells more susceptible to chemotherapy. The hypothesis of this treatment is that cancer cells are particularly dependent on anaerobic respiration and use glucose for metabolism. When glucose is removed as a food source, the cells respond by triggering a survival process called autophagy. The antimalarial drug chloroquine blocks activation of autophagy and thus makes the cancer cells extremely vulnerable to external stressors, like glucose reduction and chemotherapy. We demonstrated that this two-pronged approach of glucose deprivation combined with autophagy inhibition was extremely effective at sensitizing colon cancer cells to treatments. The next step is to test the approach in mice, to determine whether normal cells will respond to the nutrient stress/autophagy inhibition or whether they will be unaffected. Should this multi-pronged approach increase chemotherapeutic efficacy in mice, then we will proceed with clinical trials involving glucose restriction and autophagy inhibition prior to chemotherapy.

Application of Ultrafast Database Searching in Proteomics

Alexey Nesvizhskii, Ph.D.

We present a comprehensive suite of tools assisting with application of the new search engine MSFragger in a wide range of data analysis scenarios. As an ultrafast tool, MSFragger is valuable when conventional tools are too slow, e.g. for identification of endogenous peptides that requires non-specific enzyme searches. It is particularly useful for open (wide mass tolerance) searching which has become an effective strategy to look for post-translational modifications (PTMs) or, more generally, some recurring structural differences in observed molecules. By integrating multiple computational components, our pipeline facilitates the entire process from searching raw data, validation of the search results, visualization of observed mass shifts and subsequent PTM annotation to normalized TMT quantification of multiple data sets. We demonstrate the utility of our tools using several representative datasets.
**Post-Translationally Modified Proteins in Extracellular Vesicles as Biomarkers for Breast Cancer**  
*W. Andy Tao, Ph.D.*

The state of protein modification can be a key determinant of cellular physiology such as early stage cancer. Here we demonstrate, for the first time, a strategy to isolate and identify glyco- and phospho-proteins in extracellular vesicles (EVs) from human plasma as potential markers to differentiate disease from healthy states. We identified thousands of peptides with post-translational modifications (PTMs) in EVs isolated from small volumes of plasma samples. Using label-free quantitative proteomics, we identified proteins with PTMs in plasma EVs that are significantly higher in patients diagnosed with breast cancer as compared to healthy controls. Several novel biomarkers were validated in individual patients using Paralleled Reaction Monitoring for targeted quantitation. This study demonstrates that the development of PTMs in plasma EV as disease biomarkers is highly feasible and may transform cancer screening and monitoring.

**Native Mass Spectrometry: A Structural Biology Tool**  
*Vicki Wysocki, Ph.D.*

Characterization of the overall topology and inter-subunit contacts of protein complexes, and their assembly/disassembly and unfolding pathways, is critical because protein complexes regulate key biological processes, including processes important in understanding and controlling disease. Conventional structural biology methods such as X-ray crystallography and nuclear magnetic resonance provide high-resolution information on the structures of protein complexes. However, other emerging biophysical methods that provide structural data (e.g. stoichiometry and subunit connectivity) on protein complexes are also important. Native mass spectrometry is an approach that provides critical structural information with higher throughput on low sample amounts. The power of native MS increases when coupled to ion mobility (IM-MS), a technique that measures rotationally averaged collisional cross sections and thus direct information on conformational changes, or to high resolution mass spectrometry (HRMS). This presentation illustrates surface-induced dissociation/ion mobility SID/IM MS and SID HRMS for characterization of topology, intersubunit connectivity, and other structural features of multimeric protein complexes. Data for a number of protein-partner complexes are under investigation, where the partner can be small molecule ligand, protein, DNA, or RNA.

**Understanding the Molecular Defect in Cystic Fibrosis**  
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A component to understanding biological processes involves identifying the proteins expressed in cells as well as their modifications and the dynamics of processes. Several major technologies, but especially mass spectrometry, have benefited from large-scale genome sequencing of organisms. The sequence data produced by these efforts can be used to interpret mass spectrometry data of proteins and thus enables rapid and large-scale analysis of protein data from experiments. Advances in multi-dimensional separations as well as mass spectrometry have improved the scale of experiments for protein identification. This has improved the analysis of protein complexes, and more complicated protein mixtures.
Quantitative mass spectrometry can be used to study biological processes such as protein-protein interactions, development or the effects of gene mutations on pathways. Recent studies on the loss of function mutant form of the Cystic Fibrosis Transport Regulator (F508) as it progresses through the folding pathway will be presented. Through the study of protein-protein interactions and modifications that regulate maturation of CFTR, we are beginning to understand the critical interactions regulating pathways for export or destruction1.


This work was supported by National Institutes of Health grants 5R01HL079442-08 (to J.R.Y. and W.E.B.), P01AG031097 (to J.R.Y.), P41 GM103533 (to J.R.Y.), HHSN268201000035C (to J.R.Y.), and a Cystic Fibrosis Foundation mass spectrometry fellowship BALCH050X6 (to S.P. and J.R.Y.). M.L.-A. holds a postdoctoral fellowship from Fonds de recherche du Québec - Nature et technologies

RISING STAR SESSION ABSTRACTS

Towards Expanding Human Proteoform Analysis by Integrating Intact Mass Measurements with Nucleic Acid Sequencing and Bottom-up Proteomic Analysis

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Proteoforms are proteins with posttranslational modifications (PTMs) at specific positions along the amino acid sequence. These molecules perform myriad biological functions in the cell, and thus measuring intact proteoforms gives meaningful biological insights. I will present our results of expanding proteoform identifications in C2C12 mouse cell mitochondria and point to our current thrusts of research to allow such proteoform analysis in human samples. We have developed software programs named MetaMorpheus and Proteoform Suite to facilitate analyzing these top-down mass spectrometry (MS) proteomic measurements, focusing particularly on information gained from using only measured intact proteoform masses. We use global post-translational modification discovery (G-PTM-D) in the program MetaMorpheus to discover PTM sites, which are added to a protein database. We also are developing a new program Spritz that will analyze nucleic acid sequencing data to find protein sequence variations and enter those into the protein database. We use this custom protein database in Proteoform Suite to identify proteoform observations using measurements of intact mass, quantify them using measured intensities, and compare abundances between conditions. This method of identifying proteoforms increases the number of identifications by 40% over top-down fragmentation MS analysis alone. I will present the results of a study in which we analyzed myoblast and myotube differentiation in the C2C12 mouse cell line, in which Proteoform Suite reported 93 proteoforms with statistically significant changes1. These results show familiar biological changes that validate our quantification approach in a mammalian system, such as an increase in proteoforms involved with oxidative phosphorylation in myotubes.

1Leah V. Schaffer; Michael R. Shortreed; Anthony J. Cesnik; Jarred W. Rensvold; Adam Jochem; Mark Scalf; Brian L. Frey; Stefan K. Sointsev; David J. Pagliarini; Lloyd M. Smith. “Expanded Identification and Quantification of Unlabeled Mouse Mitochondrial Proteoforms in Proteoform Suite,” submitted.
The Role of Acyl-CoA Short Chain Synthetase 2 and Acetyl-CoA in Regulation of Chromatin Modifications and Gene Expression

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Post-translational modifications of proteins regulate many complex biological processes, including genome expression, chromatin dynamics, metabolism, and cell division. Reversible phosphorylation was the first modification characterized to have widespread regulatory effects and more recently, many other post-translational modifications, including protein acetylation have been identified. Protein acetylation was first characterized on histone proteins and has been well-studied in the regulation of gene expression and chromatin dynamics. Advancements in mass spectrometry-based proteomics have led the widespread identification of protein acetylation and demonstrated protein acetylation extends in the nucleus beyond histones. Chromatin modifying enzymes have been shown to utilize a variety of metabolites as co-substrates to catalyze their respective chromatin modifications, closely linking chromatin modifications, such as acetylation, and metabolism. In this study, we investigated the role of acyl-CoA short chain synthetase family member 2 (ACSS2), in the regulation of histone and chromatin protein post-translational modifications and gene expression. We reveal that under dietary stress, ACSS2 is required for changes in histone acetylation at H3K18 in the brain and transcriptional changes in the brain and liver. To investigate nuclear-specific regulation of non-histone protein acetylation, we have developed an approach using data-independent acquisition mass spectrometry in conjunction with subcellular fractionation resulting in considerably deeper coverage of the acetyl stoichiometry of nuclear protein acetylation. In the case of serum stimulation in MCF7 cells, using subcellular fractionation increased nuclear acetylation sites with quantified stoichiometry by 30 percent. Utilizing this method, we are currently investigating the role of ACSS2 in the regulation of chromatin protein acetylation.

Global Glycoproteome Analysis Reveals Site-Specific Glycan Heterogeneity

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Protein glycosylation is a highly important, yet poorly understood protein post-translational modification. Hundreds of possible glycan structures and compositions create potential for tremendous site heterogeneity and analytical challenge. Intact glycopeptide analysis can reveal this site-specific glycan microheterogeneity (i.e., several glycans modifying the same site), but large-scale analyses of intact glycopeptides have been notoriously handicapped by lack of suitable methods. This has ultimately limited our abilities to both address the degree of heterogeneity across the glycoproteome and to understand how it contributes biologically to complex systems. Here we show that glycoproteome site-specific microheterogeneity can be captured at a global level via glycopeptide profiling with activated ion electron transfer dissociation (AI-ETD), which combines simultaneous vibrational activation from IR photon bombardment and electron-driven dissociation via ETD. This approach is particularly well-suited
for intact glycopeptide fragmentation because it concomitantly capitalizes on two complementary modes of fragmentation in a single MS/MS event. Al-ETD provides information about both peptide and glycan components of intact glycopeptides and it enabled characterization of nearly 2,100 N-glycosites (> 7,500 unique N-glycopeptides) from mouse brain tissue. Moreover, we use this unprecedented scale of glycoproteomic data to develop several new visualizations that will prove useful for analyzing intact glycopeptides in future studies. Our data reveal that glycosylation profiles can differ between subcellular regions and structural domains and that glycosite heterogeneity manifests in several different forms, including dramatic differences in glycosites on the same protein. Furthermore, we are now using this technology to investigate differences in glycosylation expression in several MCF10A cancer cell lines that include known oncogenes (KRAS, HER2, MEK, BRAF, EGFR, and AKT).

Deciphering the Human Heart Proteome in Cardiac Disease and Regeneration by Top-down Proteomics

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Heart diseases remain the leading cause of death in developed countries for both men and women. Altered post-translational modifications (PTMs) and variations in amino acid sequence for many cardiac proteins have been implicated as causative factors for cardiovascular diseases. Nevertheless, the disease mechanisms are highly heterogeneous and poorly understood. To begin to understand the molecular biology underlying human heart function and dysfunction, we must obtain a global qualitative and quantitative view of the combinatorial PTM-amino acid sequence variant landscape. Mass spectrometry (MS)-based top-down proteomics (TDP) is the most powerful technology for deciphering PTM codes together with amino acid sequence variations, providing essential insight into the structure and function of proteoforms, the effectors of all biological processes. Herein, we seek to develop and implement novel TDP technologies to characterize the human heart at the proteoform level, qualitatively and quantitatively, to deepen our knowledge of the human heart proteome. We have identified previously unknown phosphoproteins in the cardiac sarcomere using our lab’s TDP technology. Using label-free quantitative top-down proteomics, we have unveiled a reversal of deleterious PTMs following cardiac injury and treatment with an induced pluripotent stem cell (iPSC) – derived patch. Additionally, a novel quantitative proteomics platform developed in our lab has allowed for label-free quantification of protein expression levels of isoforms for assessing maturation of iPSC-derived cardiomyocytes. Further developments of novel qualitative and quantitative TDP platforms will enable an increased understanding of heart disease and regeneration.

References


A Multiplexed Absolute Quantification Strategy for Candidate Biomarker Verification in Alzheimer’s Disease

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Absolute quantification in targeted proteomics is challenging due to a variety of factors, including low sensitivity and specificity in complex backgrounds, limited analytical throughput, and wide dynamic range. To address these problems, we developed a hybrid offset-triggered multiplex absolute quantification (HOTMAQ) strategy that combines cost-effective mass difference and isobaric tags to enable simultaneous construction of an internal standard curve, real-time identification of peptides, and mass offset-triggered unambiguous quantification of target proteins in up to twelve samples via a single experiment. As proof-of-concept demonstration, HOTMAQ was successfully employed to verify candidate protein biomarkers in preclinical Alzheimer’s disease with excellent sensitivity and accuracy. HOTMAQ method ideally bridges the gap between the discovery and verification phases for candidate biomarkers from large cohorts of clinical specimens. The utility of this new strategy goes beyond biomarker verification; as its greatly enhanced throughput and quantitative performance, paired with sample flexibility, gives it potential to find widespread applications in targeted peptidomics, proteomics, and phosphoproteomics.