MS.1
Improving the Robustness and Reproducibility of Mass Spectrometry Based Proteomics
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Proteomics technology has improved dramatically over the last decade. The technology developments have largely been directed around instrument hardware, where instruments have been developed that scan faster, are more sensitive, and have greater mass measurement accuracy. However, the basic workflow has remained largely unchanged – mass spectrometers are directed toward the acquisition of tandem mass spectra on the most abundant molecular species eluting from a chromatography column. More recently, efforts have been focused on the acquisition of mass spectrometry data on target peptides of interest. With improvements in instrument hardware and instrument control software, the practical experimental difference between a targeted and discovery proteomics is beginning to become blurred. These analyses are a significant change from the traditional proteomics workflow and have required the development of novel computational strategies to analyze, visualize, and interpret these data. We will present work illustrating our efforts in the development of targeted proteomics and provide a vision for challenges that still need to be overcome before these analyses become routine and replace more traditional discovery proteomics methodology.

MS.2
SWATH-MS: Principles and Applications to Quantitative Biology
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A range of proteomics technologies has been highly successful in supporting projects in molecular and cell biology and in biochemistry. For most of these projects proteomics has focused on the identification and quantification of proteins and their modifications in a small number of samples. For some fields of research, exemplified by clinical (e.g. biomarker) or systems biology studies, it is essential to accurately quantify specific sets of proteins across large numbers of samples at a high degree of reproducibility and high sample throughput.

To address these needs we recently introduced SWATH-MS. Like other Data Independent Acquisition (DIA) methods SWATH-MS essentially converts all physical specimens in a sample, in the case of proteomics the proteolytic peptides of a protein extract, into a digital file that can be perpetually interrogated for the presence and quantity of any protein that is in the detection range of the system. In the process high mass accuracy fragment ion maps are acquired for all sample analytes within a user-defined retention time and mass range window, by repeatedly cycling through consecutive precursor isolation swaths. The thus generated complete and permanent fragment ion records are then, in a second step, queried for the presence and quantity of specific peptides, using spectral libraries as prior information. Essentially, SWATH-MS combines DIA and high throughput targeted data analysis.

In the presentation we will discuss with specific applications how the favorable performance characteristics of the SWATH-MS technique translate into new biological knowledge.
MS.3
Measuring Cell Signaling and Pharmacodynamics Using Immuno-MRM
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A rapidly growing trend in protein quantification is using targeted mass spectrometry techniques like multiple reaction monitoring (MRM). The advantages of mass spectrometry compared to traditional protein measurement technologies, like Western blotting and ELISA, are the capability to multiplex, the use of internal standards (aiding reproducible quantification and cross laboratory validation), and relatively less time and cost associated with development. To improve sensitivity and throughput of mass spectrometry-based assays, we have developed immuno-MRM assays by coupling immunoaffinity enrichment of peptides and standards with quantification by mass spectrometry. Incorporating MRM and immuno-MRM assays into thematic panels, like measuring the activity of cellular pathways or the abundance of candidate biomarkers, would be enabling for a wide variety of researchers. Here, we demonstrate the utility of a panel of immuno-MRM assays and a separate panel of IMAC-enrichment MRM assays in measuring phosphorylation signaling and pharmacodynamics. Phosphorylation is a key post-translational modification and signaling event in mammalian cells. The DNA-damage response (DDR) is a critical network for maintaining genomic integrity and mutations in the DDR are among the most frequently identified in tumors. We generated anti-peptide antibodies against phosphorylated peptides in the DNA damage response network and tested their utility in a variety of samples subjected to DNA damaging agents. Working assays that were also capable of measuring the endogenous analytes were configured into an 82-plex multiplex assay. The assays were characterized by response curves to determine limits of detection and the linear range of response. Intra-day and inter-day repeatability were determined by analyzing samples at three known concentration levels using independent preparations on five separate days. The characterized assays were applied to a variety of samples to demonstrate their utility in profiling signaling events. A temporal response in phosphorylation was profiled in cell lines exposed to DNA damage (ionizing radiation or methyl methane-sulfonate, a DNA alkylating agent). A similar response was measured in peripheral blood mononuclear cells (PBMCs) and tissue specimens treated ex vivo. Changes in phosphorylation dynamics were measured in cell lines with mutations in the DNA damage response network or cells exposed to inhibitors of the DNA damage checkpoint kinase ATM. In addition to phosphorylation, we were able to develop assays to measure ubiquitination. These results show the measurement of key changes in the temporal response to DNA damage, demonstrating the feasibility of assembling a multiplexed assay to phosphopeptides and characterizing signaling dynamics in cells.

MS.4
Improving Multiplexing Limits of Parallel Reaction Monitoring (PRM) through Longer One-dimensional Chromatography and Use of Diverse Retention Time-normalized Spectral Libraries
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Targeted proteomics based on selective reaction monitoring (SRM) has been successfully applied to studies of complex biological processes such as multi-protein complex assembly and regulation of signaling networks through site-specific post-translational modifications. Despite this progress, SRM has been a low throughput methodology hindered by time-intensive assay development, relatively low specificity, and multiplexing limits. Recent introduction of parallel reaction monitoring (PRM) on quadrupole-Orbitrap™ instruments has been a major advance in targeted proteomics leading to significant gains in specificity, sensitivity, and ease of implementation. This report describes our recent work on the development of a PRM-based targeted proteomics pipeline, which takes advantage of the latest improvements in one-dimensional chromatography coupled with longer gradients and more intelligent use of available spectral libraries for initial target scheduling. As an example, a highly multiplexed PRM assay targeting over 150 human kinases will be presented.
The targeted analysis of protein biomarkers are routinely performed on triple quadrupole mass spectrometers operated in selected reaction monitoring (SRM) mode. However, the low resolution of quadrupole mass filters have limited selectivity, which is an issue for the analysis of complex samples, where the high background interferes with the analyte signals. Thus, hybrid mass spectrometers with high resolution and accurate mass (HRAM) capabilities overcome this limitation, and have opened new avenues in quantitative proteomics.

The targeted analyses of biological samples carried out using the parallel reaction monitoring (PRM) technique implemented on a quadrupole-orbitrap mass spectrometer have demonstrated a significant gain in selectivity, while the assignment of the fragment ions by accurate mass increased the confidence. The analyses in PRM mode showed better quantification performance for peptides present in low amount in bodily fluids. This translated to more consistent quantitative data for the different peptides of the same protein, and a clear discrimination between the control and patient samples.

Furthermore, optimal PRM performance was obtained through the control of the acquisition by monitoring the analytes in real-time and dynamically adjusting the parameters. The design of an instrument method called intelligent-PRM, yielded excellent results, even when applied to large sets of peptides (up to 600) in bodily fluids. Examples of application including the differentiation of isoforms will be discussed.

Proteomic analyses by mass spectrometry play an increasingly central role in the characterization of clinically relevant biological materials to elucidate biology, identify novel therapeutic targets and develop diagnostic, prognostic, and predictive disease markers. Unfortunately the effectiveness and “translatability” of proteomics-based discovery have been severely hampered by poor study design; inadequate number, suitability, and quality of samples; and technological approaches lacking sufficient sensitivity, quantitative precision, and capacity to analyze statistically relevant numbers of samples. Equally problematic has been the lack of robust methods suitably scaled to follow up on discovery studies and able to quantify proteins and modified peptides with sufficient sensitivity, specificity, reproducibility and throughput to support nuanced understanding of dynamic, protein-based biological processes.

In our discovery-to-verification proteomics studies we are addressing these serious barriers. In the discovery phase, we are employing multiplexed, quantitative MS technologies that enable analysis of larger numbers of samples with improved precision, leading to better-qualified candidates. For the verification phase, we are developing targeted mass spectrometry-based technologies to screen and quantify proteins and modified peptides in a variety of biological contexts including human tissue and plasma. These assays are sensitive and very specific, do not suffer from interferences that plague immunoassays, and can be highly multiplexed. Targeted MS based approaches are thereby helping to usher in a new era of quantitative biology in which virtually any protein or modification can be reliably measured in any biological context. In ongoing experiments we have applied these quantitative proteomic approaches in a generalizable discovery-through-verification pipeline to identify biomarkers of cardiovascular injury, breast and ovarian cancer, and multiple infectious diseases, among other clinically important conditions. The same technologies are being brought to bear to understand and predict response and intrinsic and acquired resistance to cytotoxic and targeted cancer therapies. Targeted MS approaches that we have developed are also being applied to quantification of a range of posttranslational modifications including phosphorylation, ubiquitination, and acetylation of proteins involved in various signaling networks, as well as modifications of histones that will help to elucidate epigenetic mechanisms.

The increasing application of targeted MS methods complements other developments including substantial advances in sensitivity, speed and resolution of latest-generation mass spectrometers and the development and refinement of data acquisition and analysis methodologies including data-independent acquisition approaches such as SWATH. Each of these represents an important step towards the ultimate goal of robust, comprehensive, precise quantification of complex proteomes. Together they promise to improve the effectiveness and integration of proteomic discovery and verification and ultimately to blur the distinction between unbiased discovery and targeted verification altogether.
Emerging Approaches to Monitoring Chemotherapeutic Response

Arun Wiita

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A deep understanding of how cancer responds to a chemotherapeutic insult is critical to developing new diagnostic and therapeutic strategies. Here I will discuss work on hematologic malignancies, focusing on the combined use of deep sequencing and quantitative proteomics to give a global overview of the cancer cell response to treatment. This work provides insight into mechanisms of cancer cell deconstruction after treatment as well as suggesting combination therapeutic regimens. Furthermore, I will describe the use of specific protein N-terminal labeling and targeted proteomics to define a potential novel class of biomarkers of chemotherapeutic efficacy.

Regulation of Protein Acylation by SIRT3 and SIRT5

Bradford Gibson

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Over the last few years, along with my collaborators at the Gladstone Institute, Duke and Harvard, we have investigated the role of the mitochondrial sirtuins SIRT3 and SIRT5 in metabolic regulation. These sirtuins regulate the levels of reversible lysine acetylation, succinylation, and malonylation in hundreds of proteins through their deacylation activities. Specifically, we have identified several thousand sites of protein lysine acetylation, succinylation and malonylation in mitochondria isolated from the livers of mice using antibody-based affinity enrichment approaches and MS1 label-free proteomics. Moreover, by comparing the levels of these lysine modifications in both WT and KO backgrounds, we have been able to discern a large subset of acyl-lysine sites that appear to be regulated. In this presentation, I will discuss the development and optimization of our label-free proteomics approach, with particular attention paid to some of the problems we encountered, including the difficulties posed by comparing dozens of experiments across many months of operation, and the still unresolved issue of assessing site occupancy and biological function.
1.1
Big Data and Human Disease: The New Era

Michael Snyder
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We have used large multiomics data to investigate complex diseases. Multiomics data has been used to investigate two common human diseases: Type 2 diabetes and autism. For autism we have combined genome sequencing and gene expression to reveal a novel pathway and novel parts of the brain that participate in autism. For Type 2 diabetes we have used multiomic data to follow a cohort of individuals at risk for Type 2 diabetes reveal the pathways that are activated during disease onset and weight gain. The power of multiomics data for understanding and treating complex disease will be discussed.

1.2
Mass Spectrometry Based Draft of the Human Proteome

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TUM team, SAP team, Cellzome team, JPT team

Abstract: Proteomes are characterized by large protein abundance differences, cell type and time dependent expression patterns and post-translational modifications all of which carry biological information not accessible by genomics or transcriptomics. Here, we present a mass spectrometry-based draft of the human proteome and ProteomicsDB, a public in-memory database for real time analysis of big data. The information assembled from human tissues, cell lines and body fluids allowed estimating the size of the protein coding genome, identified organ-specific proteins and a large number of translated lincRNAs. Analysis of mRNA and protein expression profiles of human tissues revealed conserved control of protein abundance, integration of drug sensitivity data allowed the identification of proteins predicting resistance or sensitivity and proteome profiles also hold considerable promise for analysing the composition and stoichiometry of protein complexes. ProteomicsDB thus enables navigation of proteomes, provides biological insight and fosters the development of proteomic technology.
1.3

Network Approaches to Aggregating Variants and Mutations

Trey Ideker
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A prime objective of genomic medicine is the identification of disease-causing mutations and the mechanisms by which such events result in disease. As most disease phenotypes arise not from single genes and proteins but from a complex network of molecular interactions, a priori knowledge about the molecular network serves as a framework for biological inference and data mining. In this talk I will describe recent developments at the interface of biological networks and mutation analysis. I will examine how mutations may be treated as a perturbation of the molecular interaction network and what insights may be gained from taking this perspective. A particularly promising approach of this type, network-based stratification (NBS), is a method to integrate somatic tumor genomes with gene networks. This approach allows for stratification of cancer into informative subtypes by clustering together patients with mutations in similar network regions. NBS has been used to define alternative stratifications of ovarian, uterine and lung cancer cohorts from The Cancer Genome Atlas.

1.4

Single Cell Network Analysis in 3D

Bernd Bodenmiller¹, Charlotte Giesen¹, Hao Wang¹, Denis Schapiro¹, Peter Schüffler¹, Joachim Buhmann¹, Peter Wild², Detlef Guenther¹

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Introduction

Tissues are complicated assemblies of multiple interacting cell types that communicate with each other to achieve physiological states. In cancer, malignant cells and cells of the tumor microenvironment (TME) facilitate tumor reprogramming, progression, and drug resistance. For an understanding of the underlying processes it is important to comprehensively investigate the components and their relationship within the TME. This necessitates imaging approaches that can simultaneously measure dozens of biomarkers to define cell types, their functional and signaling states, and spatial relationships.

Methods

For highly multiplexed tissue imaging at subcellular resolution, we have coupled immunohistochemical (IHC) methods with high resolution laser ablation and mass cytometry. In mass cytometry, rare earth metals are used as reporters on antibodies. Analysis of metal abundances using the mass cytometer allows determination of biomarker expression. In the approach presented here, tissue sections were prepared for antibody labeling using IHC protocols. Rare-earth-metal isotope tagged antibodies were selected to target proteins and protein modifications relevant to breast cancer. After antibody staining, the sample was positioned in a laser ablation chamber developed by Wang et al. (1) to minimize aerosol dispersion for high-resolution, high-throughput and highly sensitive analyses. The tissue was then ablated spot by spot, and the ablated material was transported by a mixed argon/helium stream to the CyTOF mass cytometer. After data preprocessing, the 32 transient, isotope signals were plotted using the coordinates of each single laser shot, and a high-dimensional image of the sample was generated. Single-cell features were computationally segmented and the single cell marker expression data were extracted for downstream bioinformatics analyses.

Results

Imaging mass cytometry provides high-dimensional analysis of cell type and state at subcellular resolution to study tissues and adherent cells. The novel imaging approach enabled the simultaneous visualization of 32 proteins and protein modifications, with the potential to map up to 100 markers on a single tissue section with the availability of additional isotopes. Application of imaging mass cytometry to breast cancer samples allowed delineation of cell subpopulations and cell-cell interactions, highlighting tumor heterogeneity and enabling new routes to patient classification. As such it has the potential to yield novel insights of the TME by exploiting existing large collections of FFPE tumor samples and associated clinical information.

Conclusions

Imaging mass cytometry complements existing imaging approaches and will support the transition of medicine toward individualized molecularly-targeted diagnosis and therapies.

Novel Aspect

Highly multiplexed imaging of tissue biomarkers at subcellular resolution.

1.5 Spatially-resolved Proteomic Mapping of Living Cells (with a Focus on Mitochondria) Using Engineered Peroxidase Reporters

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Microscopy and mass spectrometry (MS)-based proteomics are complementary techniques: the former provides spatiotemporal information in living cells, but only for a handful of recombinant proteins at a time, while the latter can detect thousands of endogenous proteins simultaneously, but only in lysed samples. In this talk, I will describe a new technology that combines the strengths of microscopy and MS by generating spatially and temporally-resolved proteomic maps of endogenous proteins within living cells. The method relies on a genetically targetable peroxidase enzyme that biotinylates nearby proteins, which are subsequently identified by MS. We used this approach to identify 495 proteins within the human mitochondrial matrix, 127 intermembrane space proteins, and 137 outer mitochondrial membrane proteins, including 76 proteins that have never before been assigned to mitochondria. The labeling was exceptionally specific, able to distinguish between inner membrane proteins facing the matrix versus the IMS. Our catalog also revised the sub-mitochondrial localizations for several well-studied proteins, with the new assignments confirmed by electron microscopy.

2.1 Use of Personalized Sequence Databases for Peptide MS/MS Spectrum Matching in the Proteogenomic Analysis of 105 TCGA Breast Tumors

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Quantitative measurement of patient-specific sequence features may be critical to understanding underlying disease biology. In a typical proteomics experiment, software tools match peptide MS/MS spectra against a protein sequence database (UniProt, RefSeq) derived from a reference genome. Thus any patient-specific peptides will be missed, including peptides that contain single amino acid variants, span the junctions of alternatively spliced exons or fusion genes, or are unexpectedly expressed due to frameshifts. As part of the NCI Clinical Proteomics Tumor Analysis Consortium, we accessed genome and transcriptome sequence data for each patient-derived tumor sample being analyzed by proteomics. Using iTRAQ labeling and LC-MS/MS, proteome profiles were generated with human breast cancer tumor tissue from 105 patients that include luminal A, luminal B, Her-2 enriched and basal-like subtypes. Illumina RNA-seq and whole exome sequencing data generated from portions of the tumors and accompanying germline DNA samples obtained from the TCGA network were processed by QUILTS (quilts.fenyolab.org) to generate personalized protein sequence databases through comparison of each genome and/or transcriptome to existing RefSeq based gene models. Tumor RNA-seq data was analyzed using tools for mapping (Bowtie), assembly (TopHat), and transcript quantification (Cufflinks). After cryofracturing frozen tumors, extracting protein, and digesting with trypsin, the 105 patient samples were prepared for multiplexed iTRAQ quantitation in 36 groups of 3 patient samples plus an aliquot of internal reference. High pH reversed phase peptide fractionation of each iTRAQ 4-plex into 24 fractions preceded LC-MS/MS on a Thermo QExactive instrument. The internal reference sample was a mixture of portions of 40 of the tumors with equal representation from the 4 breast cancer subtypes (luminal A, luminal B, Her-2 enriched and basal-like). Because each experiment contained the internal reference sample, the personalized databases for each patient were concatenated together and made non-redundant prior to searching. Using Spectrum Mill the MS/MS spectra were searched in two stages: all spectra against RefSeq, then the remaining unidentified spectra against the personalized sequence database. Data will be presented that correlates the observation of expressed peptide with the variant calling score and the number of observed transcript reads for splice junctions. Incompleteness of proteome/transcriptome coverage will be addressed in the context of ability to detect low abundance variants and exon splice junctions.
2.2 Proteogenomic Analysis of Human Breast Cancer Connects Genetic Alterations to Phosphorylation Networks

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The genetic landscape of human breast cancer has been well defined in The Cancer Genome Atlas (TCGA) project by using genomics technologies, including RNA sequencing, whole exome sequencing, gene expression, copy number variation, DNA methylation, and microRNA profiling. Mass spectrometry (MS)-based global proteome and phosphoproteome analyses may provide an orthogonal approach to genomic studies to further improve the molecular taxonomy and our understanding of breast cancer. Central questions in breast cancer biology that will be addressed in this study are: (1) Which genomic characteristics are executed at the protein level? (2) How is the molecular taxonomy of breast cancer reinforced and revised by protein and phosphorylation data? and (3) What phosphorylation-driven signaling networks emerge from genetic alterations?

We analyzed 105 human breast cancer samples that have been previously genetically characterized by the TCGA project. Tumor samples were cryofractured, proteins extracted with 8M urea, digested with LysC/Trypsin and iTRAQ4-plex labeled. Samples were fractionated using basic reversed-phase separation into 24 fractions for proteome analysis and 12 fractions for phosphoproteome analysis. Phosphorylated peptides were enriched using immobilized metal affinity chromatography. All mass spectrometry data was acquired using 2.5h runs on a Q Exactive instrument. In each of a total of 35 iTRAQ 4-plex experiments three tumor samples were analyzed and directly compared against an internal reference sample comprised of a mix of 40 different tumors. All MS data was analyzed in Spectrum Mill using patient-specific RNA-sequencing derived protein databases.

The breast tumor samples analyzed had equal representation of the major four subtypes of breast cancer: Her2-enriched, luminal A and B, and basal-like. In total we quantified >12,000 proteins and >70,000 phosphorylation sites, with an average of >11,000 quantified proteins and >20,000 phosphorylation-sites for each tumor. While the global mRNA protein abundance correlation was rather low (Spearman’s correlation of 0.35), we found very good correlation for most protein kinase gene amplifications for mRNA, protein and phosphoprotein abundance. Hierarchical clustering analysis of both the proteome and the phosphoproteome data yielded an overlapping set of three major clusters: a basal-like, a luminal and a new, previously uncharacterized group. We are currently in the process of identifying new protein and phosphosite classifiers for these breast cancer subtypes. The two most recurrently mutated genes in human breast cancer are PIK3CA and TP53 at frequencies of 30–40%. Comparison of PIK3CA or TP53 mutated vs non-mutated tumors highlights specific phosphorylation signaling events downstream of mutated PI3-kinase and increased phosphorylation of cell cycle check point kinases in p53- mutated tumors. Network and pathway analysis is being performed to comprehensively integrate all genetic and phospho-/proteomic alterations in one model.

2.3 Proteogenomic Analysis of Human Colon and Rectal Cancer

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We analyzed proteomes of colon and rectal tumors previously characterized by the Cancer Genome Atlas (TCGA) and performed integrated proteogenomic analyses. Somatic variants displayed reduced protein abundance compared to germline variants. mRNA transcript abundance did not reliably predict protein abundance differences between tumors. Proteomics identified five proteomic subtypes in the TCGA cohort, two of which overlapped with the TCGA “MSI/CIMP” transcriptomic subtype, but had distinct mutation, methylation, and protein expression patterns associated with different clinical outcomes. Although copy number alterations showed strong cis- and trans-effects on mRNA abundance, relatively few of these extend to the protein level. Proteomics data indicated that the chromosome 20q amplicon was associated with the largest global changes at both mRNA and protein levels; proteomics data highlighted potential 20q candidates including HNF4A, TOMM34 and SRC. Targeted parallel reaction monitoring analysis was used to profile ~75 proteins involved in metabolic pathways and revealed differences in metabolic reprogramming associated with KRAS and BRAF mutations and other genomic features. Integrated proteogenomic analysis provides functional context to interpret genomic abnormalities and affords a new paradigm for understanding cancer biology.