NewsSpots

Summer 2007

NHLBI PROTEOMICS

BOSTON TEAM BREAKS THROUGH WITH FIRST SIGNAL ON THE CRYOGENIC MALDI-FTMS

On May 18 Peter B. O'Connor, Cheng Lin, Raman Mathur, and Konstantin Aizikov reported the first signal on the cryogenic MALDI-FTMS instrument being developed at the Cardiovascular Proteomics Center (CPC) of the Boston University School of Medicine (BUSM). The instrument's design was conceived in 2001, published in mid-2002, and funded in October 2002 by the National Institutes of Health/National Heart Lung and Blood Institute as a major instrument development project of the NHLBI Proteomics Initiative. Figure 1 shows the researchers in front of the instrument.

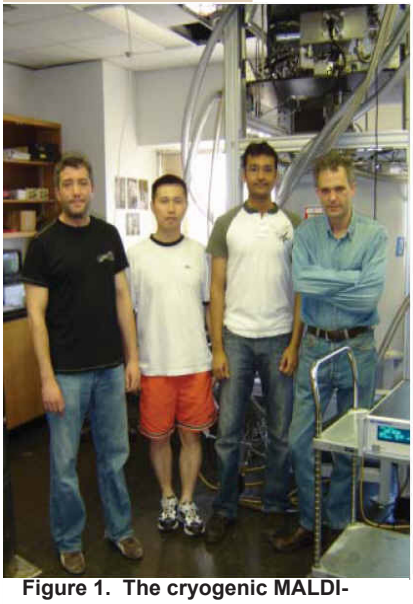

Figure 1. The cryogenic MALDI-FTMS researchers (*left to right*): Konstantine Aizikov, Dr. Cheng Lin, Raman Mathur, Prof. Peter B. O'Connor.

The Cryogenic MALDI-FTMS is a major advance in Fourier Transform Ion Cyclotron Resonance (FTICR) mass spectrometry design. It enables Matrix-Assisted Laser Desorption/Ionization-Fourier Transform Mass Spectrometry (MALDI-FTMS) at extremely low temperatures. It involves close construction and integration of an FTICR instrument with a modern cryogenic superconducting magnet design. This configuration provides three major advantages. First, the magnet bore and FTICR cell chamber become very cold, which cryopumps the chamber and decreases the base pressure. Second, because of the cryopumping, the bore tube diameter can be much smaller, allowing high homogeneity and high magnetic fields to be generated at greatly reduced cost. Third, the cold surfaces can be used to cool a preamplifier for improved signal-to-noise ratio.

The BUSM prototype instrument is designed with a 14 Tesla magnet at ~10 ppm homogeneity over the 2"x2" cylindrical ICR cell. When fully tuned, this instrument will provide performance several orders of magnitude better than existing instruments, using parts that cost about half as much and a magnet costing about four times less.

The first signal (Figure 2, *top*) is C_{60} laser desorbed, transferred down the two hexapoles, and detected in the ICR cell. Figure 2 (*bottom*) shows an optimized signal under current conditions. While many improvements remain to be made, and the amplifier now being used is at room temperature outside the vacuum, these images demonstrate a functioning instrument. The researchers have achieved the critical threshold, a signal that can be used to tune the instrument much better.

The bulk of this development project was funded by NIH/NHLBI under contract N01-HV-28178 with important components funded by NIH/NCRR grant P41 RR010888.

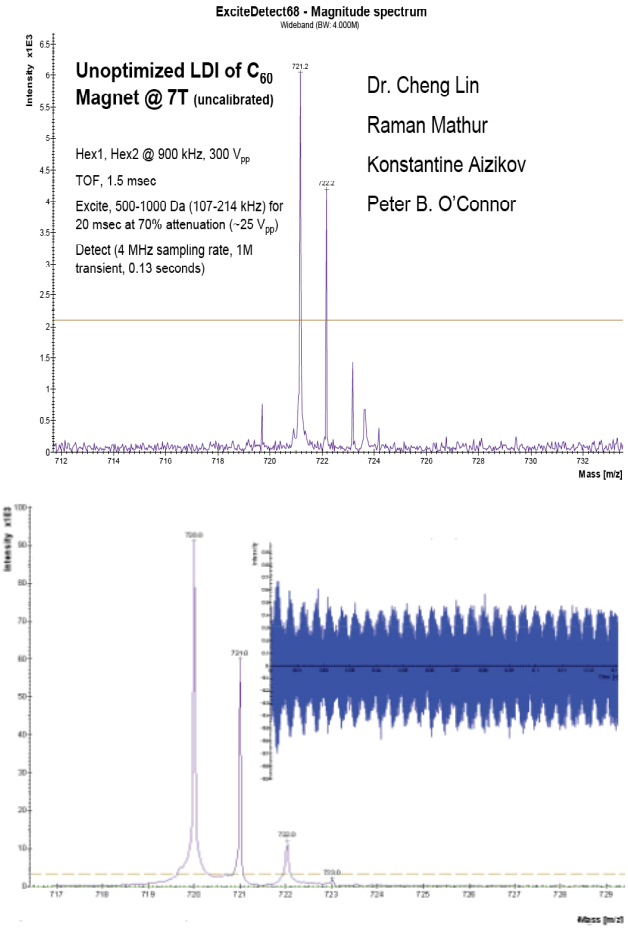

Figure 2. The first signal from the cryogenic MALDI-FTMS instrument (top), and an optimized C60 signal from the instrument (bottom). Inset shows the time domain signal.

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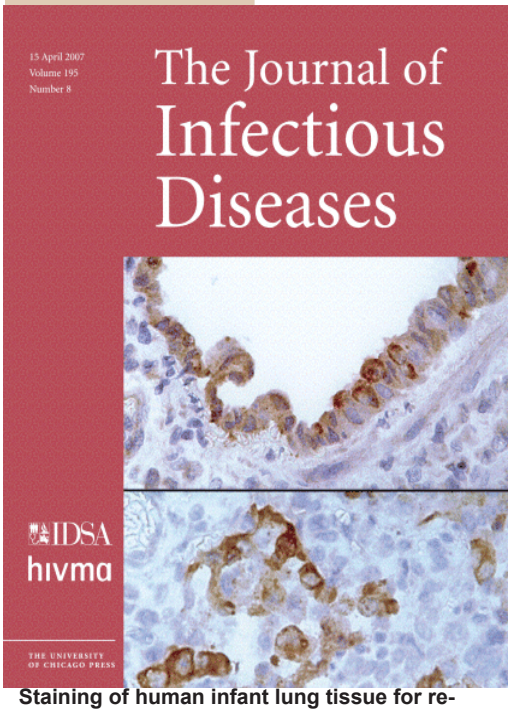
UTMB STUDY SHEDS NEW LIGHT ON COMMON CHILDHOOD RESPIRATORY INFECTION

A team of researchers led by members of the Proteomics Center at the University of Texas Medical Branch at Galveston (UTMB) recently published findings that may reverse the conventional wisdom on the mechanism underlying of severe respiratory syncytial infection.

The most common cause of infant hospitalization in the United States is respiratory syncytial virus or RSV, which infects virtually all children by age two. Normally RSV results in an upper respiratory infection similar to a common cold. However, in some babies it spreads deep into the lungs where it prompts coughing, wheezing and extreme difficulty in breathing—a clinical syndrome known as bronchiolitis. In these cases, the child's survival may depend on immediate medical attention.

Worldwide, RSV contributes (along with the influenza virus) to approximately two million infant deaths related to respiratory infection, according to the World Health Organization. To date, there is no safe and effective RSV vaccine to prevent severe respiratory infection and no specific antiviral therapy.

Until now medical scientists have believed that the most severe symptoms of RSV infection result from an overreaction in the lungs by specific immune cells known as T lymphocytes or T cells. Recently researchers at UTMB, the State University of New York at Buffalo, the University of Chile, the Hospital Roberto del Rio in Santiago, Chile, the University of Texas Southwestern Medical Center, Dallas, and MedImmune Inc. of Gaithersburg, Md., have generated data that turn that dogma on its head.



Staining of human infant lung tissue for respiratory syncytial virus (RSV) antigen in fatal cases of RSV lower respiratory tract infection. Immunohistochemistry stains demonstrate RSV in the airway epithelium (top) and in exfoliated epithelial cells occluding the terminal airway and alveoli (bottom). (Original magnification ×40)

Instead of being caused by too strong an immune response, the research team showed that severe RSV infections in the lower respiratory tract are associated with an inadequate immune reaction—a characteristic they share with fatal influenza infections, which were also studied by the group. These findings have major implications for efforts to develop therapies for RSV and perhaps other viral respiratory infections during infancy.

The *Journal of Infectious Diseases* featured this work on the cover of its April 15 issue. The findings were also reported by several online science dailies, including Science Daily on April 6 (http://www.sciencedaily.com/releases/2007/04/070405170227.htm) and Medical News Today on April 10 (http://www.medicalnewstoday.com/medicalnews.php?newsid=67411).

A UTMB news release issued prior to publication quotes one of the lead investigators, Dr. Robert Garofalo, of UTMB: "As part of our studies funded by the UTMB National Heart, Lung and Blood Institute Proteomics Center to study airway inflammation, we compared respiratory secretions from RSV-infected and influenza-infected babies, looking for proteins and cytokines—immune signaling molecules—made by T cells, and we saw no evidence that T cells had been activated in the RSV babies. In fact, irrespective of RSV or influenza infection, the cytokines we found in these infants were mostly those made by other types of cells of the lung, such as macrophages or epithelial cells."

Garofalo and his colleagues (including fellow senior author Robert Welliver of the State University of New York at Buffalo and UTMB postdoctoral research fellow Yashoda Hosakote) then put the data together with postmortem lung samples from autopsies of infant victims of severe RSV and influenza in Chile. (Although RSV causes extensive serious illness and a significant drain on medical resources in the United States, RSV fatalities in the U.S., are rare thanks to the widespread availability of advanced respiratory therapy.)

Analysis of the lung samples showed high concentrations of RSV and inflammatory signaling molecules associated with infection, but no sign of T-cell activity. "This significantly changes the way we look at how we want to intervene in terms of therapy," Garofalo said. "We all agree that killing the virus with anti-viral drugs, which we still don't have, is important. But it looks like we also need to find a way to control unwanted inflammation and boost the disease-fighting T-cell response."

Garofalo attributed the long-lived but mistaken "hyperactive T-cell" explanation for severe RSV to a "bias in the literature" influenced by studies of infections that occurred in infants who had been given a flawed experimental RSV vaccine in the mid-1960s. Instead of preventing RSV infections, that vaccine actually made them worse, resulting in the deaths of two children in 1967. "I think there was pretty strong evidence of increased T-cell response in the post-vaccine infections,

and people started to think that even the natural primary infections of unimmunized babies had these characteristics, too," Garofalo said.

The significant details of the study, "Severe Human Lower Respiratory Tract Illness Caused by Respiratory Syncytial Virus and Influenza Virus Is Characterized by the Absence of Pulmonary Cytotoxic Lymphocyte Responses," appear in vol. 195, no. 8 of the *Journal of Infectious Diseases*. It is accompanied by an editorial commentary emphasizing its significance, entitled "A New Direction in Understanding the Pathogenesis of Respiratory Syncytial Virus Bronchiolitis: How Real Infants Suffer," by John P. DeVincenzo.

The paper's authors include Timothy P. Welliver, LuAnn McKinney and Jennifer L. Reed of MedImmune; Karen H. Hintz and Pearay L. Ogra of the State University of New York at Buffalo; Luis Avendano and Katherine Sanchez of the University of Chile; Luis Velozo of Santiago's Hospital Roberto del Rio; and Hasan Jafri and Susana Chavez-Bueno of the University of Texas Southwestern Medical Center, Dallas. The study was supported by grants awarded to UTMB by the National Institute of Allergy and Infectious Diseases and the National Heart, Lung and Blood Institute.

Reference

Welliver TP, Garofalo RP, Hosakote Y, Hintz KH, Avendano L, Sanchez K, Velozo L, Jafri H, Chavez-Bueno S, Ogra PL, McKinney L, Reed JL, Welliver RC Sr. Severe human lower respiratory tract illness caused by respiratory syncytial virus and influenza virus is characterized by the absence of pulmonary cytotoxic lymphocyte responses. *J Infect Dis* 2007 Apr 15; 195(8):1126-36. Epub 2007 Mar 9.

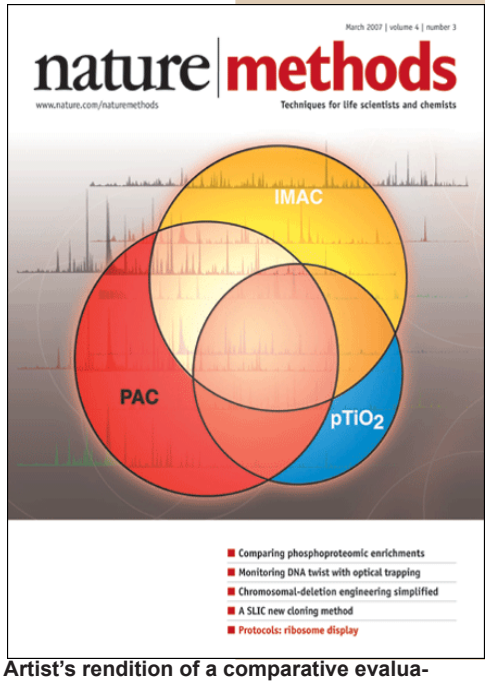
SEATTLE PROTEOME CENTER PROPOSES A COMBINED STRATEGY FOR PHOSPHOPROTEOMICS

Among the several hundred known post-translational modifications, protein phosphorylation is particularly important because it is involved in the control of essentially any biological process, and is thus involved in many diseases. The systematic and quantitative analysis of phosphorylation sites on single proteins and protein mixtures has therefore been a high priority goal of proteomics research. Because phosphopeptides usually are minor and rare components of the peptide samples generated for proteomic analyses by LC-MS/MS, they cannot be routinely analyzed without first specifically enriching them from the complex matrix of non-phosphorylated peptides.

Over the past few years several methods for the selective enrichment of phosphorylated peptides from complex sample mixtures have been developed—among them, phosphopeptides selection via phosphoramidate chemistry by the Seattle Proteome Center [1]. The availability of several methods has generated heated debate about which one might be the most suitable.

In a recent study by investigators at the Seattle Proteome Center, three common phosphopeptide isolation methods based on phosphoramidate chemistry, immobilized metal affinity chromatography and titanium dioxide were assessed with respect to their ability to reproducibly, specifically and comprehensively isolate phosphopeptides from complex mixtures. Phosphopeptides were isolated from aliquots of a tryptic digest of the cytosolic fraction of *Drosophila melanogaster* Kc167 cells and analyzed by liquid chromatography-electrospray ionization tandem mass spectrometry. The results, published as a cover article in *Nature Methods*, showed that each method reproducibly isolated phosphopeptides. However, the methods differed in the specificity of isolation and, importantly, in the set of phosphopeptides isolated.

The results suggest that the methods detect different, partially overlapping segments of the phosphoproteome and that therefore, at present, no single method by itself is sufficient for a comprehensive phosphoproteome analysis. The importance of this work lies in the recognition that phosphoproteome studies should apply several methods in parallel to obtain results of maximal significance.



Artist's rendition of a comparative evaluation of three phosphoproteomics enrichment methods followed by ms analysis.

References

1. Bodenmiller B, Mueller LN, Mueller M, Domon B, Aebersold R. Reproducible isolation of distinct, overlapping segments of the phosphoproteome. *Nat Methods* 2007 Mar; 4(3):231-7. Epub 2007 Feb 11.

2. Tao WA, Wollscheid B, O'Brien R, Eng JK, Li XJ, Bodenmiller B, Watts JD, Hood L, Aebersold R. Quantitative phosphoproteomic analysis using a dendrimer conjugation chemistry and tandem mass spectrometry. *Nat Methods* 2005 Aug; 2(8)591-8.

HOPKINS RESEARCHERS TACKLE THE NITROSOPROTEOME WITH S-FLOS: A NOVEL METHOD FOR QUANTIFYING S-NITROSYLATION SITES IN PROTEIN EXTRACTS, CELL LINES AND TISSUE

Nitric oxide (NO)-dependent cysteine S-nitrosylation (SNO) is a reversible post-translational protein modification critical for redox-sensitive cell signaling, which directly influences cell proliferation and survival. SNO modifications regulate protein function at many levels, including enzyme activity, receptor affinity or the open-state probability of ion channels. Changes in SNO levels correlate with many human diseases involving myocardial ischemia/reperfusion injury, pulmonary inflammation and sepsis. Rapid global detection of biologically relevant SNO modifications would help identify novel NO signaling pathways and potentially lead to new therapies for cardiovascular-related diseases.

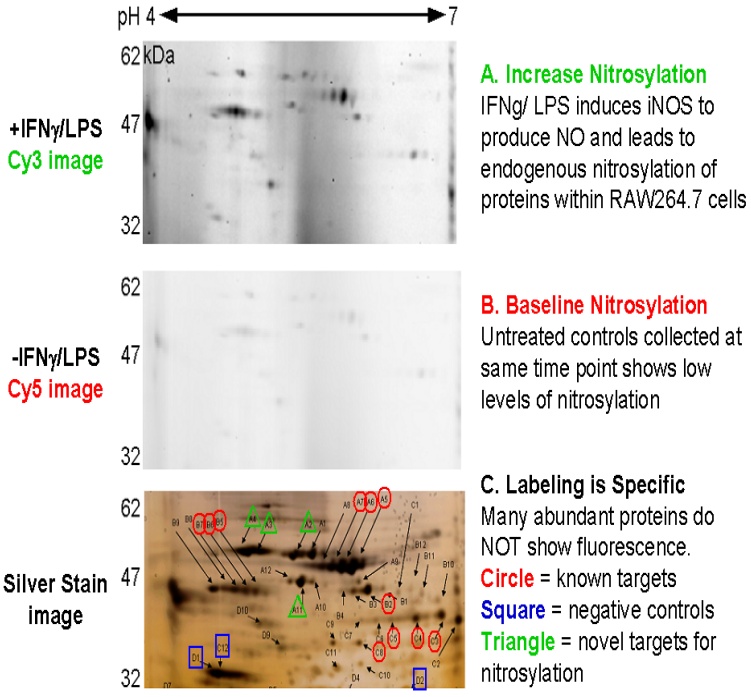
SNO is triggered either by exposure to NO from chemical or protein NO donors, or by endogenous production via NO synthases. However, SNO modifications are highly labile and thus technically challenging to study. In 2001 Jaffrey et al. [*Nat Cell Biol* 3, 193-7] published a clever method, the Biotin Switch, which rapidly became the best method for identifying nitrosylated proteins. This method replaces the SNO modification with a biotin tag that can be used for rapid detection or enrichment of SNO-containing proteins. Yet this powerful method has two major limitations: high false positive rate from endogenous biotinylated proteins and co-isolating proteins interacting with SNO proteins, and the inability to quantify directly SNO modifications in complex mixtures of proteins due to losing the biotin tag under reducing conditions. These limitations are overcome by a modified biotin switch method, termed Selective Fluorescent Labeling of S-nitrosothiols (S-FLOS) developed in the Technical Implementation and Coordination Core of the Hopkins NHLBI Proteomics Center, directed by Dr. Bob Cole, in collaboration with the lab of Dr. Dan Berkowitz in the Cardiac Anesthesiology Department at Hopkins.

The S-FLOS method modifies the Biotin Switch method and selectively labels proteins at S-nitrosylated sites with a fluorescent tag that is stable in reducing conditions. The S-FLOS method produces virtually no background and thus few or no false positives, provided blocking of free cysteines is performed carefully to completion. Compared to colorimetric Griess-Saville assay for quantifying SNO content, the S-FLOS is at least 10 times more sensitive and can detect fmol levels of S-nitrosylation. In addition, S-FLOS has great potential for mapping biologically relevant nitrosylation sites because the fluorescent-labeled peptides generate a pair of signature ions upon fragmentation. Finally, S-FLOS can be used to visualize changes in S-nitrosylation in intact cells or disease tissue slices.

Thus, the S-FLOS method is a significant improvement over the standard biotin switch method for studying the biological role of protein S-nitrosylation. This method can image, directly quantify and identify S-nitrosylation sites on individual proteins in complex mixtures, gels (see figure), cells and tissues. S-FLOS is a cross-platform method, not restricted to gel electrophoresis, mass spectrometry, or cytohistochemistry-based applications. This promises to have a significant impact in helping to uncover functional proteomic changes in disease states in which nitros-redox balance is dysregulated.

In addition to being presented at the NHBLI center meetings, the S-FLOS method has been presented at the HUPO 5th Annual World Congress (Long Beach CA) in October 2006, the Annual DIGE User Group Meeting (University of Georgia, Athens GA) in January 2007, and the AACR Special Conference on Advances in Proteomics in Cancer Research (Amelia Island FL), Feb 27-Mar 2, 2007). It will be presented at the 55th ASMS Conference on Mass Spectrometry in Indianapolis, IN (June 3-7, 2007). The method paper is currently under review and the authors have a provisional patent on the S-FLOS technique.

Detecting Nitrosylated Proteins using S-FLOS



A. Increase Nitrosylation
IFNg/ LPS induces iNOS to produce NO and leads to endogenous nitrosylation of proteins within RAW264.7 cells

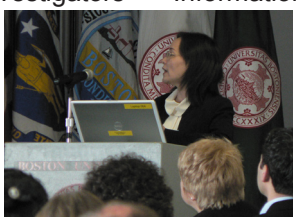
B. Baseline Nitrosylation
Untreated controls collected at same time point shows low levels of nitrosylation

C. Labeling is Specific
Many abundant proteins do NOT show fluorescence.
Circle = known targets
Square = negative controls
Triangle = novel targets for nitrosylation

BOSTON CPC HOSTS 9TH NHLBI PROTEOMICS INVESTIGATORS MEETING, APRIL 18-19

The Cardiovascular Proteomics Center (CPC) at the Boston University School of Medicine (BUSM) hosted the 9th Investigators Meeting of the NHLBI Proteomics Initiative on Wednesday and Thursday, April 18-19, 2007. Drs. Catherine Costello (PI, BUSM) and Kenneth Williams (PI, Yale University) co-chaired the program.

The Thursday session, Toward Systems Proteomics, was open to scientists from the greater Boston proteomics community. Thursday's highlights included welcoming remarks by Dr. Karen Antman, Provost and Dean of the Boston University School of Medicine, a Keynote Lecture by Dr. Joseph Loscalzo, Hersey Professor of the Theory and Practice of Physic at Harvard Medical School and Head of the Department of Medicine at the Brigham and Women's Hospital, and three Invited Lectures on Bioinformatics, Instrumentation for Proteomics, and Applications for Proteomics, respectively, by: Dr. Cathy H. Wu, Professor of Biochemistry and Molecular & Cellular Biology at Georgetown University Medical Center and Director of the Protein Information Resource; Dr. Stephen A. Martin, Senior Vice President and Chief Technical Officer of BG Medicine, Inc.; and Dr. Steven A. Carr, Director of Proteomics at the Broad Institute.



Dr. Cathy H. Wu

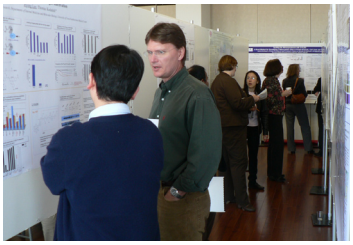
Dr. Joseph Loscalzo

Dr. Stephen A. Martin

Dr. Steven A. Carr

Other activities on Thursday included poster presentations from all ten centers, and oral presentations by individual center representatives in the three topical areas of bioinformations, instrumentation and applications.

The Investigators Meeting started officially at noon on Wednesday with lunch and informal discussion. Activities on Wednesday afternoon included four Highlight Presentations, two each from the host centers (BUSM and Yale), and round-table discussions to promote networking among the various centers. On Wednesday morning's the Center Principal Investigators met with NHLBI personnel, and other participants enjoyed lab tours and demonstrations in the BUSM CPC Core Proteomics and FTMS Labs.



Good scientific exchange during poster session and round table discussion

Highlights from young investigators Jesse Rinehart, Milind Mahajan, Raman Mathur, and David Perlman

Apropos

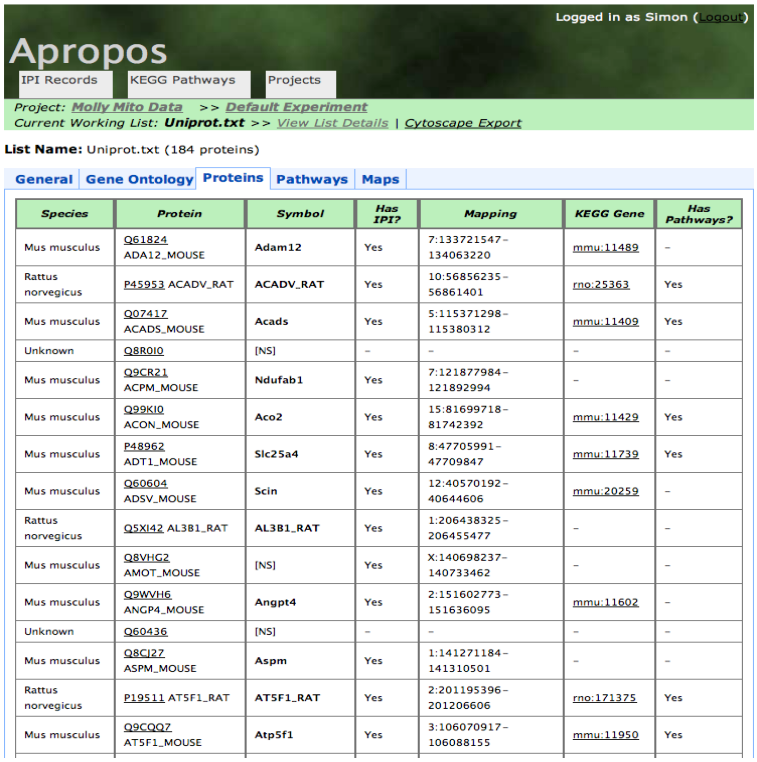
One of the major challenges facing proteomics and many other "-omic" technologies faces investigators in what should be the finishing stretch—the experiments have been run, proteins have been identified, now all that remains is to figure out what those proteins do, what pathways they are in, and how it all relates back to the experimental hypothesis. Unfortunately, big hurdles remain. It is often a relatively painful task to take a list of protein identifiers and pull out functional annotations, pathways, genome locations and other relevant data. In mammalian systems such as human, rat and mouse, you may be interested in not only the data from one species but related species as well. Any piece of information can be valuable at this stage.

To address this situation, the NHLBI Proteomics Center at the Medical College of Wisconsin (MCW) has developed a new piece of software named **Apropos**. Apropos is a web-based annotation tool that allows researchers to upload lists of identifiers for proteins or genes from rat, mouse or human and have the list annotated with a variety of functional annotations drawn from all three species.

The main experimental system used at the MCW Proteomics Center is the rat, specifically consomic (chromosome substitution) strains developed as part of the MCW PhysGen project (http://pga.mcw.edu). MCW also houses the Rat Genome Database, RGD (http://rgd.mcw.edu), and therefore has access to expertise in genome annotation and more specifically, access to annotations made using a variety of ontologies or structured vocabularies, such as the Gene Ontology, Mammalian Phenotype Ontology, a disease ontology and a pathway ontology. The Rat Genome Database uses these ontologies to annotate rat genes with similar work being done by counterparts at the Mouse Genome Database. Apropos takes the IPI protein databases for the three species and adds on top these various types of annotations along with KEGG pathway data to create a novel tri-species annotation database.

The Apropos software is open source, written using RubyOnRails and hosted on RubyForge (http://apropos.rubyforge.org). The primary installation is available at http://apropos.mcw.edu. Researchers can browse the database or register for an account and upload his or her lists of identifiers for annotation. The list of uploaded protein identifiers is searched against IPI's extensive list of cross-references to connect the identified proteins to one or more IPI protein records and their associated annotations. The orthology relationships established by the Mouse Genome Database are used to tie together IPI records from all three species into ortholog clusters. Once a protein is linked into the IPI database, Apropos draws together the annotations from all the members of the cluster containing that IPI record to create **a combined tri-species annotation report for each protein on the list.**

Once a list is annotated, the researcher has instant access to Gene Ontology annotations (listing the molecular functions, biological processes and cellular locations of identified proteins), Mammalian Phenotype annotations, KEGG pathway assignments and genome mapping data (Figure 1). The mapping data are presented across all three species for each protein and its orthologs, using the Center's FlashGViewer. This is particularly useful for groups like MCW who are focusing in on particular chromosomes. Of particular interest are any proteins that either map to one of the chromosomes that have been substituted



Species	Protein	Symbol	Has IPI?	Mapping	KEGG Gene	Has Pathways?
Mus musculus	Q61824 ADA12_MOUSE	Adam12	Yes	7:133721547-134063220	mmu:11489	-
Rattus norvegicus	P45953 ACADV_RAT	ACADV_RAT	Yes	10:56856235-56861401	rno:25363	Yes
Mus musculus	Q07417 ACADS_MOUSE	Acads	Yes	5:115371298-115380312	mmu:11409	Yes
Unknown	Q8R0I0	[NS]	-	-	-	-
Mus musculus	Q9CR21 ACPM_MOUSE	Ndufab1	Yes	7:121877984-121892994	-	-
Mus musculus	Q99KI0 ACON_MOUSE	Aco2	Yes	15:81699718-81742392	mmu:11429	Yes
Mus musculus	P48962 ADT1_MOUSE	Slc25a4	Yes	8:47705991-47709847	mmu:11739	Yes
Mus musculus	Q60604 ADSV_MOUSE	Scin	Yes	12:40570192-40644606	mmu:20259	-
Rattus norvegicus	Q5XI42 AL3B1_RAT	AL3B1_RAT	Yes	1:206438325-206455477	-	-
Mus musculus	Q8VHG2 AMOT_MOUSE	[NS]	Yes	X:140698237-140733462	-	-
Mus musculus	Q9WVH6 ANGP4_MOUSE	Angpt4	Yes	2:151602773-151636095	mmu:11602	-
Unknown	Q60436	[NS]	-	-	-	-
Mus musculus	Q8CJ27 ASPM_MOUSE	Aspm	Yes	1:141271184-141310501	-	-
Rattus norvegicus	P19511 AT5F1_RAT	AT5F1_RAT	Yes	2:201195396-201206606	rno:171375	Yes
Mus musculus	Q9CQQ7 AT5F1_MOUSE	Atp5f1	Yes	3:106070917-106088155	mmu:11950	Yes

Figure 1. Gene Ontology annotations in Apropos

in the consomic strains or that are involved in a pathway that has one or more proteins mapped to that same chromosome. The GViewer shows both a genome-wide view and a chromosome view, and provides links to individual protein reports and out to popular genome browsers for more high-resolution browsing (Figure 2).

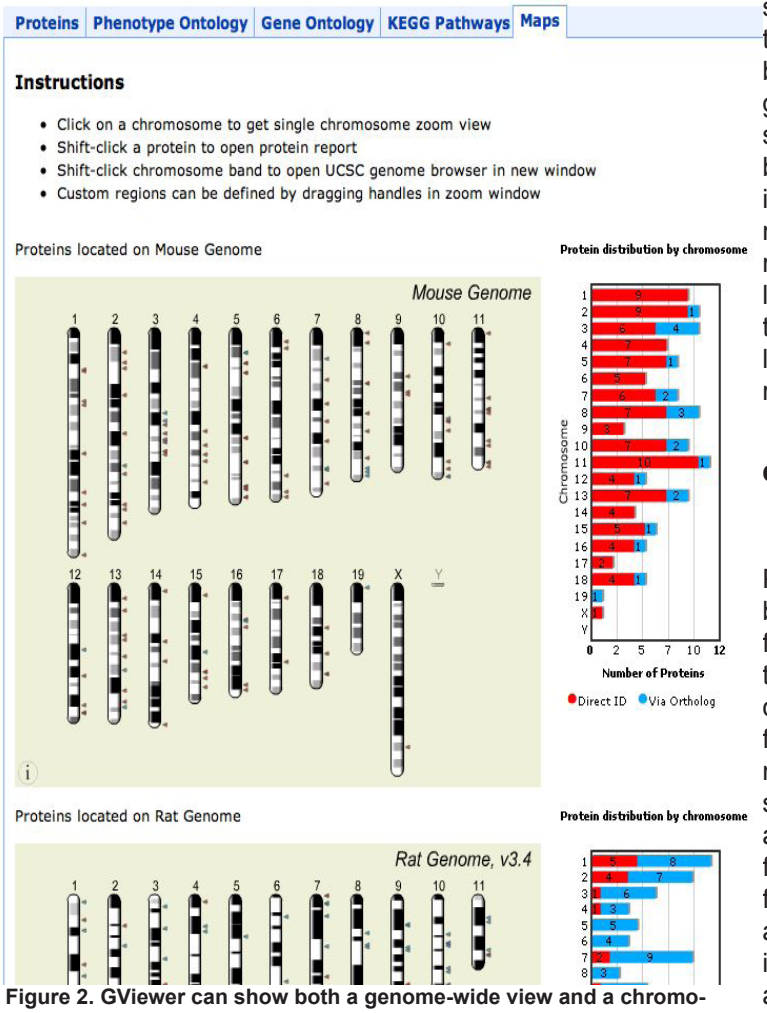


Figure 2. GViewer can show both a genome-wide view and a chromosome view and provides links to individual protein reports and to popular genome browsers.

Another novel graphical view is used to show the composite Gene Ontology annotations for each protein. Annotations are shown on a simplified version of the Gene Ontology annotation hierarchy with each annotation colored according to the species that contain that annotation or a more specific version of that annotation. The resulting graph (Figure 3) indicates which annotations have broad support from all three species and which may be unique to a specific species—hence what might have been missed had the annotation data been taken from just a single species.

Apropos can also export data in a Cytoscape-compatible format. This allows proteins and their annotations to be visualized as a network, permitting the researcher to quickly see connections and relationships that are hard to see in other ways. One can spot proteins that are located in the same part of the cell or that belong to the same pathway or give the same phenotype. At this stage, any biological connections between the proteins may be informative, and Apropos aims to make it as easy as possible for researchers to get from a protein list to the point where they are thinking about the biology with as little bioinformatics-induced interruption as possible on the way.

ez2

In addition, components of the MCW Suite of Proteomics Analysis Tools have been updated to include new features. A new data storage file type, the **.ez2** file, has been introduced. While keeping the best features of the .ezf file format, namely, compression to save on size and extensibility to allow the addition of different data, the .ez2 file permits more efficient and faster access to the data while also moving to an XML based internal format. Both the Visualize and ZoomQuant applications have been upgraded to be able to open the new .ez2 file type. Both can be found as part of the ZoomQuant application under http://proteomics.mcw.edu/zoomquant/.

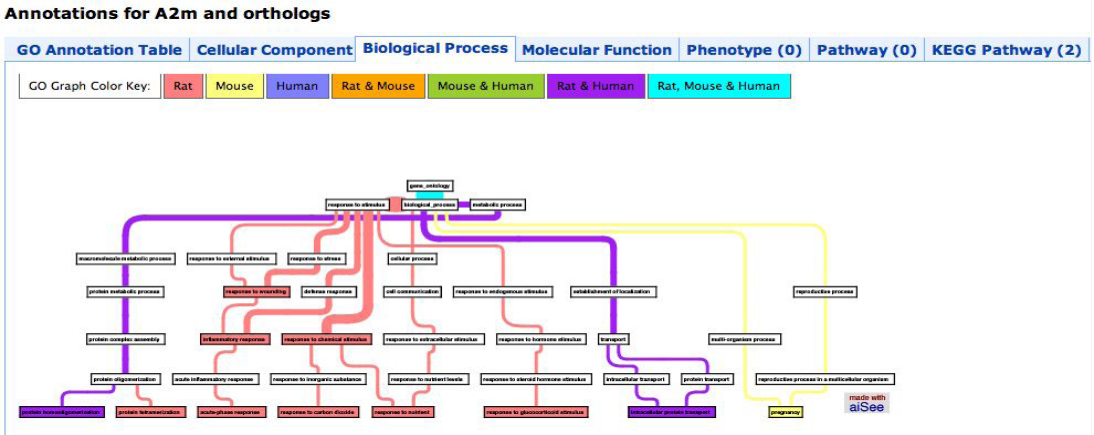


Figure 3. Annotations are shown on a simplified version of the Gene Ontology annotation hierarchy with each annotation colored according to the species that contain that annotation or a more specific version of that annotation.

Zoomquant

In addition to compatibility with the .ez2 filetype, **ZoomQuant** has been upgraded with new features. The user interface has been improved by changing the layout to better accommodate users with smaller (<17") displays. Also, to increase the efficiency and speed of workflow, additional keyboard shortcuts for moving from spectrum to spectrum and adjusting spectra have been added. An important new feature is the ability to save the state of the program in a .zsf file. This allows the user to save an analysis at any point and then later restore the ZoomQuant program to that exact point. In addition to allowing users to save their work and complete it at a later time, this feature lets users reopen an analysis and save outputs in different formats. In addition, the .zsf file format can serve as a convenient method to save both the spectral data and search data in a single, compressed file. A feature requested by users was ability to include the chemical formula of a modification into the calculation for isotopic distribution. Since most search engines do not specify the formula of a post-translational modification, just its mass and target amino acid, it was impossible to include the modification in the calculation of the isotopic distribution. Now, the user can include this information in a modified profile file (.lsp) and it will be used in the isotopic distribution calculations. In addition, new output formats have been defined, including an XML format compatible with the Proteus LIMS system (Figure 4).

Visualize

Visualize has also been upgraded with both improvements to the user interface and 'under the hood' changes that increase performance (Figure 5). One major upgrade is a switch to the PeptideProphet/ProteinProphet algorithms for ranking and filtering peptide and protein identifications. Epitomize now calculates the probability scores for peptide and protein identification using a Bayesian classifier, and Visualize incorporates this information into its display. It is also possible to run Epitomize from within Visualize, permitting a consistent user interface. New result files can also be combined from subsets of existing files or combining files. When combining replicate runs, the Visualize program automatically calculates the new protein probabilities based on the independent observations derived from multiple replicates. Files can also be compared, and Visualize now produces a sophisticated interactive display of the relative ratios of proteins present in samples (Figure 6). Comparisons can be made between a reference sample and a group of experimental samples or within a group of experimental samples based on the maximum or total values observed. The comparison results can also be formatted for dynamic display using the TreeMap tool based on either the GO ontologies or the KEGG pathways. Individual proteins can be linked to UniProt, NCBI and other web based tools. Lists of proteins can be exported for additional annotation in the Apropos tool (described above) or for visualization as a pseudo 2D gel by the ProMoST tool.

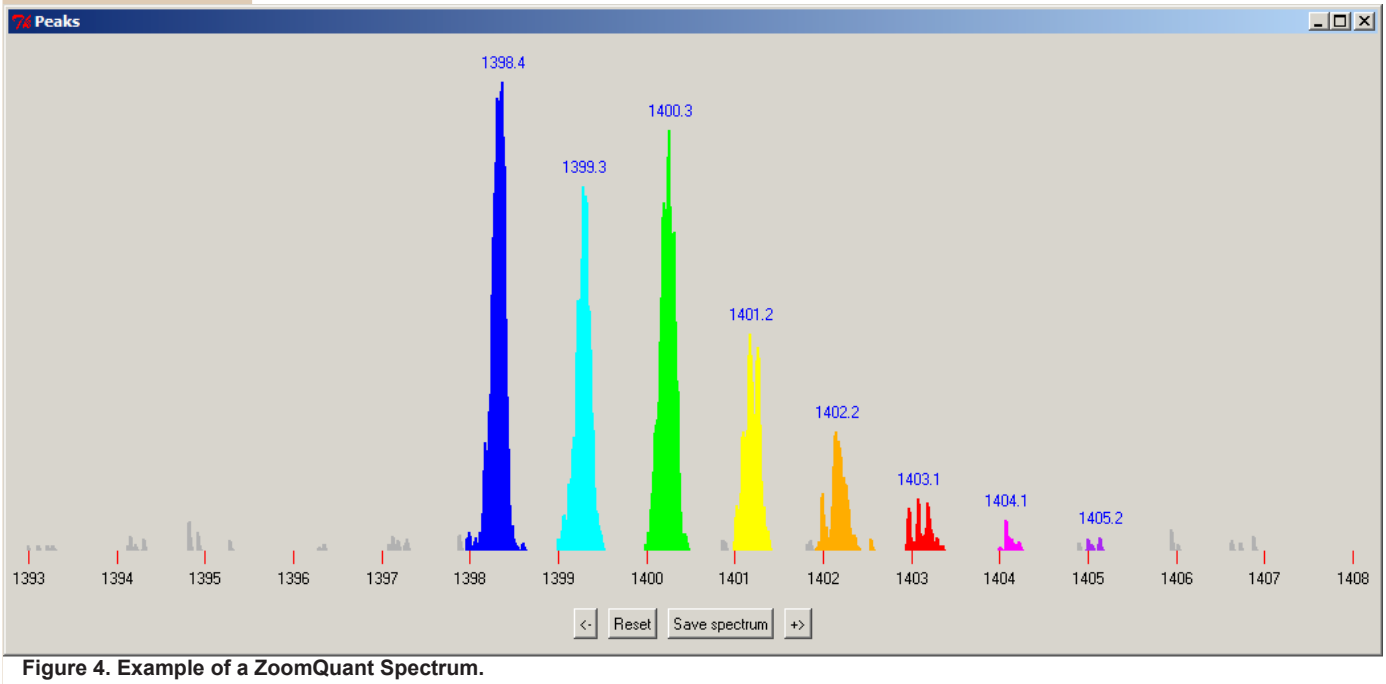


Figure 4. Example of a ZoomQuant Spectrum.

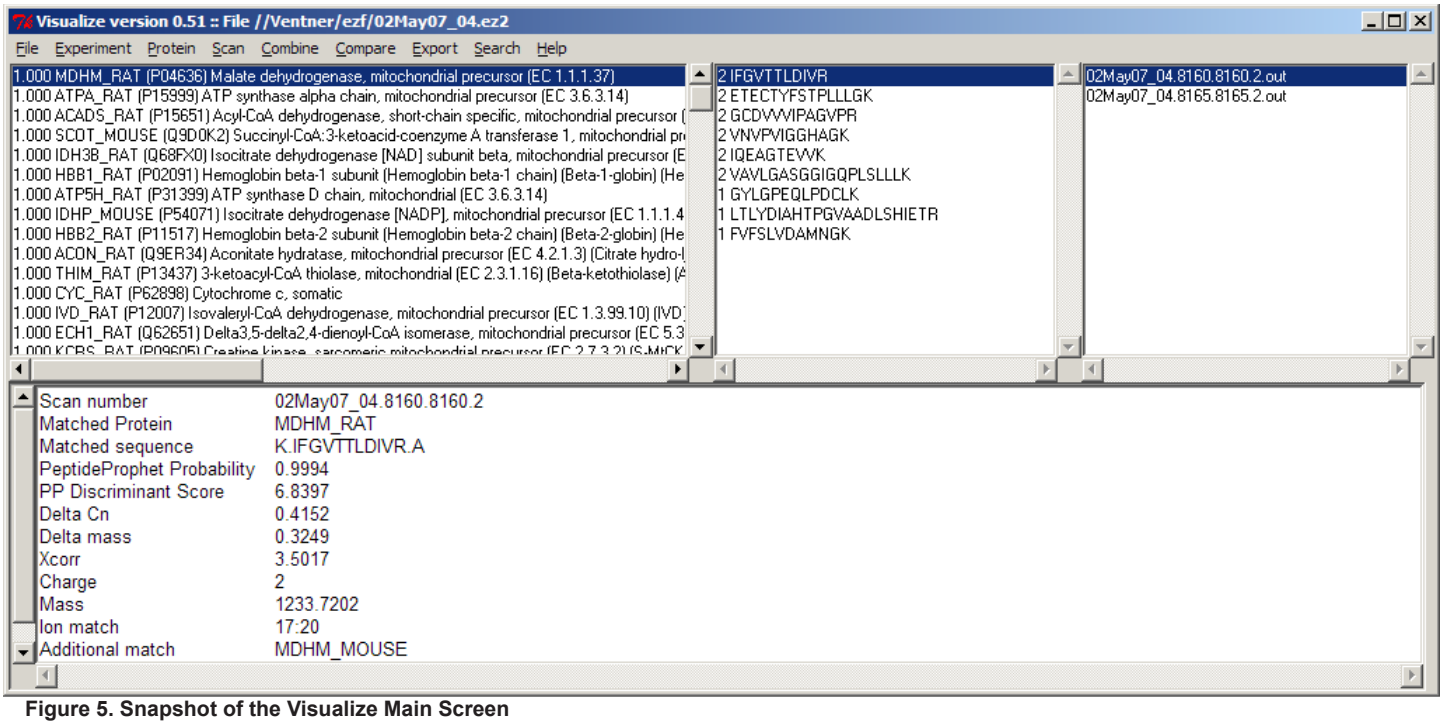


Figure 5. Snapshot of the Visualize Main Screen

Figure 6. Example for the sophisticated Visualize Comparison tool

BUSM SCIENTISTS SHOW REDOX MODULATION MAY BE IMPORTANT TO P21RAS GENE FUNCTION

Although increased oxidants contribute to many aspects of cardiovascular disease, scientists do not fully understand how oxidants regulate the function of a protein. Researchers at Boston University School of Medicine's Cardiovascular Proteomics Center recently quantified an increase in activity of the p21ras GTPase protein and correlated the increase with modification of one of the cysteine thiols on the surface of the protein [1].

The analytical approach uses a novel ICAT labeling method that can quantify both reversible and irreversible modifications. Cysteine-118 and terminal cysteines were the targets of reversible modification by the antioxidant, glutathione, and irreversible modification by the oxidant, peroxynitrite. Using this method as well as Fourier transform mass spectrometry [2], the research team also showed that of all the cysteines on this protein, cysteine-118 was the most sensitive to oxidants.

Although it is termed an "oncogene" because it is the most commonly mutated gene in a wide variety of tumors, p21ras also may be a factor in cardiovascular disease. Other studies by the BUSM group demonstrated that the glutathione modification of p21ras contributed to insulin resistance in endothelial cells exposed to oxidized lipoprotein [3], a factor associated with atherosclerosis. This suggests that the modification of p21ras may be a marker of metabolic syndrome and associated vascular disease.

Using new labeling tools, scientists now routinely quantify protein modifications, hoping to identify and understand other biomarkers with potential relevance to human diseases. The human proteome has yet to be completely elucidated. Researchers, however, are beginning to answer the practical 'how much' and 'what is the consequence' of changes in the proteome caused by oxidative modifications of proteins.

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PANDEY GROUP AT HOPKINS ADVANCES METHODS AND BIOINFORMATICS FOR PHOSPHOPROTEOMICS

The research group led by Dr. Akhilesh Pandey at the Johns Hopkins University NHLBI Protoemics Center recently published two papers contributing significantly to new methods and bioinformatic approaches for phosphoproteomic analyses.

In a recent paper in *Proceedings of the National Academy of Science*, the Pandey lab reports the use of electron transfer dissociation (ETD) tandem mass spectrometry to perform a global phosphoproteome analysis of HEK 293T cells. They identified the phosphorylated amino acid residue of a total of 1,435 phosphopeptides, 80% of which were not previously reported. A detailed comparison of ETD and collision-induced dissociation (CID) modes showed that ETD identified significantly more phosphopeptides than CID. Although ETD was found to be superior to CID for phosphopeptide analysis, the two methods could be effectively combined for a more comprehensive analysis. This work suggests that ETD should be an essential strategy in global phosphoprotein analysis.

Dr. Pandey and colleagues at Johns Hopkins and international collaborators have also added a new wrinkle to the human protein reference database to help researchers identify phosphorylation sites in proteins, PhosphoMotif Finder (http://www.hprd.org/PhosphoMotif_finder). This is not a putative motif searcher, rather it catalogs known substrates as well as binding motifs that are curated from the published literature. PhosphoMotif includes 16,000 phosphorylation sites described in the literature and allows investigators to find potential phosphorylation sites in any protein of interest. This new site is described in a recent report in *Nature Biotechnology* [2].

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SEATTLE PROTEOME CENTER DEVELOPS NEW LABELING TECHNIQUE FOR QUANTITATIVE PROTEOMICS

The identification and accurate quantification of proteins in high throughput analysis are essential components of proteomic strategies for biomarker discovery and studying cellular functions and processes. A common chemical approach for quantitative proteomics is the use of small chemical reagents (e.g., ICAT) or a solid phase reagent to simultaneously capture and label proteins in solution for analysis.

Supported as a pilot project of the NHLBI Seattle Proteome Center, Andy Tao and colleagues at Purdue University, West Lafayette, have developed a new strategy for quantitative proteomics called soluble polymer-based isotopic labeling (SoPIL). The method uses a soluble nanopolymer, highly branched dendrimer, to bind and deliver an isotope label to cysteine residues in a complex protein mixture. An alkyne group on the dendrimer then acts as a handle to join the dendrimer to a solid phase reagent, allowing them to be removed easily from the solution. The tagged proteins/peptides can then be released from the solid and analyzed by mass spectrometry. Compared to the established single-step solid phase capture and tagging method, the SoPIL two-step strategy labels proteins much more quickly and recovers the labeled peptides in a higher yield.

In the first application of SoPIL [1], the research team quantified the differences in protein levels and enzyme activity between two different samples of snake venom. Snake venoms contain complex mixtures of proteins and other pharmacologically active molecules, and have great implications for biomedical applications. The hemorrhagic, hemolytic, clotting ability and fibrinogenolytic activities of crude venoms were measured and correlated with difference in protein abundance as determined by SoPIL analysis.

A wide range of applications, including probing various protein modifications in biological systems, should be possible by choosing different reactive groups on the dendrimer. One important direction is *in vivo* proteomics. Today, most proteomics approaches require physical disruption of cells before *in vitro* analysis, which could affect protein function and distributions. Used essentially as delivery vehicles, SoPIL agents can readily permeate cell barriers and interact with target proteins through functional groups on the surface. This would give a more accurate indication of the proteins in the living cell.

This pioneering study drew immediate attention from multiple scientific media. In particular, Earth and Sky Radio featured the technique on its 90-second program on March 22, 2007 [2], and *ProteoMonitor* ran a feature article reporting it as a pioneering proteomics technology [3]. The research was also reported by numerous online science magazines.

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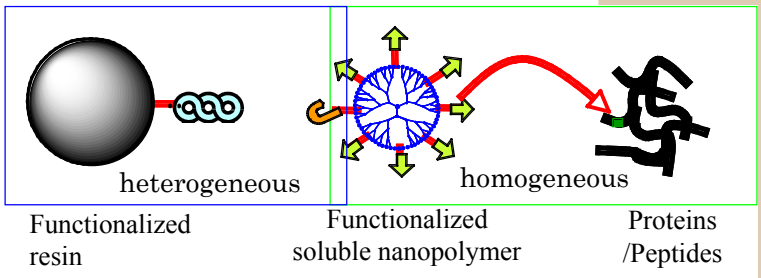


NIH PROTIG WILL CONTINUE

Based on the enthusiastic responses received to an email survey, Dr. Sanford Markey announced in mid-May that the NIH Proteomics Interest Group, or ProtIG, will continue its webcast series during 2007-08. The ProtIG Steering Committee will select speakers during the summer. The schedule will be released in the fall.

Anyone wishing to nominate a speaker for the webcast seminar series should spend a brief description of the individual and his/her work to Dr. Markey at markeys@mail.nih.gov. Nominees in the fields of proteomics for clinical or basic biological applications, bioinformatics, instrumentation and method developments are welcome.

YALE PROTEOMICS CENTER INVESTIGATORS REVEAL NEW WNK KINASE PHOSPHORYLATION SITES USING ROBUST MS-BASED PHOSPHOPEPTIDE ENRICHMENT STRATEGIES

Pursuing highly promising proteomic research into WNK kinases—signaling proteins essential for normal blood pressure regulation—researchers in Dr. Richard Lifton's laboratory at Yale University have uncovered several new sites of phosphorylation in multiple members of this family of regulatory kinases. Their work was made possible by the development in the Yale/NHLBI Proteomics Center of a simple and robust method of phosphopeptide enrichment using TiO_2-based technology coupled with MS detection.

The kidney regulates blood pressure by controlling the balance of sodium retained in the blood or excreted in the urine. Mutations in genes that normally control this process cause hypertension. Thus, researchers believe that understanding the dynamic state of kidney proteins in normotensive and hypertensive mouse models will uncover molecular mechanisms of blood pressure control. They also note that because of the often poor correlation between mRNA and protein expression—with differential rates of translation of individual mRNAs and differing turn-over rates *in vivo* of individual proteins, perhaps contributing to the relatively poor correlation—research is best carried out at the protein rather than at the mRNA expression level. Additionally, mRNA expression profiling cannot provide information about protein post-translational modifications such as phosphorylation that often play key roles in modulating protein function.

The power of the MS-based phosphopeptide detection approach derives not only from the absolute identification of biologically relevant sites, but also from the subsequent quantitative analysis of the extent of phosphorylation at each site of interest that can be carried out once the sites have been identified. In addition, multiple sites of phosphorylation can be analyzed simultaneously.

Using improved TiO_2 phosphopeptide enrichment methodologies, researchers in the Lifton lab recently identified 11 novel phosphorylation sites in the kinase WNK2 (Figure 1), and 5 sites in the kinase WNK4. This strategy has allowed the Yale team to begin to uncover the molecular mechanisms unique to the regulation of this important family of kinases. WNK4 is of particular interest as it is part of a novel signaling network involved in blood pressure homeostasis. Figure 2 highlights preliminary mass spectral results for one of the identified phosphopeptides in WNK4 before and after the phosphopeptide enrichment process.

WNK4 is an active kinase and is likely part of an intracellular signaling cascade that alters the phosphorylation state of multiple proteins, hence the importance of improved phosphoproteomic technologies to characterize this signaling pathway. A detailed understanding of regulatory phosphorylation sites on WNK4 would provide clues to the unknown players in the WNK signaling network. No WNK4 phospho-specific antibodies are currently available. The large number of predicted phospho-sites within WNK4 makes an experimental determination of the sites essential. An antibody-based approach has a lower probability of success and cannot differentiate complex phosphorylation events. By coupling phosphopeptide enrichment with the use of

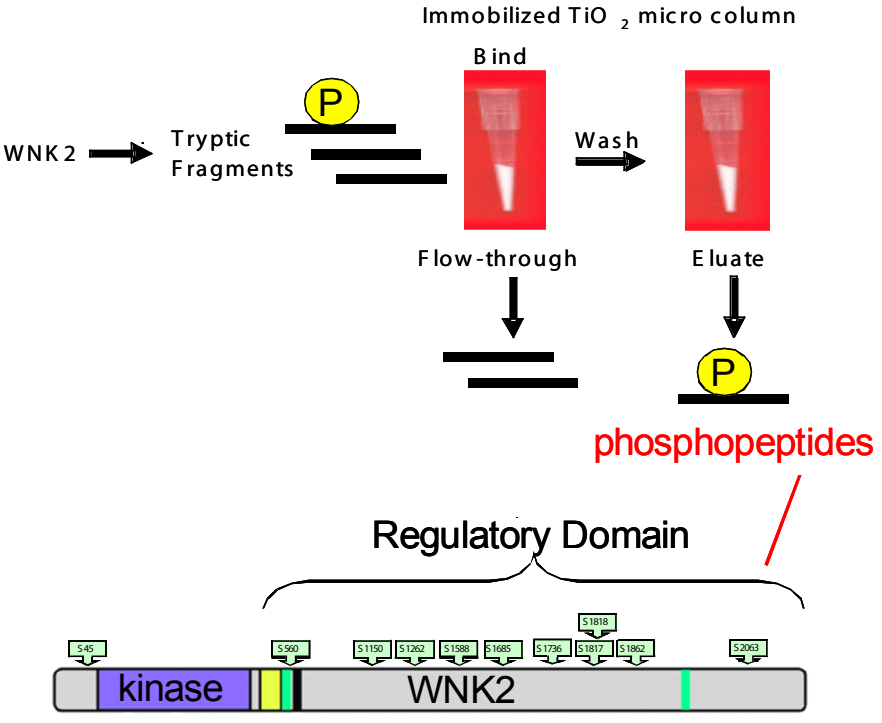


Figure 1: Schematic presentation of the phosphopeptide enrichment process using TiO2 microcolumns (top). Depiction of all of the sites of phosphorylation detected on WNK2 kinase (bottom).

synthetic, stable isotope-labeled, internal standard phosphopeptides, the Yale group hopes to be able to quantify the level of phosphorylation at *individual* WNK4 sites in response to physiological stimuli. This information could then be used to test novel aspects of WNK4 signaling in two WNK4 mouse models currently being studied in the Lifton Laboratory. This research should ultimately shed light on the molecular mechanism(s) of blood pressure regulation and related diseases such as hypertension.

Recent technological advances in the Proteomics Core of the Yale/NHLBI Proteomics Center make it potentially feasible to carry out global analysis of phosphoproteomes. However, to take maximum advantage of the tryptic phosphopeptide enrichment and other newly developed/improved phosphoproteomic methodologies requires a more sophisticated MS platform than is now available in the Yale/NHLBI Proteomics Core. For this reason the Proteomics Core is making a very concerted effort to acquire a more powerful mass spectrometer that will have the higher acquisition speed, higher mass accuracy (i.e. 2-5 ppm), higher resolving power (i.e. >60,000), and the MS^n fragmentation capability needed to confidently identify far larger numbers of phosphopeptides from tryptic digests of complex cell or tissue extracts than is possible on their existing MS platforms.

For more information, please contact Jesse Rinehart (jesse.rinehart@yale.edu) or Erol Gulcicek (erol.gulcicek@yale.edu).

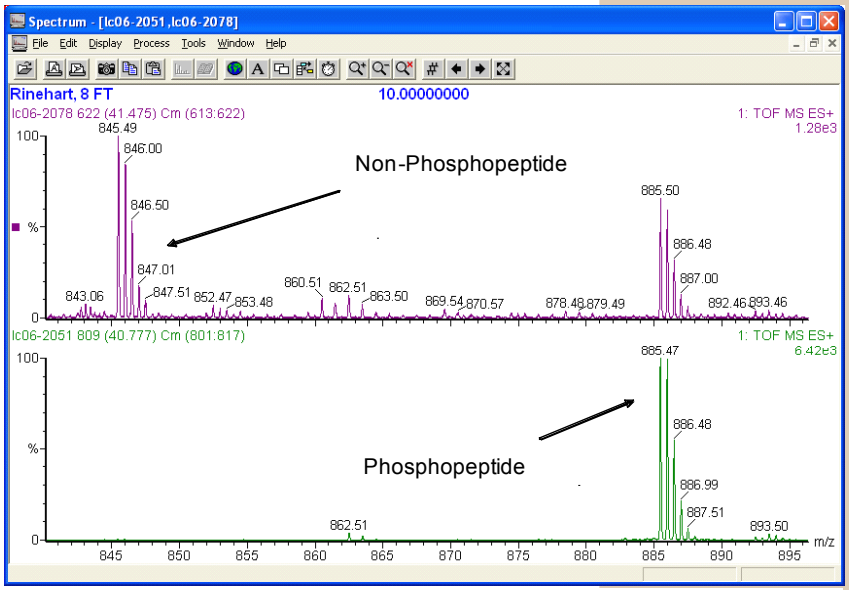


Figure 2: Mass spectra showing both the unmodified and modified forms of WNK4 peptides before (top), and only the phosphorylated peptide after the TiO_2 enrichment (bottom).

HOPKINS CENTER INVESTIGATORS RECEIVE NIH HIGH END INSTRUMENTATION GRANT

On April 26, 2007 the Johns Hopkins School of Medicine received a grant of $928,365 from the NIH High End Instrumentation (HEI) Program for the purchase of a Fourier transform (FT) mass spectrometer (FTMS). The instrument, selected by the group of nine investigators who make up the user group, is a Thermo LTQ/FTMS, a hybrid linear ion trap and FT mass spectrometer, equipped with electron capture dissociation (ECD) and infrared multiphoton dissociation (IRMPD).

Jennifer Van Eyk, Robert J. Cotter and Raimond Winslow, members of the Johns Hopkins NHLBI Proteomics Center, will benefit from the high mass accuracy of this instrument (1-5 ppm) for the identification of cardiac biomarkers, as will the work of Avindra Nath on biomarkers from CSF for HIV dementia and multiple sclerosis.

Other investigators affiliated with the NLHBI Proteomics Center at Hopkins will benefit as well. Electron capture dissociation is well known for its ability to preserve post-translational modifications while fragmenting the peptide bond, and will be invaluable in elucidating phosphorylated protein structures (Akhilesh Pandey) and proteins containing O-GlcNAc (Gerald Hart). Post-translational modifications to histones, histone acetyltransfereases (HATs) and histone deacetylases are a major interest to members of the NIH Roadmap Technology Center for Networks and Pathways (TCNP) of Lysine Modifications—Jef Boeke, Robert J. Cotter, Philip Cole and Shelley Berger (Wistar Institute). High resolution (in excess of one part in 300,000) will enable these investigators to distinguish acetylation and trimethylation sites, while ECD will be used to develop “top down” fragmentation of intact histones to observe the concurrence of multiple modifications on the same molecule.

The Principal Investigator for the grant is Robert J. Cotter (rcotter@jhmi.edu). The instrument will be located in the Middle Atlantic Mass Spectrometry Laboratory at the Johns Hopkins University.

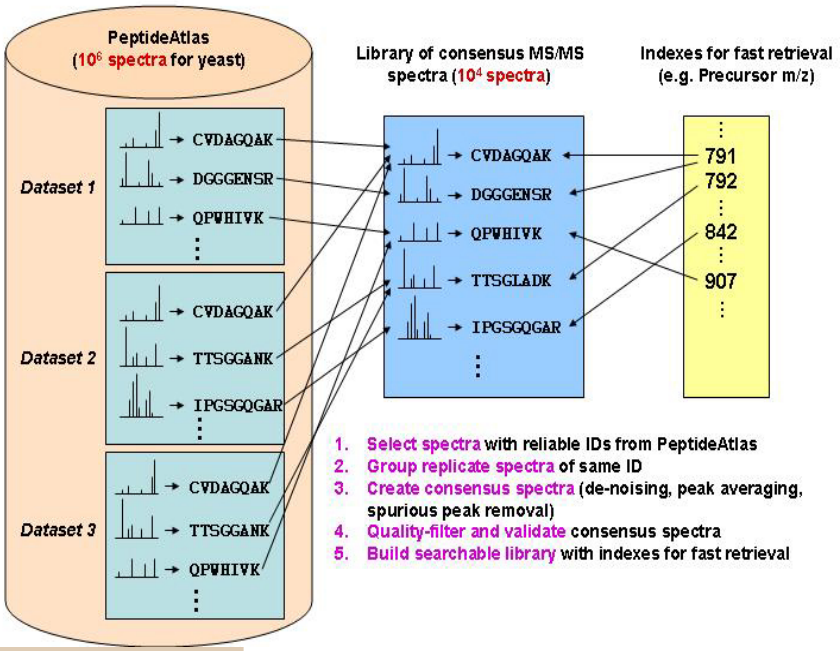
SEATTLE PROTEOME CENTER DEVELOPS SUPERCHARGED DATABASE SEARCH ENGINE

A notable inefficiency of shotgun proteomics experiments lies in the repeated rediscovery of the same identifiable peptides by sequence database searching methods, which often are time-consuming and error-prone. A more precise and efficient method, in which previously observed and identified peptide MS/MS spectra are catalogued and condensed into searchable spectral libraries to allow new identifications by spectral matching, is seen as a promising alternative, especially for targeted proteomics applications. This approach takes advantage of the rapidly growing amount of peptide MS/MS spectra held in public data repositories, such as the PeptideAtlas of the Seattle Proteomics Center. Repeated identifications of each observed peptide ion from traditional sequence searching are combined to form a high-quality and representative "consensus" spectrum. A library of these consensus spectra is carefully filtered to remove incorrectly identified and low-quality spectra, then indexed for fast searching. Subsequent peptide identification from a query MS/MS spectrum is then achieved by fast indexed searching against this library, using a spectral similarity scoring function.

Scientists at the NHLBI Seattle Proteome Center have developed an open-source, functionally complete, high-throughput and readily extensible software tool, SpectraST, to perform the tasks of spectral library building and searching [1]. As proof of principle, a consensus spectral library of more than 30,000 peptide ions was created from over 1.4 million confidently identified spectra in 22 datasets in the Human Plasma PeptideAtlas [2]. The same datasets were then re-searched against this library using SpectraST. Compared to the sequence search engine SEQUEST, SpectraST was about 500 times faster and achieved much better discrimination between the correct and incorrect hits. Consequently, SpectraST identified >60% more spectra at the same probability threshold of 0.9, compared to those made by SEQUEST, at vastly improved sensitivity and error rates.

In order to facilitate user adaptation, SpectraST was seamlessly integrated into the popular Trans Proteomic Pipeline (TPP) suite of software, which adopts standardized file formats and provides full workflow support, including such functionalities as automatic validation, protein assignment, quantitation and data visualization. It is predicted that spectral searching will replace traditional sequence searching in most proteomics workflows in the near future, providing more accurate data and reducing the computational overhead of shotgun proteomics by orders of magnitude.

Creating a Spectral Library of Previously Observed Peptides



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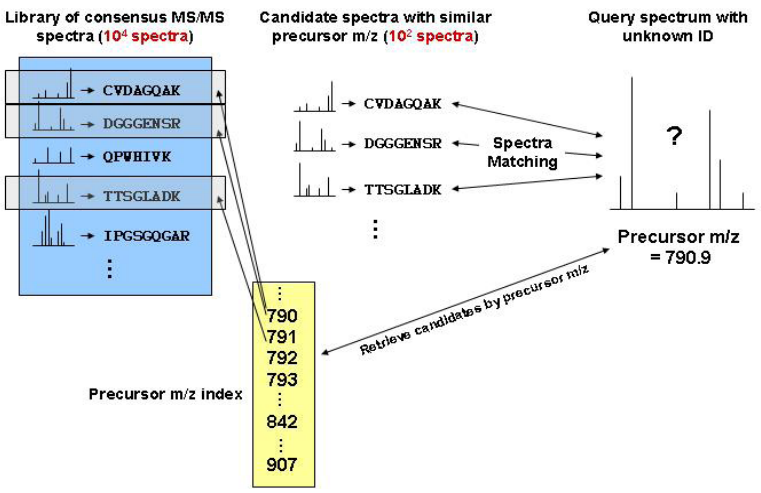
Peptide Identification by Library Searching



UPCOMING CONFERENCES

June 3 - 7, 2007— 55th ASMS Conference on Mass Spectrometry, Indiana Convention Center Indianapolis, IN

The program will begin with tutorial lectures at 5:00 pm on Sunday, June 3, followed by the conference mixer in the exhibit hall. Monday through Thursday will be full program days of concurrent oral sessions, poster sessions, and workshops. For more information visit https://www.asms.org/Default.aspx?tabid=43.

September 19 - 20, 2007—10th NHLBI Proteomics Initiative Investigators Meeting, Bethesda, MD

For more information visit https://www.nhlbi-proteomics.org/meetings/

October 6 - 10, 2007—HUPO 6th Annual World Congress, Seoul Korea

Program theme is Proteomics: From Technology Development to Biomarker Applications

For more information visit http://www.hupo2007.com/

Oct 23 - 27, 2007 —The American Society of Human Genetics 57th Annual Meeting, San Diego, CA

The objective of this meeting is to provide a forum for the exchange of new research by scientists and investigators. At the completion of the meeting, participants should be able to: 1) discuss the research underway and/or the current topics relevant to their areas of interest in human genetics, 2) demonstrate a gained level of insight into the methods being used by researchers and practitioners in this field, and 3) describe a personal exposure to several stimulating areas of inquiry with speakers in related areas.

For more information visit http://www.ashg.org/genetics/ashg/menu-annmeet.shtml

November 3 - 7, 2007— AHA Scientific Sessions, Orlando, FL

Scientific Sessions encompasses five days of invited lectures and investigative reports. Simultaneous presentations represent all fields of cardiovascular and related disciplines.

This year's programming includes:

- Over 4,000 basic, clinical and population science abstract presentations
- Over 1,000 world-renowned faculty presenting cutting-edge science
- Clinical practice sessions focusing on current standards of care for practicing clinicians
- Translational science sessions that bring together basic scientists and clinicians
- Increased programming for interventional cardiologists and electrophysiologists
- Increased international focus on stem cells and myocardial regeneration
- Early Career Awards/Competitions on Sunday afternoon

For more information visit http://scientificsessions.americanheart.org/portal/scientificsessions/ss/

March 16 - 19, 2008—4th Annual US HUPO Conference, at the Bethesda North Marriott Hotel & Conference Center, North Bethesda, Maryland.

Conference themes:

- Proteomic Technology/Mass Spectrometry
- Proteomic Applications/Systems Biology
- Clinical Proteomics
- Proteomic Bioinformatics

For more information visit http://www.ushupo.org/

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