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Speaker Abstracts

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INVITED ABSTRACTS

Advancing biomedical research via innovation in mass spectrometry-based approaches

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Comprehensive characterization of all signaling molecules in a biological system with chemical, spatial and temporal information is often critical to deciphering their functional roles, yet it poses a daunting challenge. In this presentation, I will present our recent progress on the development of a multi-faceted mass spectrometry (MS)-based analytical platform to probe neuronal signaling with enhanced sensitivity and selectivity. By combining chemical labeling, micro-scale separation, and tandem MS sequencing techniques, we discovered more than 300 novel neuropeptides in several model organisms. Moreover, both mass spectrometric imaging (MSI) technology and in vivo microdialysis sampling tools have been developed and implemented to follow neuropeptide distribution and secretion with unprecedented details. To further enhance the chemical information extracted from in situ MALDI MSI experiments, we report on a multiplex-MSI method, which combines high-resolution accurate mass (HRAM) MSI technology with data dependent acquisition (DDA) tandem MS analysis in a single experiment. Additionally, several in situ chemical derivatization strategies have been developed to enable spatial mapping of various biomolecules including lipids and glycans in complex biological samples, such as human cell lines and cancer tissue samples.

Furthermore, we are developing multiplexed isobaric and isotopic tagging strategies to discover, identify and evaluate candidate biomarkers of Alzheimer's disease (AD) in cerebrospinal fluids (CSFs) obtained from asymptomatic cognitively-healthy middle-aged adults, older cognitively-normal adults, and patients with mild cognitive impairment (MCI) and AD. A large-scale comparative glycoproteomic analysis via the 12-plex DiLeu (N,N-dimethyl leucine) tagging strategy revealed distinct glycosylation patterns and dynamic changes of certain glycoforms in CSF samples collected from the control, MCI, and AD patients. Additionally, we report on a multiplexed quantitation method for simultaneous proteomics and amine metabolomics analyses via nanoflow reversed phase LC-MS/MS, exploiting mass defect-based DiLeu (mdDiLeu) labeling. Several on-going efforts and future perspectives provided by these enabling technologies will be highlighted and discussed.

Machine Learning Methods for Proteomics

Brian Searle, PhD
The Ohio State University

Shotgun proteomics using mass spectrometry is enabling a revolution in the study of large-scale systems biology. Data-independent acquisition (DIA) is a mass spectrometry technique that regularly samples co-fragmented ions produced from multiple peptides falling within a specified mass range. While comprehensive, this approach results in highly complex mass spectra requiring the reconstruction of which fragment ions were produced from which peptides. Here we discuss machine learning methods to make those assignments and assess our confidence in that data.

Mass spectrometry based approaches for interrogation of epitranscriptomic RNA modifications

Benjamin Garcia, PhD
Washington University in St. Louis

Post-transcriptional modifications of RNA are associated with fundamental biological processes such as RNA splicing, translation, and degradation, as well as human physiology and disease such as cancer or viral infection. Over 140 modifications have been identified across species and RNA types, with the highest density and diversity of modifications found in tRNA. Nevertheless, the analysis of ribonucleoside modifications is hampered by the hydrophilicity of the ribonucleoside molecules, and difficulty to fully characterize and quantify them by mass spectrometry (MS) based methods. To improve both mononucleoside and oligonucleotide analyses, we have developed a variety of chemical, sample prep, MS and chromatography and computational advances. We have adapted chemical derivatization approaches to improve the retention of RNA mononucleosides on C18 based columns and enhance quantification. Additionally, we have developed data independent acquisition (DIA) methodology which has allowed us to quantify over 70 RNA modification types. For oligonucleotide analysis, we have combined two orthogonal modes of RNA ion separation before MS quantification: high-field asymmetric ion mobility separation (FAIMS) and electrochemically modulated liquid chromatography (EMLC). FAIMS RNA MS increases the depth of coverage and throughput, while the EMLC LC-MS orthogonally separates RNA of different length and charge. These two methods combined offer a broadly applicable platform to improve length and depth of MS-based RNA sequencing while providing contextual access to the analysis of RNA modifications. Overall, these approaches will significantly improve the characterization of RNA moelcules by MS.

Harnessing the power of microextraction in metabolomics for targeted and untargeted characterization of complex systems

Emanuela Gionfriddo, PhD
University of Toledo

The separation of small molecules from complex samples often poses the ultimate challenge to any analytical method development process, whether for targeted or non-targeted analysis. The complexity of matrices that can be used for metabolomics studies combined with the considerable chemical diversity of the metabolites that constitute the metabolome, create a significant need for the development of optimized workflows that guarantee reproducible and reliable analytical determinations. Microextraction methodologies have demonstrated their usefulness in the analysis of a broad range of metabolites in a variety of samples. This talk will discuss the use of microextraction for targeted and untargeted metabolomics and its suitability for in vivo and nondestructive sampling in order to capture a representative snapshot of the metabolome. The use of biocompatible extraction phases and alternative SPME geometries will also be discussed to address specific analytical needs and guarantee minimal disturbance of partition equilibria during the extraction process.

NMR-based stable isotope resolved metabolomics from cells to organisms

Andrew Lane, PhD
University of Kentucky

Stable Isotope Resolved Metabolomics (SIRM) is an approach that determines isotopomer and isotopologue distributions downstream of an enriched precursor administered to the cells, tissues or organisms. This enables reconstruction of pathway utilization and response to external perturbations, and provides the raw information and data for flux analyses. Despite its relatively modest sensitivity, NMR has several desirable capabilities including the ability to determine structures and conformations, atomic level details of isotope labeling, isotope editing and accurate quantification in unfractionated mixtures. New developments in chemoselection, multiplexed isotope labeling and isotope editing schemes will be presented, in the context of cancer biochemistry in cells, organoids, tissues and organisms.

3D metabolomics-enabled insights into infectious disease pathogenesis and drug development

Laura-Isobel McCall, PhD
University of Oklahoma

Infectious diseases are structured spatially: infected cells adjoin uninfected cells, symptoms develop in specific tissue sites or organs, and following pathogen clearance, some tissue sites recover while others show poor resilience. Metabolism is at the core of these processes, so spatial metabolomics approaches are necessary. My group combines liquid chromatography-mass spectrometry-based metabolomics with 3D modeling and microbiology tools, to define local sites of metabolic perturbation during infection. Results are helping us understand why symptoms develop where they do, and mechanisms of treatment failure. Affected pathways can also be targeted for drug development, leading to therapeutic candidates with novel mechanisms of action. Here, I will showcase how my group has applied 3D metabolomics to address these questions in the context of parasitic infection, as well as the broader applicability of this approach.

The periodic table of food initiative: First steps towards development of a democratized foodomics technology platform

Jessica Prenni, PhD
Colorado State University

Food is at the center of the world's most urgent challenges, however, our scientific understanding of the biochemical composition of food is still rudimentary. The Periodic Table of Food Initiative (PTFI) is a global participatory effort to create a composition reference database of the food we eat. Key to this effort is the development of standardized, fully democratized technology platforms for generating food composition data that will support the growth of a reference database now and into the future. As a first step, we performed a baseline experiment to evaluate the variability of analytical platforms, methods, and data analysis pipelines across multiple established metabolomics laboratories. The results revealed that while data were highly reproducible within laboratories the results were not comparable between laboratories because there was little overlap of analyte identifications. As an example, a total of 927 named compounds were returned by three laboratories from the analysis of an identical apple sample, however, only 14 of these compounds were reported by all three laboratories. The results

demonstrate (perhaps not surprisingly) that while the analytical technology is highly developed there is significant variation between laboratories, especially within the data pipeline and annotation tools. This presentation will introduce the PTFI strategy for addressing this challenge using standardized reagents and a unified cloud-based data processing pipeline. Ultimately, the PTFI toolbox will enable acquisition of next generation food composition data in any laboratory across globe that is both publicly accessible and fully comparable.

Leveraging LC-MS metabolomics to elucidate dietary bioactive interactions

Rachel Kopec, PhD
The Ohio State University

The effects of essential nutrients and dietary bioactive compounds, including drugs, are still largely studied in silos. However, interactions among these compounds arise during food processing, mixed meal consumption and digestion, and post-absorption within tissues. The Kopec laboratory employs targeted and untargeted liquid chromatography-mass spectrometry (LC-MS)-based metabolomics to elucidate these interactions, with special emphasis on those occurring in the gastrointestinal tract and in the brain. In particular, the Kopec group focuses on interactions arising between fat soluble vitamins (A, D, E, and K), long-chain omega-3 fatty acids, carotenoids, chlorophylls, iron, solid tumor chemotherapy drugs, and other dietary and tissue-containing lipids. Her group also employs lipidomics/metabolomics (LC-HRMS) techniques for unknown metabolite identification. In an effort to “make more meaning” from the highly dimensional omics data she collects, she is also collaborating with NMR spectroscopists, microbiome scientists, and bioinformaticians. Ultimately, better understanding of the role these bioactives play at multiple levels of biology is essential to establish evidence for “precision” nutrition recommendations.

SPONSORED ABSTRACTS (LUNCH AND LEARN)

Collision induced unfolding: Rapid, sensitive, information rich stability measurements (Sponsored by Agilent)

Brandon Ruotolo, PhD

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The next generation of medicines will rely heavily upon our ability to quickly assess the structures and stabilities of complex macromolecular machines, as well as the influence of large libraries of conformationally-selective small molecule binders and protein-based biotherapeutics. Such endeavours are nearly insurmountable with current tools. In this presentation, I discuss recent developments surrounding collision induced unfolding (CIU) methods that aim to bridge this technology gap. CIU uses ion mobility-mass spectrometry (IM-MS) to measure the stability and unfolding pathways of gas-phase proteins, without the need for covalent labels or tagging, and consuming 10-100 times less sample than almost any other label-free technology. Recent developments in high-throughput CIU screening methods, their ability to track alterations in biomolecular structure as a function of stress, and software developments that seek to enhance CIU information content will be discussed. Furthermore, our efforts that have led to the development of the Agilent 6560C drift tube IM-MS platform will be discussed, focusing on the performance of this platform for applications associated with native IM-MS and CIU.

Expanding the Horizon! Analysis of Various Compounds by UPLC/MS/MS Detection Using a UniSpray Ion Source
(Sponsored by Waters)

Stuart Oehrle, PhD

Waters Corporation

Liquid Chromatography/Tandem-Mass Spectrometry (LC/MS/MS) is a powerful tool for the analysis of various analytes in a wide variety of matrices. What is especially attractive about LC/MS/MS is its sensitivity and selectivity. Microcystins, in particular, represent an emerging class of algal toxins of concern to the drinking water industry. Recognizing the potential health risk, the World Health Organization, and other nations throughout the world have established guidelines for the amount of microcystins permissible in drinking water.

Typically, electrospray ionization (ESI) is the mode of ionization used for these compounds. In this paper we investigate the use of a new and novel ion source (called UniSpray) which allows for enhanced sensitivity of these compounds (as well as a variety of other compounds). This source allows for greater ionization efficiency as well as providing a rugged interface. Comparison of algal toxins in various matrices to traditional ESI and Unispray ionization will be shown. Data from various toxin blooms in the United States will be shown and discussed using both techniques. In addition, examples of other compounds such as PFAS, herbicides, steroids, and peptide digest of COVID will be shown and discussed.

4D mass spectrometry for metabolomics and food analysis
(Sponsored by Bruker)

Artem Filipenko, PhD, and Savannah Snyder, PhD
Bruker

We will introduce an innovative mass spectrometer platform and corresponding workflows for targeted and untargeted analysis. The timsTOF MS incorporates an additional dimension of separation by ion mobility for enhanced analytical sensitivity, speed, and identification confidence. Advancements in data analysis solutions facilitate simplified unknown profiling and statistical analysis, as well as streamlined transfer to sensitive targeted and screening workflows. High resolution ion mobility provided by the timsTOF is well suited for separating and identifying co-eluting isomeric and isobaric lipids. The instrument's speed and sensitivity increase the breadth and quality of the data – and with this the total number of compounds that can be annotated and quantified. The instrument is supported by standard acquisition and analysis methods to ensure time-to-results is minimized. We will also explore application for food analysis and the synergies between targeted and untargeted workflows. The use of ion mobility can simplify complex analytical challenges and reduce analysis time. New developments provide the sensitivity of quadrupole systems while retaining the benefits of high-resolution data and retrospective analysis delivered by accurate mass instruments. Integrated solutions for both unknown and known data analysis provide a straightforward solution for transferring methods from a discovery to a production/QC environment.

Initial Impressions of Electron Activated Dissociation on a New QTOF Platform
(Sponsored by SCIEX)

Benjamin Garcia, PhD
University of Washington in St. Louis

Post-translational modifications (PTMs) of proteins are important biological modules of regulation that influence structure and function in a variety of biological processes. PTMs are vital to many cellular events, including transcription, intercellular signaling and cytoskeletal movement, just to name a few. Mass spectrometry (MS) has played a crucial role in characterization of protein PTMs over many decades. Nonetheless, many PTMs remain challenging to detect and quantify, due to being labile in the gas phase, or lost in sample preparation. Here we present a first glimpse from my lab utilizing the new Sciex ZenoTOF 7600 system for the analysis of highly modified proteins. Examples using glycoproteins and the combinatorially modified histone proteins will be presented. Results from Bottom Up and Top Down approaches demonstrate the unique capabilities of this new instrument to impact investigations on highly modified proteins and proteomes.

An automated sample preparation solution for mass spectrometry-based proteomics (Sponsored by Thermo Fisher)

Maowei Dou

Thermo Fisher Scientific

The proteomics field lacks standardized reagents and methods for sample preparation. Current methods are tedious, time-consuming, highly variable, unsuitable for processing large numbers of samples, and tend to negatively affect the robustness of the LC-MS instrumentation. Therefore, we developed an automated sample preparation solution that simplifies and standardizes mass spectrometry (MS) sample preparation. The new automated sample preparation platform offers integrated proteomics workflow solutions from designing experiments to managing the entire sample preparation process delivering high-quality samples ready for LC-MS analysis.

With factory-supplied reagents and kits and step-by-step on-screen instructions that include both label-free and Tandem Mass Tag multiplexing strategies (TMT™ tags), the platform makes short work of complex tasks with online protein digestion, TMT labeling, pooling, clean-up, UV peptide measurement, and integrated solvent dilution capabilities.

The automated system can process up to 36 samples containing 10-100 µg of initial protein within 4-6 hours. Reagents have been optimized to prepare cells, tissue, biofluids, and purified proteins for high-quality data acquisition. The final peptides have minimum missed peptide cleavage of less than 10%, complete cysteine reduction/alkylation (>99%), and most importantly, can be readily analyzed by LC-MS with a known concentration. The sample preparation process is very robust and reproducible. Less than 10% CVs in terms of peptide/protein identifications and quantification between sample replicates from 3 different users with 3 different systems were achieved. Combined with Thermo Scientific™ TMT™ 11plex or TMTpro™ 16plex isobaric multiplex labeling approaches, the platform maximizes laboratory productivity with high-quality samples. High labeling efficiency of >99% and high quantification precision and accuracy with less than 10% CV were obtained.

Designed with a unique experimental design experience, the automated platform is optimized to fit into the Thermo Scientific™ Orbitrap™ MS ecosystem delivering confident results with speed, accuracy, and high reproducibility, allowing researchers to spend more time on more valuable tasks.

ORAL ABSTRACTS (submitted abstracts)

Assessing Poly(styrene sulfonic acid) as A Noncovalent Protein Probe for Characterizing Locations of Surface Accessible Basic Residues on Folded Proteins

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Protein footprinting using a selective noncovalent adducting protein probe (SNAPP) for ESI-MS analysis is useful in determining stoichiometric information of basic residues; the Wesdemiotis group has recently been investigating the use of anionic polyelectrolytes for SNAPP due to their increased binding affinities. The presented work aims to develop multidimensional mass spectrometry methods that demonstrate the benefits of this polyelectrolyte on proteins with molecular masses ranging from 2-20 kDa. Five proteins have been investigated so far with varying sizes, structures, and isoelectric points: Melittin (2.8 kDa, pI ~12.1), Glucagon (3.4 kDa, pI ~6.8), Epidermal Growth Factor (6.3 kDa, pI 4.8), Ubiquitin—N and A state (8.6 kDa, pI ~6.8), and Myoglobin—Apo state (17 kDa, pI ~7.4). Proteins were incubated with PSS under biological conditions prior to direct infusion electrospray ionization mass spectrometry (ESI-MS). Complex ions were separated via ion mobility (IM) if necessary, then subjected to collisionally activated dissociation (CAD) in the presence of Ar(g). Results from the top-down method described will be discussed and compared to corresponding PDB files. In addition to sequencing for individual basic residues, the distance between basic sites on protein structures was also predicted using molecular dynamics simulations of the oligomeric tags. Radial probability distributions of the sulfonate residues on the PSS tetramer and hexamer were simulated to obtain approximate bond distances. PDB files of PSS were compared to the PDB files of protein structures to predict the regions of salt bridging based on the distance between amino groups, the size of the PSS tag, and the complexed peptide fragments observed. The method thus far has shown to agree with the model proteins' known structures so far and could provide a simple, rapid method for mapping both the location of individual basic amino acids in addition to their proximity to one another.

Investigation of thiol-yne comb polymer architectures through the utilization of tandem mass spectrometry and ion mobility techniques

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Thiol-yne copolymers were initially analyzed via traditional mass spectrometry to determine the potential copolymer architectures that may exist within the mixture. Samples analyzed in these preliminary experiments showed two main distributions. Initial hypothesis was that the low-mass distribution was a tadpole species containing one thioether chain as the cyclic head and one poly(ethylene oxide) pendant chain, and the higher-mass distribution a comb species with a poly(thioether) chain as the backbone and additional poly(ethylene oxide) pendant chains. In order to confirm these architectures tandem mass spectrometry (MS/MS) and ion mobility

spectrometry (IMS) experiments were carried out on the copolymer mixture. ESI-MS/MS was utilized to investigate the low-mass species and successfully confirm its tadpole structure based on three distinct fragments from the dissociation of the tadpole tail. Similar analysis of the larger oligomers resulted in differing fragmentation products than those from the smaller tadpole species, indicating the presence of a different architecture. The fragmentation products observed from the higher-mass oligomers were indicative of unsaturated linear, or cyclized comb species. Using IMS, drift time data were collected for both the saturated combs which were forced to be linear as a result of having two dithiol end groups, and unsaturated combs which could be linear or cyclized due to the absence of one end group. The unsaturated comb polymers consistently had lower drift times than the saturated linear species, indicating that the unsaturated comb had undergone cyclization. Using proper calibration methods, collisional cross sections (CCS) were derived for both the molecules with known and unknown architectures. Comparison of these experimental CCS values to ones derived through molecular modeling, provided further evidence that the unsaturated linear combs undergo cyclization. Overall, this work shows the benefits of combining various techniques in order to gain a complete understanding of polymeric architectures within a mixture.

Investigating Differentially Expressed Epithelial-to-Mesenchymal Biomarkers in 1st and 2nd Generation Spheroids

Justin C. Rabe, Emily R. Sekera, Brian D. Fries, Philip H. Lindhorst, and Amanda B. Hummon
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Multicellular Tumor Spheroids (MCTS) are a three-dimensional cancer model system that better mimics the physiological microenvironment of in vivo tumors compared to two-dimensional cell culture. As spheroids grow radially from a central core of cells, different chemical and biological gradients begin to form. Due to these gradients, spheroids contain three distinct layers including an outer proliferating layer, a middle quiescent layer, and an apoptotic core. Cells in the different layers can be separated by a method known as 'serial trypsinization'. Serial trypsinization removes the cells in a sequential manner analogous to 'peeling an onion'. After the separation, the cells from each layer can be regrown, either as two-dimensional monolayers or as second-generation three-dimensional spheroids. Cells from different layers regrown in two-dimensional cell culture have been shown to retain some phenotypical traits related to their origin layer. Cells from distinct regions have been shown to have different drug resistance compared to two-dimensional culture before spheroid growth. Second-generation spheroids regrown from different layers also have different characteristics depending on the layer of origin. After 14 days of growth the second-generation spheroids underwent bottom-up proteomic analysis to compare whole second-generation spheroids grown from distinct layers of origin to their precursor spheroids. This analysis revealed differentially expressed proteins in all three types of second-generation compared to the first-generation spheroids. Second-generation spheroids originating from the outer and middle layers have a significant upregulation of vimentin and a significant down regulation of EpCAM, while the spheroids originating from the core have a

significant down-regulation of vimentin. Both the upregulation of vimentin and downregulation in vimentin are indicators of the epithelial-to-mesenchymal transition. Second-generation spheroids could be helpful in better understanding the EMT transition along with helping in drug discovery in drug resistant tumors.

Database-Assisted Globally Optimized Targeted Secondary Electrospray Ionization High Resolution Mass Spectrometry (dGOT-SESI-HRMS) Enhanced Volatilomics Analysis of Bacterial Metabolites

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Secondary electrospray ionization high-resolution mass spectrometry (SESI-HRMS) is an innovative analytical technique for the ambient analysis of volatile organic compounds (VOCs). However, ion suppression in the SESI source has hindered feature detection stability and reproducibility of SESI-HRMS in untargeted volatilomics. To address this, we have developed and optimized a novel targeted MS approach, database-assisted globally optimized targeted (dGOT)-SESI-HRMS, using the microbial-VOC (mVOC) database to enhance metabolite coverage in headspace of bacterial cultures. We cultured representative strains of bacteria in modified Gifu anaerobic broth (mGAM) supplemented with either fructose or sucrose as substrates for growth. Cultures were left in anaerobic conditions overnight to allow for volatiles to accumulate. Headspace volatiles were expelled into a commercially available SUPER-SESI source coupled to a QExactive™ high resolution mass spectrometry. Volatiles were assed using different analytical methods; full scan with DDA, conventional parallel reaction monitoring (PRM), and scheduled PRM experiments were performed on MS peaks corresponding to mVOC reported compounds. Scheduled PRM-based dGOT-SESI-HRMS were staggered to reflect unique m/z windows for fragmentation. The analysis method proportionally fragmented peaks with respect to the target windows, with a total of 147 VOCs fragmented from 306 targeted compounds. Of the collected spectra, 91 features were confirmed as culture derived volatiles with respect to media blanks. Annotation was also achieved with a total of 77 metabolites referenced to standards and *in silico* fragmentation databases allowing for biological interpretation. Partial least squares-discriminant analysis (PLS-DA) revealed distinct clustering of strains suggesting unique composition of headspace volatiles. Collectively, our dGOT-SESI-HRMS method afforded more robust capability of differentiating bacterial strains and culture conditions when compared to conventional PRM and full scan mode suggesting the newly developed approach can serve as a more reliable analytical method for the sensitive monitoring of gut microbial metabolism.

Elucidating the catalytic mechanism of a bacterial deglycase essential for utilization of fructose-lysine, an Amadori product

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Amadori products are stable sugar-amino acid conjugates that are formed non-enzymatically via the Maillard reaction that takes place during preparation of foods. Fructose-lysine (F-Lys) is one of the most abundant Amadori compounds in processed foods and is a key intermediate in the formation of advanced glycation end products, which in turn are implicated in inflammation and disease. Either during or after bacterial uptake, F-Lys is phosphorylated to form 6-phosphofructose-lysine (6-P-F-Lys). FrlB, a deglycase, converts 6-P-F-Lys to L-lysine and glucose-6-phosphate, with the latter feeding into glycolysis. Since the catalytic mechanism of FrlB has not been studied, we sought to obtain a high-resolution structure of *Salmonella* FrlB ± 6-P-F-Lys and identify the active-site residues essential for catalysis.

After overexpression and purification of recombinant *Salmonella* FrlB, we obtained its 1.9Å crystal structure in the absence of substrate and overlaid it with the structure of *Escherichia coli* glucosamine-6-phosphate synthase (GlmS) in complex with fructose-6-phosphate. Based on these comparative analyses, seven active-site residues in FrlB were individually mutated to either alanine or an amino acid that corresponds to a conservative substitution, and the variants purified using immobilized metal-chelate affinity chromatography. Our differential scanning fluorimetry studies revealed that all the mutants exhibit thermal stability nearly identical to wild-type FrlB; importantly, our native mass spectrometry (nMS) studies confirmed that the mutations did not impair the ability of these mutants to form a stable dimer. A spectrophotometric coupled assay was employed to measure the activity of FrlB and the mutants. When a mutation dampened or eliminated deglycase activity, nMS was used to make the crucial distinction between a defect in substrate binding versus cleavage. Collectively, the integrated structural biology approach involving nMS has led to a deeper understanding of the active site and the catalytic mechanism of FrlB and expect similar payoffs in structure-function studies of other enzymes.

A mass spectrometry-based approach for quantification of steroidal alkaloids in pig plasma after tomato consumption

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Tomato consumption is linked to decreased chronic disease risk, with dietary carotenoids (e.g., lycopene) typically being investigated as the agent of action. However, whole tomato consumption is found to be more effective than lycopene alone against prostate cancer in rats, UV-induced skin inflammation in humans, and cardiovascular disease risk factors in humans. These results suggests that other phytochemicals may play a role in the health properties of tomatoes. Our group has determined that tomato steroidal alkaloids (tSAs) are deposited in tissues after tomato consumption, where previous reports indicate these phytochemicals were not absorbed. Isolated tSAs have been found to exhibit bioactive properties *in vitro* and *in vivo*. To understand what role tSAs may play in tomato bioactivity, accurate quantitative methods for tSAs and their metabolites in biofluids must be developed. This work describes the first method for quantification of tSAs and their *in vivo* metabolites in blood plasma. Twenty piglets (n=10/group) were provided either a control or tomato powder supplemented diet for 2 weeks and blood plasma was collected for analysis. Samples were extracted with methanol and spike recovery experiments ensured quantitative liberation from the matrix. Analytes were quantified using a matrix-matched calibration curve to address matrix interferences. Analytes (acquired from 28 peaks, representing 9 unique tSA masses) along with two internal standards were chromatographically separated on a C_{18} column and quantified using UHPLC-QTOF-MS and UHPLC-MS/MS. Total tSA concentrations averaged to 110.6 nmol/L pig plasma. Putative tSA metabolites hypothesized to form after phase I metabolism were found at the highest concentrations, making up 64% of total detected tSAs in plasma. These results indicate tSAs and their putative metabolites are present at physiological concentrations after short-term tomato consumption. Our methodology and findings are crucial for further investigations of tSA bioactivity using physiologically appropriate levels.

Mass Spectrometry and Flow Cytometry: Complimentary Techniques for Proteomics of HCT 116 Spheroids

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Proteomics is a common application for mass spectrometry because high mass resolution allows for identification of multiple proteins in a sample. However, the cost of high resolution is long experiment time, particularly in experiments with multiple samples. In comparison, flow cytometry provides a faster analysis of proteins in a sample, but requires a labelled target, making it unsuitable for discovery experiments. As a result, these techniques are complementary

because mass spectrometry can be used to identify analytes for flow cytometry. To show these techniques complement one another, we are conducting proteomic analyses of HCT 116 spheroids. HCT 116 is a colon cancer cell line that can be grown into 3D cell cultures called spheroids. Spheroids possess more biological similarities to in vivo cancer tumors than 2D cell cultures, such as nutritional and chemical gradients that result in different cell populations. To characterize these populations, spheroids have to be dissociated using a technique called serial trypsinization, which slowly removes cells from the outside of the spheroid and results in single cell suspensions representing different areas of the spheroid. The combination of mass spectrometry and flow cytometry is capable of identifying and characterizing protein markers for each of these suspensions and their constituent populations. As an example, Ki67 is a well-characterized proliferation marker in humans. An untargeted proteomics analysis of HCT 116 spheroids showed Ki67 is upregulated by a factor of 2.5 in the outermost cells of these cultures, typically considered an area of proliferation. Furthermore, a Ki67 flow cytometry assay comparing the levels of Ki67 in the different areas of the spheroid showed the highest amount of Ki67 present in the outermost cell suspension, with decreasing amounts toward the core. Going forward, we expect to identify and characterize more protein markers for each cell population in the HCT 116 spheroid model.

Establishing robust calibration curves for mass photometry-based measurements of RNAs and ribonucleoprotein complexes

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Mass photometry (MP) measures masses of biomolecules using interferometric scattering microscopy[1]. This method can be used to rapidly assess sample heterogeneity, composition, and macromolecular interactions. Typically, molecules with known masses and similar optical properties as the analytes (e.g., proteins, nucleic acids) are used for calibration. However, calibrations generated with either RNA or protein may lead to incorrect mass assignments for ribonucleoprotein (RNP) complexes[2]. We sought to develop alternatives that would allow greater accuracy during MP studies of RNPs; mass measurements were independently validated by high-resolution native mass spectrometry (nMS). Preliminary studies were conducted using archaeal RNase P, an essential, multi-subunit, catalytic RNP[3-5], consisting of one RNase P RNA (RPR) and five RNase P proteins (RPPs). RPR can be reconstituted with different suites of RPPs, to form RNPs of varying RNA:protein ratios. First, protein- and RNA-only calibrations were generated from the ratiometric contrasts obtained by MP-based measurements of biomolecules with known molecular masses. β-amylase and thyroglobulin (present as multimeric complexes) were used for protein calibration. *In vitro* transcribed RNAs of known sizes (including some larger RNAs generated by RNA engineering) were used for RNA-based calibrations. Masses of protein and RNA standards were measured with MP using appropriate calibrations and were found to be in good agreement (within 0-6%) of expected and nMS-generated masses. To evaluate the suitability and appropriateness of RNA and protein calibrations in estimating masses of RNP

complexes, RPR was reconstituted with either two or five RPPs (67% and 49% RNA content respectively). Despite significant fractional mass contribution of the RPR, protein calibration provided more accurate mass measurements (0.5 – 2.6% of expected mass) compared to the RNA calibration (5 – 7% of expected mass). Studies with a more structurally diverse set of RNPs, followed by mass validation using nMS, are needed to establish optimal mass calibration protocols for RNPs.

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Protein complex prediction using Rosetta, AlphaFold, and mass spectrometry covalent labeling

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Structural mass spectrometry offers several techniques for the characterization of protein structures. Covalent labeling (CL) in combination with mass spectrometry can be used as an analytical tool to study and determine structural properties of protein-protein complexes. Degrees of modification obtained from CL experiments for specific labeled residues can be compared between the unbound and bound states of complexes. This analysis can yield insights into structural features of these protein assemblies, specifically the proximity of specific residues to the protein-protein interface. However, this data is sparse and does not unambiguously elucidate protein structure. Thus, computational algorithms are needed to deduce structure from the CL data. In this work we present a novel hybrid method that combines models of protein complex subunits generated with AlphaFold with differential CL data via a CL-guided protein-protein docking in Rosetta. In a benchmark set, the RMSD (root-mean-square deviation) of the best-scoring models was below 3.6 Å for 5/5 complexes with inclusion of CL data, whereas the same quality was only achieved for 1/5 complexes without CL data. The average improvement in RMSD observed upon inclusion of CL data was 5.2 Å. This study suggests that our integrated approach can successfully use data obtained from CL experiments to distinguish between nativelike and non-nativelike models.

Glutaminolysis messages lipogenesis via ammonia

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Tumorigenesis is associated with elevated glucose and glutamine consumption, but how cancer cells can sense their levels to activate lipid synthesis for tumor growth is unknown. Here, we reveal that ammonia, released from glutamine, acts in concert with glucose to promote lipogenesis via activation of sterol regulatory element-binding proteins (SREBPs), endoplasmic reticulum (ER)-bound transcription factors that play a central role in lipid metabolism. Ammonia activates the dissociation of glucose-regulated, *N*-glycosylated SREBP cleavage-activating protein (SCAP) from Insig, an ER-retention protein, via its binding to SCAP aspartate 428 (D428) and serine 326/330 residues, which triggers sequential conformational changes of SCAP, eventually leading to SREBP translocation and lipogenic gene expression. Interestingly, 25-hydroxycholesterol prevents ammonia to access its binding site on SCAP, thereby blocking binding to SCAP and suppressing SCAP/Insig dissociation. Mutating D428 to alanine (D428A) also prevents ammonia binding to SCAP, and ensuing conformational changes, abolishes SREBP-1 activation, and suppresses tumor growth. Our study characterizes the unknown role, opposite to sterols, of ammonia as a key activator that stimulates SCAP/Insig dissociation and SREBP-1 activation to promote tumor growth, and demonstrates that SCAP is a critical sensor of glutamine, glucose and sterol levels to precisely control lipid synthesis.

Integrative multi-omic analysis of cellular responses to EZH2 mutations found in leukemia and lymphoma

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Enhancer of zeste homolog 2 (EZH2) is a specific histone H3 lysine 27 (H3K27) methyltransferase frequently mutated in several hematological malignancies, such as diffuse large B-cell lymphoma and myeloid leukemia. Mutated EZH2 generates cancer methylation patterns in the repressive

epigenetic mark H3K27 and other non-canonical targets. Uncharacterized cellular networks governed by EZH2 and other chromatin remodelers have limited its use as a therapeutic target in outpatient care. This study aims to characterize the cellular downstream networks affected by EZH2 with point mutations of clinical relevance via multi-omics.

Four EZH2 stable transfected cells were analyzed: $EZH2^{WT}$, $EZH2^{H689A/F667I}$, $EZH2^{A677G}$ and $EZH2^{Y641F}$. EZH2 and H3K27me3 levels were monitored by an MS-based histone profiling assay and immunoblotting. Subsequently, a comprehensive multi-omics analysis was carried out involving: 1) ATACseq and 2) transcriptomics, performed using *3'QuantSeq* on an *Illumina Hiseq 3000* 3) label-free whole-cell proteomics, acquired with *Bruker timsTOF Pro HPLC-MS/MS with Ion Mobility*, and 4) MS-based untargeted metabolomics, in positive and negative ionization mode, acquired with *Agilent 6545 QTOF* and HILIC. Effector pathway analysis combining omics data revealed distinctive reprogramming effects for each mutant. A general dysregulation of mitochondrial processes, including TCA cycle and β-oxidation was common in all mutants. For $EZH2^{A677G}$ and $EZH2^{Y641F}$, alterations in the methionine salvage pathway were characteristic, while NAD^+ pathways were highly disrupted in $EZH2^{H689A/F667I}$. Additionally, important nuclear interactors were found dysregulated, including SMYD3, NSD2, and CHD7, suggesting a cooperative network of chromatin remodelers for gene expression reprogramming. This system biology-based analysis used provides a greater understanding of how EZH2 alterations lead to cellular transformation and provides insights into the role of epigenetics in cancer and developmental diseases. This understanding provides novel targets for improved diagnostics and treatments in clinical diseases driven by aberrant EZH2 activity.

More than Meets the Eye: Untargeted Metabolomics and Lipidomics Reveal Complex Pathways Spurred by Activation of Acid Resistance Mechanisms in *Escherichia coli*

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Escherichia coli (*E. coli*) is a ubiquitous group of bacteria that can be either commensal gut microbes or enterohemorrhagic food-borne pathogens. Regardless, both forms must survive acidic environments in the stomach and intestines to reach and colonize the gut, a process that partially relies on amino acid dependent acid resistance (AR) mechanisms and modifications to membrane phospholipids. However, only the basic tenets of these mechanisms have been elucidated by previous research. Our study aimed to conduct the first known full-scale metabolic and lipidomic characterization of *E. coli's* adaptations to acid stress to further understand the contribution of acid stress to its major metabolic pathways. We hypothesized that our use of untargeted metabolomics and lipidomics would reveal mechanisms downstream of AR processes that provide novel contributions to acid stress survival. Upon untargeted investigation, we detected significant differences in the extracellular metabolome and the lipidome induced by amino acid supplementation (glutamine, arginine, or lysine) and were able to contextualize these results with genes related to canonical acid resistance pathways using RT-qPCR. We additionally identified distinct metabolic and lipidomic pathways modulated by differential amino acid

supplementation. These results demonstrate AR may not be simply a set of basic mechanisms but rather a coordinated acid resistant metabolic phenotype. Future studies may use our analysis to elucidate distinct targets for prebiotic supplements to cultivate commensal strains or therapies to combat pathogenic ones.