Quantitative Phosphorylation in (FACS) Purified CD4T+Human Thymocytes as a Function of IGF-1 Stimulation
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PTK7, a catalytically inactive RTK, is a novel marker for human MHC-classII-restricted CD4+ RTEs and highly expressed during human and murine intrathymic development. It is implicated in neuroepithelial development, planar cell polarity and overexpressed in several cancers. Although surface PTK7 is useful in defining the phenotype of viable human CD4+ RTEs, its function in human T-cell development remains unclear. To decipher a mechanistic role of PTK7 in RTEs, we performed RNAi-mediated gene silencing in T-lineage tumor cells, primary human peripheral T-cells and thymocytes. Reduction in PTK7 consistently decreased cell survival alongside Caspase-3 activation. Survival dependent on AKT phosphorylation downstream of PTK7 together with IGF-1R associated PI3kinase activity were reduced upon PTK7 knockdown, suggesting its essential role in survival of RTEs and developing thymocytes involving PI3K/AKT pathway associated phosphorylation events.

Glutathionylation is a Critical Signaling Mechanism for Protein Tyrosine Phosphatases
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Mass spectrometry based proteomic technologies have shed light on the multifaceted nature of cell signaling mechanisms for such post translational modifications (PTMs) as phosphorylation, acetylation, ubiquitination and methylation in both a qualitative and quantitative fashion. These breakthroughs have been reliant principally on a “bottom up” strategy in which complex protein matrices, such as whole cell lysates, are reduced, alkylated and digested to peptides and analyzed by the mass spectrometer. The in vivo modification of the amino acid cysteine within proteins is known to play a role as a signaling effector, but is largely unexplored. The sample workflow for proteomic studies, in particular reduction and alkylation, is not conducive to the global characterization of cysteine PTMs. In this work we take into account the aforementioned conditions, and show that glutathiolation of the active site cysteine of the PTP protein SHP2 is the molecular response to cellular oxido-reductive stress created by knockout of the intracellular antioxidant Glutathione Peroxidase-1 (GPX1).

Absolute Quantitation of Aminoglycoside Antibiotics in Mouse Plasma by a HILIC-based LC-MS/MS Method
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A search for the least ototoxic and nephrotoxic aminoglycoside antibiotics is an ongoing effort. N2 is a recently synthesized broad-spectrum aminoglycoside antibiotic structurally closely related to the naturally occurring sisomicin. The determination of the pharmacokinetic (PK) properties of these two antibiotics in mice is an essential part of the screening effort.

From an analytical perspective, aminoglycosides present several challenges: in general, aminoglycosides are very polar and they exhibit high ionic binding to plasma proteins; these characteristics require special attention to chromatographic and sample preparation conditions, respectively. For this project in particular, both high-dose and low-dose PK studies were conducted, and method modifications were required to cover this wide dynamic range.

In summary, quantification of aminoglycosides in mouse plasma using a simple TCA/methanol protein precipitation procedure and newly developed HILIC based LC-MS/MS methods with high/low sensitivity was achieved. SRM of the singly charged ion was applied to de-optimize the method performance for investigation of plasma profiles following high dose (175 mg/kg) administration (calibration range 100 –
10,000 ng/mL). For analysis of plasma samples following a low dose of aminoglycosides (0.65 mg/kg), SRM of the doubly charged ion was applied to achieve high method sensitivity (calibration range 2 – 2,000 ng/mL). The lower limit of quantification (LLOQ) was 2 ng/mL for N2, and 10 ng/mL for sisomicin.

Stanford Nano Shared Facilities
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Nanoscale Science & Engineering (NSE) research crosses departmental boundaries and is a driving force for multidisciplinary collaborations across campus. However, many of the tools that enable NSE are not economically feasible in individual research labs. One of the goals of the Stanford Nano Shared Facilities (SNSF) is to make these tools available, together with staff for operations, development, maintenance, and training and enable the many research groups on campus with nano-relevant interests to pursue the most cutting-edge NSE research.

Top-Down Venom Analysis with Byonic Software
Marshall Bern (1), Doron Kletter (1), David Fenyo (2), David Morgenstern (2), Beatrix Ueberheide (2), Nicholas Bern (1), Wilfred Tang (1), Yong J. Kil (1), Christopher Becker (1)
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Venoms from snails, spiders, snakes, and other organisms represent a rich source of bioactive compounds with potential as drug leads and research tools. Venom presents a number of special challenges to proteomics analysis: (1) crude venom may contain 100 or more individual toxins, some of quite low abundance; (2) protein databases are often incomplete or missing altogether; (3) toxins have masses up to ~10 kDa so good fragmentation coverage may be difficult to obtain; (4) toxins contain multiple disulfide bonds, and in the case of marine snails, numerous posttranslational modifications. One successful data acquisition method derivatizes cysteines to increase charge and then employs ETD fragmentation on the highly charged precursors. This method produces complex spectra that in turn pose challenges for data analysis. Here we show how we used Byonic software in the analysis of venom from a variety of organisms.

Progress on Glycopeptide Identification with Byonic Software
Marshall Bern (1), Katalin Medzihradszky (2), Nicholas Bern (1), Wilfred Tang (1), Yong Kil (1), Christopher Becker (1)
(1) Protein Metrics Inc., (2) UCSF

Glycosylation is one of the most important and prevalent posttranslational modifications of proteins, yet it is also one of the most difficult to study, due to its extreme complexity and the great difficulty of assaying protein and carbohydrate components simultaneously. However, with recent developments in mass spectrometry, glycoproteomics—the study of samples complex in both protein and glycan content—has become increasingly feasible. One of the greatest remaining challenges, however, is assignment of mass spectra.

Here we evaluate the performance of automatic, high-throughput analysis using ByonicTM on a large ETD data set and a small HCD data set on a sample of mouse synaptosome. We also show selected examples from other data sets.

We show that automatic analysis of intact glycopeptides can be quite sensitive and accurate, even on a data set containing > 350 glycoproteins and ~100 N-glycan masses. Automatic analysis, however, requires skill on the part of the researcher to iteratively design / discover a search that fits the data set well and gives clean interpretable results. We offer practical advice on how to obtain the best results from Byonic’s automatic analysis.
Case Study: Regulatory Considerations in the Analysis of Human Patient Samples in an Academic Core Lab

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Stanford University

Analytical challenge: Absolute quantitation of propofol in 1,600 human plasma samples

Regulatory considerations: Before our core lab could accept this project, regulatory and safety aspects had to be taken into account -- For clinical samples, would CLIA or GLP certification be required? Would HIPAA apply? Is propofol (infamous as the drug that killed Michael Jackson) a Controlled Substance? What about biosafety regulations? These issues will be defined and explored. In brief for this particular case study, CLIA was not required because results would not be reported back to patients or used for diagnosis. GLP was not required because data would not be used in FDA or other regulatory submissions. HIPAA did not apply because samples and data were not personally identifiable. Propofol is not classified as a controlled substance; if it were, many controlled substances can be obtained as analytical standards (in solution at low concentration) without triggering federal DEA regulations. As for biosafety, the patients were not known to have any infectious diseases, and samples were handled under "universal precautions".

Scientific method: Human plasma samples were spiked with D17-propofol (internal standard) and cleaned up via liquid-liquid extraction. The heptane extracts were analyzed without derivatization on a Scion TQ GC-triple quadrupole mass spectrometer (Bruker Daltonics) using electron ionization and SRM mode. An isocratic oven program (195C) minimized cycle time: total injection-to-injection time was 2.5 minutes. Four transitions were monitored, 2 each for propofol and D17-propofol.

Results: The method proved to be reliable for the analysis of 1,600 plasma samples spread over 2 months. Each 100-vial autosampler tray of samples took just over 4 hours to analyze. Calibration curves and QCs were run with each batch of samples and demonstrated consistent method performance over time. LLOQ was 4nM (400 amol on column), LLOD 2nM; response between 1nM and 4mM was linear.

Optimized Analytical Procedures for the Untargeted Metabolomic Profiling of Human Urine and Plasma by Combining HILIC- and RPLC-MS

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Metabolic profiling of body fluids is crucial for monitoring and discovering markers of health and disease and for providing insights into human physiology.

Urine and plasma each contain an enormous diversity of metabolites with a broad range of physicochemical properties such as size and hydrophobicity. As a consequence, single liquid chromatography systems coupled to mass spectrometry (MS) do not allow a full coverage of the metabolome. Hydrophilic interaction liquid chromatography (HILIC) offers complementary information to reverse-phase liquid chromatography (RPLC) by enabling the analysis of polar metabolites.

To maximize the metabolome coverage in an untargeted fashion, we systematically investigated the performance of various HILIC and RPLC columns when coupled to MS using standards and biological samples. The performance of five HILIC columns operated at different pH (acidic, neutral, basic) and five C18 silica RPLC columns were compared. The zwitterionic column ZIC-HILIC operated at neutral pH gave the optimal results (HILIC-MS) while Hypersil GOLD and Zorbax SB aq performed the best for urine and plasma samples (RPLC-MS), respectively. Intra-batch reproducibility of the optimized HILIC-MS analytical procedure was excellent and similar to RPLC-MS in term of retention time (CV < 1%) and peak area (CV < 12%). Long-term inter-batch (40 days apart) reproducibility of the HILIC-MS procedure was good with a retention time CV < 1.25% and a peak area CV < 22%. Combining the optimal HILIC- and RPLC-MS approaches greatly expanded metabolome coverage with the detection of 44% and 99% new metabolic features on urine and plasma samples, respectively compared to RPLC alone.

Overall, the use of the optimized combined approaches enables the monitoring of more than 25,000 unique metabolic features from urine and plasma and enables unprecedented coverage of the human metabolome.
Heat Stabilization Preserves Sample Quality and Enables Identification/Monitoring of Peptide Biomarkers
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Denator AB

Introduction: The action of proteolytic and other protein-modifying enzymes rapidly change the composition of the proteome and post translational modifications (PTM) after sampling. Subsequent analytical results reflect a mix of the in vivo molecular status and degradation products and display increased inter-sample variation. Effective enzyme inactivation and standardization of sample handling eliminate this problem.

Methods: A heat-stabilization system (Stabilizor T1, Denator AB) has been used to generate rapid, homogenous thermal denaturation of enzymes to stop degradation in tissues. Comparisons were made to snap-freezing and inhibitors, and in time study manner, compared with different post-mortem intervals. Using mass spectrometry the protein and peptide content, including PTM’s were examined.

Results and discussion: The results show rapid changes in the number of detected peptides only minutes after sampling indicating degradation of protein content. Protein fragments from, for example hemoglobin, stathmin, cytochrome C oxidase, NADH dehydrogenase, beta actin, increase with post mortem times. Conversely, amounts and identities of the detected proteins/peptides in heat-stabilized samples show maintained integrity. The fragment from stathmin (stathmin 2-20) was detected in increasing levels post mortem both in human, bovine, and mouse tissue. The correlation with the general level of tissue degradation suggests stathmin to be suitable as a quality indicator a sample.

Conclusions: Post sampling changes may distort our view of protein expression. The ability to study close to in vivo distribution and state of proteins, peptides and their modifications can have a major impact on the understanding, early detection, or the ability to identify and monitor biomarkers or targets of therapy for several diseases.

The effects of obesity on anesthetic drug distribution – absolute quantitation of propofol in human plasma by GC-MS/MS
Karolina M. Krasinska, Jerry Ingrande, Hendrikus J.M. Lemmens, Allis S. Chien
Stanford University

Dosing of anesthetics in obese patients is challenging due to the physiologic and anthropometric changes associated with obesity. Increases in fat mass may alter the distribution of lipophilic drugs such as propofol; increased cardiac output may alter pharmacokinetics; increased prevalence of sleep apnea and CO2 retention may alter pharmacodynamics. Propofol is the most commonly used anesthetic induction agent in both lean and obese subjects presenting for surgical procedures. To date, little information about propofol PK/PD in the obese population exists because these patients are often excluded in PK and PD studies. This study aims to determine propofol pharmacokinetics in the obese population. Our methodology included high-resolution sampling to capture the early drug distribution kinetics, and subsequent GC-MS/MS quantitation.

Comparison of three platforms for absolute quantitation of oxysterols: LC-MS/MS, GC-MS/MS, GC-MS
Karolina M. Krasinska, Navdar Sever, Allis Chien
Stanford University

GC-MS historically has been the most common technique for analysis of steroids, but it is increasingly being replaced by LC-MS/MS. Now with GC-MS/MS emerging on the market, researchers have even more analytical choices to perform analysis of steroids. All these approaches come with advantages and disadvantages, making selection of the most suitable method complicated (when not simply limited to instrumentation availability).
This study compares three different analytical platforms – single quad GC-MS, triple quad LC-MS/MS, and
triple quad GC-MS/MS – for quantitative analysis of a dozen oxysterols, cholesterol and its derivatives in particular. Criteria include sensitivity, selectivity, chromatographic resolution, speed of analysis and sample preparation, as evaluated through obtaining standard calibration curves with isotope dilution in biological matrices. Matrix-free standards are used for initial method development and preliminary data; the full study evaluates samples in complex biological matrices.

- Platform 1: underivatized oxysterols analyzed by triple quadrupole UPLC-MS/MS (TSQ Vantage, Thermo Fisher Scientific); BEH C18 1.7 um particle column (Waters), APCI ionization, SRM mode.
- Platform 2: Silylated oxysterols analyzed by triple quadrupole GC-MS/MS (Scion TQ, Bruker Daltonics); Bruker BR-5ms column, EI ionization, SRM mode.
- Platform 3: Silylated oxysterols analyzed by single quadrupole GC-MS (7890/5975, Agilent); Agilent DB-5ms UI column, EI ionization, full scan and SIM modes.

**NMR and Optical Spectroscopy Facility**
Stephen Lynch
*Stanford University*

The NMR and Optical Spectroscopy facility houses 9 instruments: 5 Varian/Agilent NMR instruments, 2 Horiba Jobin-Yvon Fluorimeters, 1 Varian/Agilent Cary UV-Vis/NIR and 1 Bruker FTIR/Raman. The NMR instruments can detect any frequency from 15N to 1H for 1D or 2D NMR. Experiments include the ability to correlate 1H through-bond to any other nucleus or through-space to other 1H or 19F resonances, allowing for assignment of covalent structure and stereoisomers. The optical spectroscopy instruments can detect over the full range from UV to the Far IR range with a variety of accessories for sample detection and for temperature control and rapid kinetics.

**Providing Insight into Complex Disease: Metabolomics Links Genetic Loci to Phenotype**
Robert P. Mohney, Michael V. Milburn
*Metabolon*

Biomarker discovery and the search for determinants of disease are critical for fulfilling the promise of personalized medicine and the design and deployment of the therapies of the future. Recent work integrating metabolomics and GWAS has provided unprecedented insight into how genetic variation influences human metabolism, complex disease, and drug response. By combining metabolomics with GWAS, we have generated a metabolic network that includes a large number of novel associations and provides a link between genes and disease phenotype. These results illustrate how metabolomics provides a powerful tool for understanding the molecular events that contribute to disease and establish the groundwork for personalized medicine approaches that provide a snapshot of the health status of the individual and risk for disease.

**Imaging Resources: Stanford's Cell Sciences Imaging Facility**
Jon Mulholland
*Stanford University*

Overview of imaging resources at the Cell Sciences Imaging Facility. CSIF offers state-of-the-art confocal, multi-photon, widefield, FLIM, super resolution, transmission electron and scanning electron microscopy.
Proteomic MS analysis of FASP-processed FFPE Leptomeningeal Amyloid Brain Tissue Identifies Transthyretin where Immunohistochemical Staining Fails

Anna Okumu, Michael Greicius, Edward Plowey, Yuxi Wu, Allis Chien, Chris Adams
Stanford University

Transthyretin misfolding and aggregation is known to be associated with amyloid diseases and senile systemic amyloidosis. We obtained rare post mortem FFPE tissue from a patient who suffered periods of memory loss/dementia, seizures and schizophrenia, and was under extensive clinical care. Immunohistological staining on this tissue was positive for amyloid proteins but had failed to identify transthyretin, a specific amyloid protein associated with the symptoms the patient exhibited. FASP MS of carefully excised amyloid plaque regions identified transthyretin as the most prominent amyloid protein in the plaques; transthyretin was absent in control (healthy) brain regions from the same patient. In this work we demonstrate that FASP MS of human FFPE brain tissue provides superior qualitative and quantitative proteomic profiling. Proteomic MS analysis of FASP-processed FFPE brain tissue requires only a small amount of tissue and can provide a significant amount of pertinent data to inform the elucidation of undiagnosed amyloid diseases.

Using PeakInvestigator™ Software to Reveal Hidden Peak Pairs in a LC/MS Dataset

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One of the persistent challenges in mass spectrometry (MS) is the separation of nearly-isobaric peaks. To address this challenge, MS manufacturers continue to develop instruments providing greater resolving power. However, greater resolving power comes with increased cost and often with lost sensitivity. Approaches that do not require an instrument upgrade or replacement, and which can be applied to improve the effective resolution of existing MS analyzers at any resolution, are highly desirable. We present a software solution (PeakInvestigator™) that detects, deconvolves and centroids peaks in mass spectra up to 4x better than current software offerings. Using a dataset from a 102 patient clinical metabolomic study conducted on an Agilent 6530 MS, we compared PeakInvestigator to Agilent’s MassHunter software for the separation of a nearly-isobaric lipid peak pair known to be present in the patient samples. In 98% of the patient samples. PeakInvestigator was able to detect and deconvolve both lipids in one or more MS scans from the LC/MS run, whereas MassHunter was unable to detect the presence of the hidden lipid pair in any scan for any patient. The ability to detect and accurately deconvolve hidden peaks in a mass spectrum acquired at any resolution has broad utility for biomarker discovery.