High-Performance Mass Spectrometry Facility

The High-Performance Mass Spectrometry Facility (HPMSF) provides state-of-the-art mass spectrometry (MS) and separations instrumentation that has been refined for leading-edge analysis of biological problems with a primary emphasis on proteomics. Challenging research in proteomics, cell signaling, cellular molecular machines, and high-molecular weight systems receive the highest priority for access to the facility. Current research activities in the HPMSF include proteomic analyses of whole cell lysates, analyses of organic macro-molecules and protein complexes, quantification using isotopically labeled growth media, targeted proteomics analyses of subcellular fractions, and detection of post-translational modifications (PTM) such as phosphorylation and ubiquination. More than a dozen microbial systems are currently being studied in HPMSF by researchers from throughout the country. In addition, there are several ongoing projects in higher order systems (including mammalian systems) that are investigating a broad range of biological questions from cancer screening to infectious diseases to fundamental questions of post-translational modifications and protein-protein interactions.

Capabilities

The facility features state-of-the-art liquid chromatographic (LC) separations capabilities coupled to a complete suite of MSs for proteomics analysis. The available instruments range from a group of nine ion trap spectrometers for tandem mass spectrometry (MS/MS) work to very high-sensitivity and high-resolution Fourier transform ion cyclotron resonance (FTICR) spectrometers that offer 1 ppm mass measurement accuracy. Proteomics analysis on these FTICR spectrometers is complemented by a quadrupole time-of-flight (QTOF) spectrometer that combines MS/MS analysis with mass measurement accuracy. These spectrometers are coupled with very high-resolution separations (500 peak capacity) that are highly beneficial to these areas of research. Focused research projects into biomolecular complexes and macro-molecules are supported on the facility's 7-tesla (T) FTICR spectrometer, a flexible instrument that can be configured in many different ways.

Instrumentation & Capabilities

Mass Spectrometers

- QTOF
- Five ion trap spectrometers
- Four linear ion trap mass spectrometers

FTICR

- 7, 9.4, 11.5 and 12-T FTICR spectrometers

FT Spectrometer

- Orbitrap™

Additional Capabilities

- Twelve custom high-performance liquid chromatography (HPLC) systems
- Agilent capillary HPLC system

The HPMSF is committed to maintaining state-of-the-art MS and separations capabilities. To this end, the facility's staff work to develop and implement new capabilities such as the ion funnel, dynamic range enhancements applied to MS (Dynamic Range Enhancement

Applied to Mass Spectrometry [DREAMS]), and data analysis tools, which are incorporated into the capabilities of the facility as they become available. As part of this commitment the facility has recently added a 12-T FTICR spectrometer that is being optimized for work on intact proteins for the identification of post-translational modifications and protein isoforms. In addition, an Orbitrap™ MS has just been added to the facility. This instrument provides performance similar to the FTICR spectrometers but without the need for a superconducting magnet. The members of the facility staff are highly skilled in all areas required for proteomics research, from sample preparation to analysis and data interpretation, and they are available to help develop methodologies to tackle these challenging problems. As needed, scientific staff not assigned to the facility can be accessed as matrixed members of the facility. Since its inception, more than 100 separate user projects have been undertaken in the facility, with some spanning over a year in duration.

The HPMSF has developed state-of-the-art software for the acquisition and analysis of FTICR mass spectra. This software package is called ICR-2LS. It is a Microsoft Windows-based application that enables many of the unique instrument control functions developed in the W.R. Wiley Environmental Molecular Sciences Laboratory (EMSL). This same software allows automated spectral interpretation of raw FTICR data. The spectral interpretation features are integrated into our application and, thus, are not easily transferred. The data acquired in the facility is stored and managed in an in-house-developed relational database.

Mass Spectrometry Research Capabilities. Nine ion trap MSs from ThermoElectron are available in the facility: two Finnigan LCQ Classics, two Finnigan LCQ Duos that provide improved sensitivity, one Finnigan LCQ DECAXP, and four LTQs that provide the latest in sensitivity improvements from Finnigan. An example of one of these instruments is shown in Figure 1.

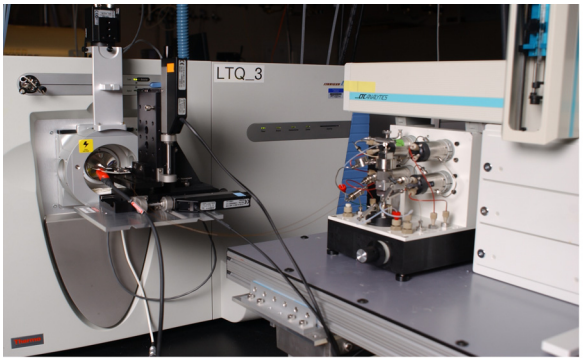

Figure 1. Ion trap MS in an HPMSF laboratory.

The ion trap instruments have either a three-dimensional quadrupole ion-trap or a two-dimensional elongated trap designed for use with electrospray ionization sources. These instruments are well-suited to MS/MS spectrometer experiments because of their very high collection efficiency for product ions. The mass range of this instrument is 150 to 2000 m/z, but the range can be extended to 4000 m/z for some applications. The ion trap instruments have a maximum resolution of 10,000 in the zoom-scan mode, and 4000 in full-scan mode. In addition, the system is easily operated in either positive or negative ion mode with the addition of SF_6 as a sheath gas.

Fourier Transform Ion Cyclotron Resonance Mass Spectrometers. This ultrahigh-performance MS uses a wide-bore (205 mm), passively shielded 11.5-T (Figure 2) superconducting magnet. The spectrometer is equipped with an electrospray ionization source and an ion funnel. The 11.5-T FTICR has a resolution of 150,000 at m/z 60,000 and a mass accuracy of 1 ppm for peptide samples with molecular weights ranging from 500 to 2000 Da. Ions are collected external to the magnet in a series of quadrupoles that allow the researcher to eliminate irrelevant ions before analysis in the FTICR spectrometer. DREAMS is a unique capability of this facility. The 11.5-T FTICR instrument can be fitted with an HPLC system and is equipped with an infrared laser for multiphoton dissociation of samples for MS/MS in the ion cyclotron resonance (ICR) cell.

Figure 2. Wide-bore, passively shielded 11.5-T FTICR MS.

The 9.4-T FTICR spectrometer (Figure 3) is a 150-mm-bore, actively shielded Bruker Daltonics APEX III. The original ion source has been replaced with a custom source that incorporates a dual-channel ion funnel for simultaneous introduction of calibrant ions, DREAMS technology, and automatic gain control. These modifications permit maximum use of the spectrometer's capabilities by maintaining the optimum number of ions in the ICR cell throughout an LC separation. The outstanding resolution of over 60,000 is maintained throughout the separation with a sensitivity comparable to the other FTICR instruments in the facility. This system is seamlessly integrated with the facility's automated HPLC (5000 psi) system for unattended operation 24 hours a day, 7 days a week.

Figure 3. 150-mm-bore, actively shielded 9.4-T FTICR instrument.

The Waters' Micromass Ultima application program interface (API) QTOF instrument is an orthogonal extraction-TOF MS that has enabled automated exact mass measurement with the ultimate performance (Figure 4) in MS and MS/MS. The instrument features optimized resolution, enhanced ion optics for enhanced sensitivity, a quadrupole mass filter, and a collision cell for MS/MS analyses. These features are also accessible over an enhanced linear dynamic range, enabling rapid and reliable quantification. This powerful combination of capabilities delivers simple exact mass measurement of precursor and fragment ions with maximum sensitivity to yield the highest confidence in structural elucidation and databank search results.

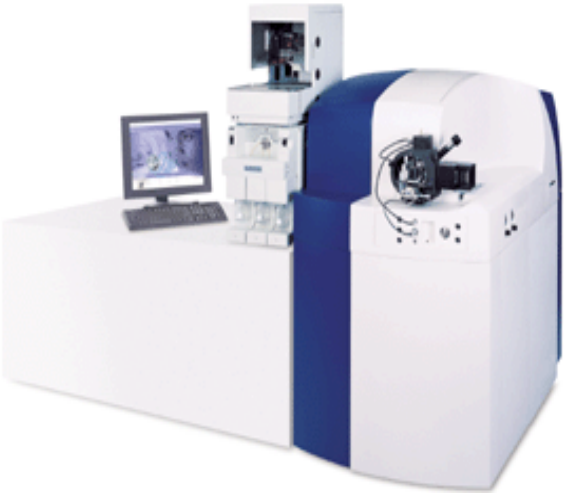

Figure 4. QTOF instrument in the HPMSF.

A signature capability of the facility is the efficient coupling of capillary separations (Figure 5) to our MSs. Instruments for both LC and capillary electrophoretic separations are available. Two ABI 270A electrophoresis systems are available along with one Agilent capillary LC and one Shimadzu capillary LC system. Unique to this facility are the in-house-developed LC systems. These systems deliver constant-pressure gradient separations at up to 5000 psi. Continuing developments of high-pressure versions of these LC systems feature a PAL autosampler with cooled sample holder, VALCO high-pressure valves, and ISCO syringe pumps. Computer software has been developed that allows the system to be configured with any of our spectrometers through DCOM communication protocols.

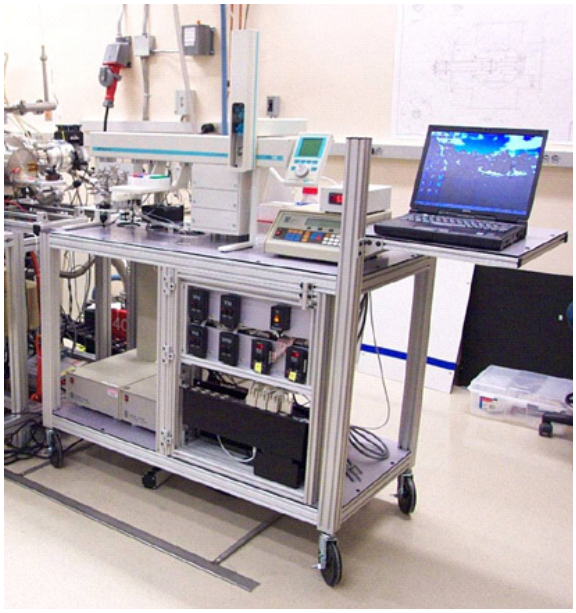

Figure 5. Capillary ultrahigh pressure separations capability.

The 7-T FTICR spectrometer is based on a 160-mm-bore, superconducting magnet and is equipped with a custom electrospray ionization source (Figure 6). This instrument has high-mass-resolving power (e.g., a mass-resolution greater than 2,000,000 has been obtained for insulin), while unit resolution is routinely achievable during online capillary isoelectric focusing (CIEF) experiments for proteins with mass resolutions of less than 30,000 μ; mass accuracy less than 5 ppm is typical for peptide/protein samples with molecular masses ranging from 500 to 30,000 μ. A detection limit of approximately 10 attomoles has been obtained with online LC and CIEF separations.

Figure 6. 7-T FTICR spectrometer.

The 12-T FTICR (Figure 7) has a 100-mm-bore magnet that is actively shielded. The original ion optics have been upgraded to include an ion funnel and additional quadrupole stages for enhanced ion transmission and future implementation of data-directed ion selection for MS/MS studies. The system is being optimized for high-throughput, proteome-wide, high-accuracy measurements of the molecular weight of intact proteins. Additionally a "top-down" protein characterization using a variety of dissociation schemes, such as collisionally induced and electron capture dissociation, can be performed on this spectrometer. Protein-separation schemes based on reversed-phase capillary LC, CIEF, and high-field asymmetric ion mobility spectrometry are in use and under development with this system.

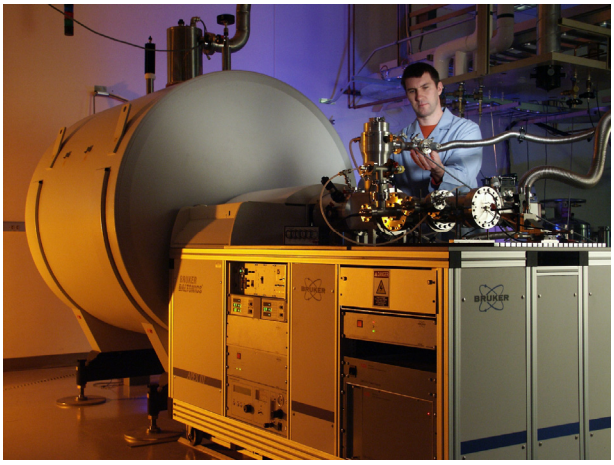

Figure 7. 12-T FTICR spectrometer.

Upgrades

In FY05, the following capability developments occurred in HPMSF:

- The Proteomics Research Information Storage and Management System (PRISM), an integrated data management and analysis environment created by researchers from EMSL's Instrument Development Laboratory, was upgraded with six new compute servers whose purpose is to perform analysis on datasets, including SEQUEST, ICR-2LS, MASIC, and others. A further six new compute servers were added as part of the processing pipeline for dataset-capturing processes. Specifically, these servers preprocess FTICR datasets, breaking the data up into smaller subfolders that allow storage and subsequent processing to occur. Previously, this task had occurred on the storage servers themselves, which had potential implications for timely processing of the data as the dataset sizes had grown. One new storage server was added for the Fungal Proteomics effort. Finally, a new database server was added to the mass tag system to allow processing of FTICR results under a newer database schema put together this year.

- PRISM software was also enhanced. The software package MASIC was placed into the automated analysis system on diluted magnetic semiconductors. This software provides chromatographic profiling of datasets, including base peak trace, total ion current traces, and specific m/s extracted ion current traces. The normalized elution time algorithm was incorporated into the analysis, allowing for precise elution time prediction for peptides that are compared to high-resolution LC-MS analyses. The following software packages were developed and implemented over the year to provide vital functions for the support staff for the PRISM system: Concatenated Text File Splitter, Mass Lynx Data File Reader, Molecular Weight Calculator, MultiAlign, Phosphopeptide Viewer, Protein Coverage Summarizer, Protein Digestion Simulator, QCTrends, QRollupExport, Spequal, STARsuite Extractor, and VennDiagram Plotter.

- A new linear ion-trap MS was acquired to increase the high-throughput proteomics capabilities. This instrument adds to the growing capability to acquire MS/MS data more efficiently, providing additional capability for working on new biological systems.

- A 12-T system has been adapted for the analysis of intact proteins. Development of fractionation schemes has been taken to the point where samples of 100 proteins can be created and subsequently analyzed with up to one-half of the proteins identified directly from their molecular weight.

- A new Orbitrap™ MS will be delivered to the facility in September 2006. This is the first fundamentally new type of MS to be developed in the last 20 years. It traps ions and detects their harmonic oscillations, much as a FTICR spectrometer does, but it does not require a magnet.

- The three linear ion MSs acquired last year and a new instrument acquired this year have been fully integrated into the operation of the facility. These instruments provide greater dynamic range and faster spectra acquisition when compared to the existing ion-trap MSs, and have now generally replaced the older instruments in our standard proteomics analysis.

- The new laboratory space of 900 square feet that was added to the facility last year has been fully integrated into the operations of HPMSF. It currently houses the LTQ-FT MS, two of the LTQ MSs, and the sample preparation robot, and will hold the new Orbitrap MS.

Two additional fully automated LC stations were added. They have been constructed for EMSL's ultrahigh-pressure LC separation system, and are now being routinely used on MSs within HPMSF. In addition, the capabilities of some of the existing LC systems have been significantly upgraded. One system has been successfully converted into a four-column system that allows 14 complete analyses per day. Two other LC systems have been converted to smaller inner-diameter capillary columns, 50 μm, that improve the sensitivity and accuracy of the analysis. The development of a LC system for phosphor-peptide analysis has been completed and the construction of an automated system is underway.

Future Directions

The facility expects to support at least 50 user projects this year comprising 75 distinct users, seven of whom would be distinguished users. These projections are based on the assumption that not all of the proteomics work done for funded programs will be counted as user work. Every effort will be made to encourage the submittal of a user project for work under these funded programs that is done with external collaborators as this would allow us to make more of the resource available to them.

Continued efforts will be made to develop tailored operations software for maximizing MS data assimilation, reduction, and visualization. To sustain state-of-the-art capabilities for proteomics analysis, our staff members will continue to develop LC separations technologies that can operate at higher pressures than commercially available instruments and to improve ion sampling efficiencies. It will also continue to automate the entire analysis procedure.

To further extend dynamic range and sensitivity in proteome studies, we developed and demonstrated the DREAMS approach for high-efficiency capillary LC separations with FTICR. In an initial application, this approach approximately doubled the number of detected peptides from microbial proteomes for which quantitative information can be obtained. The results indicated that the overall dynamic range of measurements was increased by at least an order of magnitude (to ~10^5 or larger). For this demonstration, a 1:1 mixture of $^{14}N/^{15}N$ labeled *Shewanella oneidensis* cells was analyzed. In the normal set of spectra, 2485 peptides pairs were identified, which corresponded to 1272 open reading frames (ORFs). The DREAMS approach allowed the identification of 1299 additional pairs (>50% increase), pointing to 481 new ORFs (~38% increase) not previously identified. Thus, using the accurate mass and time tag approach, we observed (and quantified) 1753 *S. oneidensis* proteins (>36% of the predicted *S. oneidensis* proteome) as peptide pairs in a single DREAMS LC-FTICR analysis. This coverage compares favorably to typical coverage achievable using shotgun proteomics. Similarly, for *Deinococcus. radiodurans*, a single DREAMS LC-FTICR analysis identified 2244 peptides, covering 965 ORFs in the normal set of spectra and 2259 peptides, covering 1000 ORFs in the additional DREAMS set of spectra, for a total of 1244 non-redundant ORFs or 279 (30 percent) additional ORFs through the use of DREAMS.

Another important improvement in FTICR performance is provided by automated gain control (AGC), a capability for data-dependent adjustment of ion accumulation time during an LC separation so as to maintain ion populations in the FTICR cell not exceeding a level that causes excessive space charge effects. AGC also helps eliminate m/z discrimination in the external ion trap and improves the dynamic range of measurements and/or the mass measurement accuracy. The AGC capability is now commercially available and is included in the LTQ-FT purchased last year with program funds.

The most important capability that the facility will develop is an ability to detect post-translational modifications of proteins. The ultimate goal will be to do this in a high-throughput fashion analogous to the existing capability for peptide detection. This will require the continued development of the new "top-down" proteomics capability that was started this year. The "top-down" approach starts with intact proteins and systematically fragments them in the MS. This is the emerging method of choice for identifying PTMs, which are very important in protein activation. This information complements the peptide-based "bottom-up" approach, which is superior for identifying the presence of protein families in a high-throughput fashion. Developing and implementing a high-throughput capability is a significant challenge that will require programmatic funding and EMSL funds. As the development is underway, the ability to analyze intact proteins in more target studies will be made available to users.

In addition to the high-throughput capability based on the 12-T FTICR, the facility will look to develop a "shotgun" PTM analysis capability that parallels the existing "bottom-up shotgun" proteomics capability. Recent developments presented at the 2005 American Society for Mass Spectrometry meeting in San Antonio, Texas, highlighted progress being made in this area by various research groups around the country. Currently, funding for in-house development of a comparable capability is beyond the funding, time, and personnel resources of the facility. A more cost-effective strategy, as we have employed with the existing capability, will be to acquire the best commercially available equipment. We acquired a commercial PTM analyzer at the beginning of calendar year 2006. The acquisition of such a platform, coupled with the development of the 12-T FTICR intact protein analysis and integrated with our informatics infrastructure, gives the facility a world-leading capability in the analysis of PTMs and protein isoforms to determine protein activation that will complement the existing protein identification capability.

The facility will continue efforts to attract strategically targeted users during fiscal year 2006.

Global Quantitative Proteome Analysis of HIV-1 Infection in CD4+-T-cells by $^{16}O/^{18}O$ Labeling and Accurate Mass and Time Tag Strategy

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This research represents the first large-scale quantitative proteome analysis of HIV-1 infection in human cell lines. The protein expression data obtained from this research is expected to provide new insights into the biological pathways regulated by HIV-1 infection.

Human immunodeficiency virus type 1 (HIV-1) is an enveloped retrovirus that causes severe depletion of the human immune system. The ~9-kb HIV-1 genome encodes nine open reading frames, consisting of fifteen distinct proteins that participate in different steps of the viral replication cycle. However, actions of these viral proteins alone cannot completely account for how HIV-1 efficiently replicates in a susceptible host. HIV-1 is expected to co-opt the host cell machinery to facilitate viral replication. In fact, many cellular proteins have been found to be incorporated into virions or otherwise interact with HIV-1. Although numerous interactions between HIV and host cell proteins have been described, the global effects of viral infection on the host cell proteome remains to be determined.

In this work, we demonstrate the first large-scale global quantitative proteome study of the primary effects of HIV-1 infection on host cellular protein expression by using $^{16}O/^{18}O$ stable isotope labeling, quantitative cysteinyl-peptide enrichment, and the accurate mass and time- (AMT)-tag strategy. The overall quantitation strategy is shown in Figure 1.

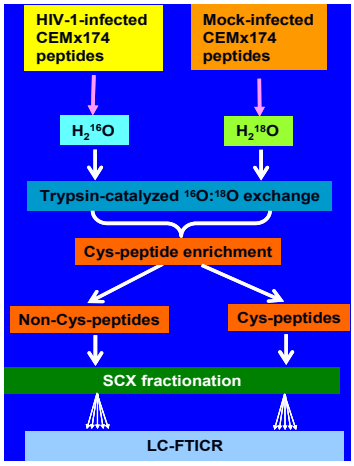

Figure 1. Quantitation strategy for isotpically labeled cystein peptides.

Through this research, initial generations of AMT-tag databases for cysteinyl-peptide and non-cysteinyl-peptides have resulted in identification of greater than 30,000 unique peptides covering ~6000 different proteins. Quantitative liquid chromatography-Fourier-transform ion cyclotron resonance mass spectrometry analyses has resulted in greater than 3000 proteins quantified with the relative abundance differences between the HIV-1-infected and mock-infected cells accurately determined. More than 300 proteins exhibited significant changes in protein abundance following HIV-1 infection, and many proteins have been previously shown to interact with HIV-1 viral proteins. Figure 2 shows an example of upregulated protein GTPase RAN, which has been found to aid in the formation of HIV-1 protein Rev-associated complexes.

This work represents the most comprehensive global protein profiling study for an HIV-1 infection model system to date. We anticipate the large-scale protein expression data will provide new insights into the biological pathways regulated by HIV-1 infection.

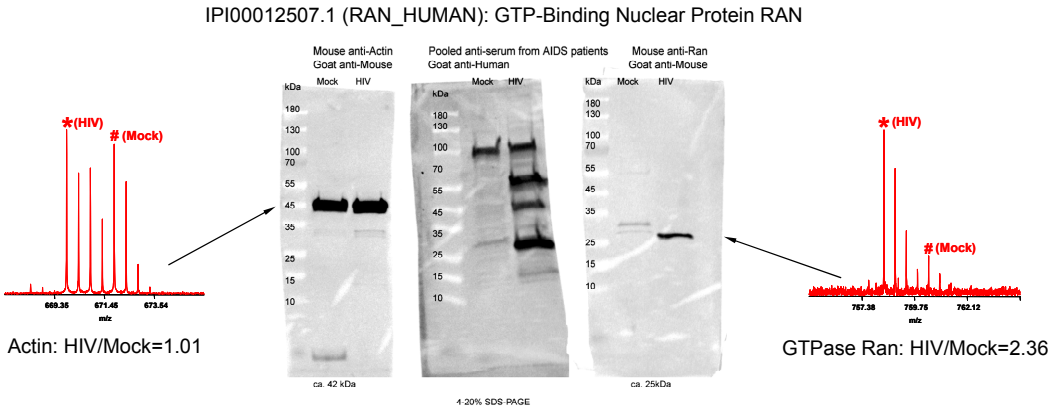

Figure 2. Many proteins with changes in abundance have been shown to interact with HIV-1 viral proteins, as annotated in the HIV-1 Human Protein Interaction Database. The GTPase Run, which aids in the formation of Rev-associated complexes, was found to be up-regulated.

Quantitative Proteome Analysis of Breast Cancer Cell Lines

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This research is helping to identify proteins that are changed in breast cancer cells. These changed proteins may be suitable as markers for the detection of breast cancer at an earlier stage, or they may provide a guide for the development of drugs that will attack only the cancer cells.

Breast cancer is the most frequently diagnosed cancer in women and accounts for 30 percent of all cancers diagnosed in the United States. Molecular profiling of proteins that play a role in cancer phenotypes can be studied using high-throughput nucleic acid-based microarrays or through measurements of the cancer cell proteome. However, it is increasingly recognized that transcriptional mechanisms do not always mirror translation, and researchers at several laboratories have shown that nucleic acid-based microarray measurements do not always correlate with protein expression (Harrison et al. 2002). The goal of quantitative proteomics is to produce a list of proteins along with a quantitative measurement, as an amount or concentration, of each of these proteins. Abnormalities in protein expression can contribute to the transformation of a normal cell to a cancer cell; drug-based therapies can be designed to prevent this transformation.

Quantitative proteomics was carried out using a $^{16}O/^{18}O$ stable isotope labeling method where an unnatural isotope of oxygen (having an atomic mass of 18) is incorporated in samples of human mammary epithelial cells. This method is compatible with the W.R. Wiley Environmental Molecular Sciences Laboratory Fourier transform ion cyclotron resonance mass spectrometers and allows for the determination of relative protein-expression measurements of two samples grown under different conditions (in this case, cancer versus normal). The data analysis was performed using the accurate mass and time- (AMT)-tag database developed at the Pacific Northwest National Laboratory (PNNL), requiring ion-trap tandem mass spectrometry identification of two-dimensional liquid chromatography-separated samples. This database provided a total of 2299 protein identifications, including 514 that were quantified using the AMT-tag database and $^{16}O/^{18}O$ method. Eighty-six proteins showed at least a threefold protein abundance change between cancer and non-cancer cell lines.

Nucleic acid microarray expression data were also available for the same cells studied by quantitative proteomics, and the correlation between the two techniques was of interest to researchers at the University of California, San Francisco, and PNNL. Nucleic acid microarray data were available for 70 of the 514 genes already quantified by AMT-tag measurements and, consequently, comparison can only be performed using these 70 proteins. When the relative expression of each gene was compared, the correlation between the two sets of values was low ($r^2 = 0.10$). A smaller list of 21 proteins was

examined because they showed marked change (at least a threefold change) in cancer versus normal expression. For 18 of the 21 proteins, nucleic acid microarray and proteome measurements were consistent in that they both indicated overexpression (or underexpression) of a gene product (Table 1). For example, keratin-18 protein should be overexpressed by a factor of 3.42 according to micro-array data, whereas proteome measurements also indicate overexpression of this protein, but by a factor of 7.82. These strategies allowed us to compare hundreds of proteins among several cell lines simultaneously and facilitated one of the largest quantitative proteomic studies of breast cancer to date.

Table 1. List of 21 proteins showing at least a threefold difference in both protein and mRNA expression between cancer and normal cells (red is greater than a threefold increase and green is greater than a threefold decrease relative to normal).

	Cancer/Normal		SPN	Description	Cell Line
	mRNA	Protein			
Congruent expression	0.120	0.253	O95573	acyl-CoA synthetase long-chain family member 3	BT-474
	0.002	0.269	P00338	lactate dehydrogenase A	
	0.251	0.248	P02570	actin, beta	
	3.420	7.824	P05783	keratin 18	
	0.196	0.310	P14625	tumor rejection antigen 1	
	0.066	0.280	P23526	S-adenosylhomocysteine hydrolase	
	0.321	0.282	P26640	valyl-tRNA synthetase 2	
	0.046	0.258	Q01813	phosphofructokinase, platelet	
	0.001	0.127	P14786	pyruvate kinase	MDA-231
	0.191	0.284	P35214	tyrosine 3-monooxygenase activation protein, gamma	
	0.004	0.063	P46940	Ras GTPase-activating-like protein IQGAP1	
	0.018	0.163	P53396	ATP citrate lyase	
	0.217	0.155	Q00765	Polyposis locus protein 1	
	4.592	11.170	P01118	v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog; Transforming protein p21b	SKBR-3
	0.230	0.311	P02570	actin, beta	
	0.024	0.221	P09960	leukotriene A4 hydrolase	
	0.187	0.284	P28288	ATP-binding cassette, sub-family D (ALD), member 3	
	0.002	0.314	P35579	myosin, heavy polypeptide 9	
Incongruent expression	41.687	0.291	O00159	myosin IC	SKBR-3
	12.445	0.226	P07339	cathepsin D	
	9.226	0.183	P15311	villin 2	

Citation

Harrison PM, A Kumar, N Lang, M Snyder, and M Gerstein. 2002. "A Question of Size: The Eukaryotic Proteome and the Problems in Defining It." *Nucleic Acids Research* 30(5):1083-1090.

Protein Expression Profiling of Wild-Type *Caenorhabditis elegans* During Development

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Understanding how different cell types, such as skin and nerve cells, are created from the same genome is one of biology's great unsolved mysteries. This work will describe proteins of individual cell types during the development in Caenorhabditis elegans. The data obtained in this study will be combined with other existing information to provide details of protein-expression profiles and an increased understanding of how different cells are generated from the same genome.

Proteomics is the leading technology used for high-throughput analysis of protein expression on a genome-wide scale. The availability of complete genomic sequences and technologies that allow comprehensive analysis of global expression profiles of messenger RNA have greatly expanded our ability to monitor the internal state of a cell. However, biological systems need to be explained in terms of their protein activity, as mRNA by itself is an imperfect monitor of cellular activity. In development, much gene regulation occurs post-transcriptionally, and these levels of gene regulation are inaccessible when one studies only steady-state levels of transcripts. A study of the proteome of *Caenorhabditis elegans*, an organism with a sequenced genome and extensive data on whole animal and specific cellular mRNA expression, is an excellent test case for current proteome technology. In one case, the identification of a protein in a tissue where a transcript has been identified will affirm the expression of the gene in that tissue. On the other hand, we, as well as other researchers, have identified thousands of transcripts within a tissue or a given cell type. This data will provide a benchmark to measure the success and sensitivity of the proteomics approach.

Currently, we are investigating protein expression patterns in the 550-cell whole embryo and isolated FAC-sorted myo-3::GFP labeled muscle cells. The transcriptomes for each of these test samples are rich, extending to many thousands of different genes. We are using a combination of enzymatic digestion, high-resolution liquid chromatograph-Fourier transform ion cyclotron resonance (LC-FTICR), and the accurate mass and time- (AMT)-tag strategy to build a snapshot of the proteome in these two samples. Initially, a peptide AMT-tag database was generated using tandem mass spectrometry (MS/MS) following extensive multidimensional LC separations; this database will serve as a "look-up" table for peptide identification in all subsequent studies. The generation of an AMT-tag database largely obviates the need for subsequent MS/MS analyses and, thus, should facilitate high-throughput analyses. Furthermore, the higher sensitivity of the FTICR analysis will increase sampling depth, allowing detection of low-abundance proteins in the complex and small-sized samples. This approach will allow us to gain a detailed picture of not only the whole embryo proteome, but also individual cellular proteomes.

For generating the AMT-tag database, proteins from the readily available wild-type embryo lysates were digested, fractionated, and analyzed using LC-MS/MS. This analysis identified 19,803 different peptides covering 4297 proteins. Eighty percent of these proteins were in concordance with the 7000 different genes found expressed in the embryo (SAGE data); thus, this AMT-tag database will provide a solid foundation for proceeding with high-throughput and highly sensitive proteome profiling of individual cell types. Proteins isolated from 580,000 muscle cells (with a cell diameter of only 1/10 of that of a typical mammalian cell) isolated by FACS analysis of disrupted embryos were digested and directly analyzed using a single LC-FTICR analysis. As a result, by searching the FTICR data in the AMT-tag database, more than 1000 muscle proteins were identified (Figure 1). In addition, we were able to identify a large number of predicted and partially confirmed proteins from both embryos and muscle cells. Shown in Figure 2 is a partial list of these proteins showing the number of peptides that were detected for each protein.

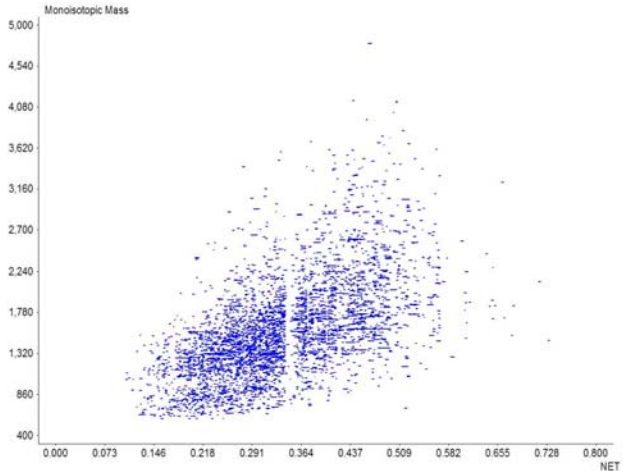

Figure 1. Two-dimensional display of LC-FTICR analysis of the tryptic peptides from 580,000 isolated FAC sorted myo-3::GFP labeled muscle cells. More than 3000 different peptides covering 1071 non-redundant proteins were identified.

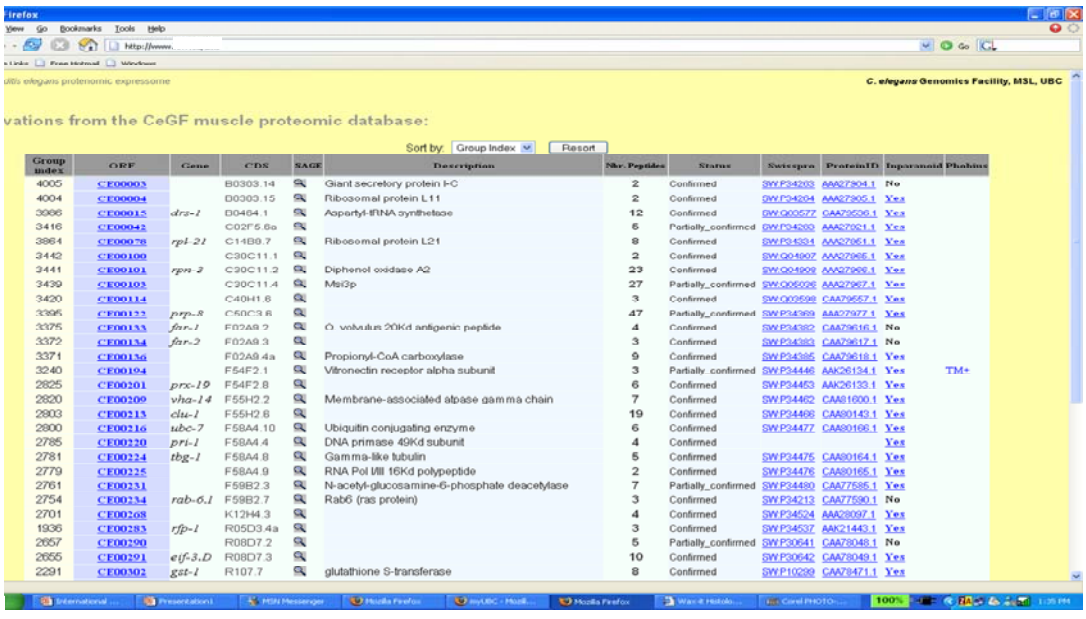

Figure 2. Some of the proteins detected with *C. elegans* proteome database information.

Proteomic Analysis of Potential Bioterrorism Agents

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This work will identify therapeutic targets for the causative agents of typhoid fever and smallpox. The methodologies developed should dramatically reduce the time required to characterize and respond to emerging infectious agents and bioterrorism threats.

Infection and pathogenicity are complicated, highly integrated processes that are unlikely to depend on any one protein product because of the availability of redundant pathways within the physiological repertoire of a pathogen. A systems biology approach that uses high-throughput global proteomics is being employed to identify new, more effective vaccines and therapies.

In the past year, researchers from the High-Performance Mass Spectrometry Facility, in collaboration with F Heffron and S Wong of the Oregon Health & Science University, have generated the data for foundational mass and time-tag databases for *Salmonella typhimurium*, *S. typhi*, *Vaccinia* virus, and Monkeypox virus. These extensive data resources will be used to allow for higher throughput and reduced sample requirements for future proteomic measurements.

The most extensive of these databases has been developed for *S. typhimurium*. This database has been initially data mined to compare to known biological knowledge and to identify previously unknown biological information. These growth conditions include a typical logarithm growth state, a stationary growth state, and a growth condition that is meant to mimic an infectious environment. These results also give the research team a baseline as the experimental results of *S. typhimurium* in a host-cell environment become more complex.

Ongoing projects include analysis of *S. typhimurium* and *S. typhi* with various regulators of pathogenesis knocked out by mutagenesis. For *Vaccinia* and Monkeypox viruses, future efforts are meant to characterize more complex portions of the viral life-cycle.

Proteome of the *Yersinia pestis* Type III Secretion System: Insights into the Injectisome

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We are becoming ever more concerned with the reemergence of pandemic diseases, such as that expected with avian influenza, the so-called "bird flu." Our most effective means of protecting ourselves will be to gain better understanding of how infecting organisms cause diseases. Yersinia pestis is the causative agent of the "Black Death" or bubonic plague, and our work with this bacterium is an important step to understanding which proteins are used by the microbe to infect other cells.

The type-III secretion system (TTSS) is an essential virulence mechanism of pathogenic *Yersinia* spp (Cornelis 2002a; Mota and Cornelis 2005). TTSS is composed of a syringe-like secretion structure (injectisome) made up of a basal protein complex that provides a portal through the inner membrane and periplasmic space (Cornelis 2002b), an outer membrane channel protein (YscC) (Burghout et al. 2004), and the needle that grows to pierce the host cell membrane through successive polymerization of the protein YscF (Hoiczyk and Blobel 2001). Once constructed, TTSS participates as a channel to direct effector proteins, known as Yops, into the host cell cytoplasm (Viboud and Bliska 2005). Yops then act on the host cell to promote pathogenesis by disrupting the cytoskeleton, preventing phagocytosis, and blocking the production of proinflammatory cytokines (Cornelis 2002a). This assault on the host allows *Y. pestis* to easily propagate with minimal detection or interference (Cornelis et al. 2002a).

Y. pestis was grown in four conditions that mimicked growth either in the flea vector (26°C with or without calcium) or a mammalian host (non-contact state: 37°C with calcium and a host cell contact state, 37°C without calcium) (Fowler and Brubaker 1994). Current hypotheses theorize that the injectisome is already preformed before host cell contact at the onset of the temperature increase (Cornelis 2002a; Edqvist et al. 2003). However, we only identified components of the injectisome under conditions that mimic host cell attachment (37°C without calcium).

Figure 1 shows the injectisome components identified using the accurate mass and time-(AMT)-tag approach to proteomics. Figures 1A-D show the theoretical assembled components of the injectisome in conditions that mimic growth in the flea or mammalian host. The indication of these results is that the injectisome assembly does not occur in any appreciable amount until the bacteria attach themselves directly to the mammalian cell. Attachment then triggers the formation of the injectisome and allows for translocation of the Yop proteins responsible for host cell destruction and host immune system diversion.

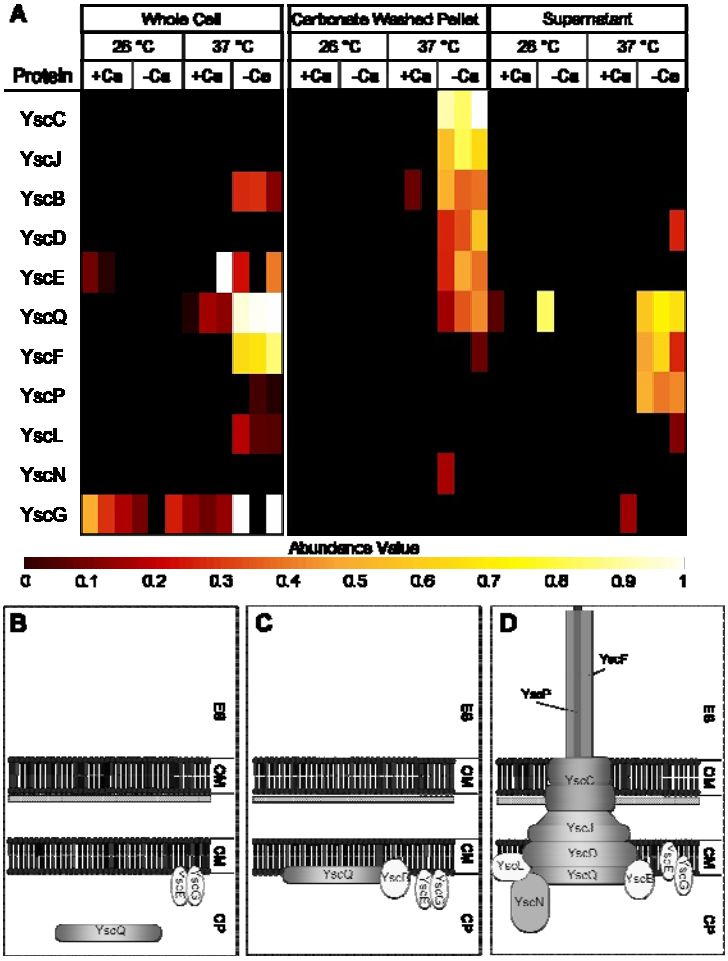

Figure 1. Abundance value comparisons of 11 injectisome components measured by the AMT-tag proteomics methods for four growth conditions representing *Y. pestis* growth in a flea or mammalian host. A: Heat-map representation of the abundance value. B-D: Schematics of the theoretical assembled component of the injectisome at B (26 °C), C (37°C with Ca^{2+}), and D (37 °C without Ca^{2+}). ES, extracellular; OM, outer membrane; CM, cytoplasmic membrane; CP, cytoplasm.

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Initial Proteomic Profiling of Human T-Cell Populations Using High-Throughput Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS)

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(b) University of Florida, Gainesville, Florida

Proteomics analysis of specific blood cell populations, such as T-cells from human patients, provides the opportunity for comprehensive monitoring of the dynamic changes in gene products responding to diseases. The identification of novel regulatory genes/proteins may provide diagnostic or predictive values for diseases and disease outcomes.

Quantitative proteomic analysis of human blood leukocyte populations with specific functional perturbations from patients will lead to the identification of novel regulatory genes/proteins that may provide predictive values for clinical outcomes. To provide the basis for use of the Pacific Northwest National Laboratory- (PNNL)-developed, high-throughput accurate mass and time- (AMT)-tag-based proteomics strategy for quantitative analysis of trauma patient leukocyte populations, initial proteomic profiling was applied to enriched human T-cell populations with the combined purposes of providing an initial catalog of proteins present, evaluating the sample processing techniques for human blood cells, and establishing an initial AMT-tag database specific to the human T-cell sub-population to facilitate subsequent quantitative analysis.

Isolation of T-cell subpopulations was performed at the University of Florida, and three T-cell samples from normal subjects (Sample ID 170611, 170612, and 170616) were shipped to PNNL for proteomic profiling. All three samples were subjected to cell lysis in the presence of detergent, 0.1% RapiGest (Waters Corporation). Extracted proteins were denatured by boiling for 5 minutes, reduced with 5-mM dithiothreitol and digested by trypsin at 37°C for 5 hours using a 1:50 enzyme/protein ratio. Approximately 20-μg aliquots of peptides from each sample were used for a comparison among the three samples by LC-MS/MS analysis. The remaining portions of the three samples were pooled and subsequently fractionated by strong cation exchange chromatography into 25 fractions, and each fraction was analyzed by LC-MS/MS (i.e., two-dimensional LC-MS/MS). Peptide MS/MS fragmentation spectra were identified by database searching against the human International Protein Index database using the SEQUEST algorithm. The final list of peptide/protein identifications was obtained after applying stringent filtering criteria to ensure a high-confidence level for peptide identifications (Qian et al. 2005). Table 1 provides a summary of the peptide and protein identification results from single analyses of the three individual samples. Comparable numbers of peptide and protein identifications were obtained from all three samples with approximately 70 percent of proteins common to each sample. The results suggest overall good reproducibility for the analytical strategy.

Table 1. Summary of the Peptide and Protein Identification Results from Single Analyses of the Three Individual Samples

Sample ID	170611	170612	170616
Number of cells (millions)	16	8.4	6.9
Amount of peptide recovered (µg)	115	90	56
Quantity of peptide used for analysis (µg)	20	20	20
Peptides identified	3549	3325	3349
Proteins identified	1097	1036	1020
Common peptides and proteins in all three samples	1775 peptides and 705 proteins		

Improved proteome coverage was achieved when two-dimensional LC-MS/MS was applied to the pooled samples. A total of 3206 non-redundant proteins was identified with high confidence (>95 percent) from combined one-dimensional and two-dimensional LC-MS/MS analyses. Figure 1 graphically depicts the Gene Ontology (GO) analysis results based on cellular component and biological process using the GoMiner software tool (Zeeburg et al. 2003); 2344 out of 3206 non-redundant proteins (73 percent) have associated GO terms. Proteins from almost all cellular components were identified, with the majority of these proteins from the plasma membrane, nucleus, protein complex, mitochondria, and other intracellular organelles. Similarly, proteins identified cover a variety of different biological processes. While metabolism is the largest observed category, nearly 10 percent and 8 percent of these proteins were categorized as cell communication and response to stimulus, respectively.

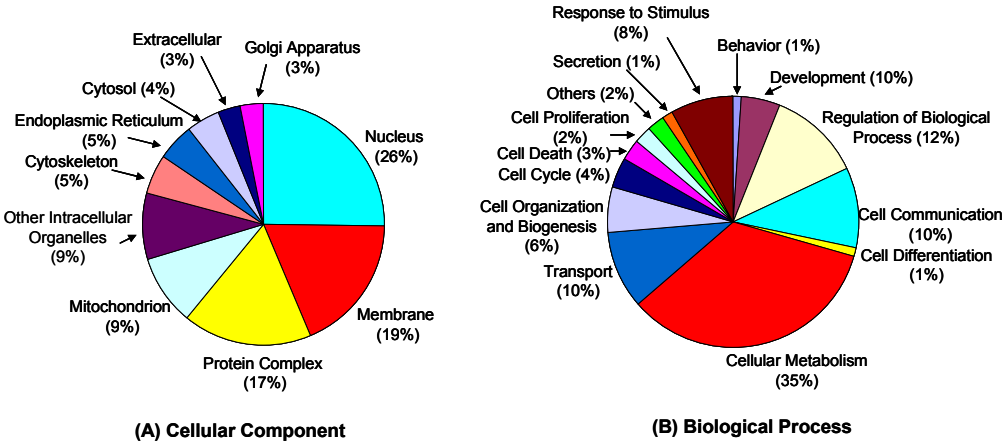

Figure 1. Categories of protein identifications based on GO analysis. (Note that GO categories overlap for some of the proteins.)

Overall, these results indicate that the set of T-cell proteins identified using the present approach provide a substantially unbiased representation of the T-cell proteome with regard to cellular component and biological process. Interestingly, based on the GO analysis results, six out of seven members of the T-cell receptor complex and 79 cell surface receptor proteins linked to signal transduction (listed in Table 2) were identified in this dataset, suggesting overall extensive coverage of the proteome, including low-abundance signaling membrane proteins.

Table 2. Gene names for the 79 identified cell surface receptor proteins linked to signal transduction as revealed by GO analysis.

CD2	CD3D	CD3E	CD3G	CD3Z	CD4	CD5
CD7	CD8A	CD8B1	CD47	CD59	CD97	CD99
IL7R	HPRP8BP	DGKA	GIT2	ITGAM	AZU1	MULK
DGKQ	LAT	CSNK2A2	GNA13	LCP2	PTPRJ	GOLGA5
ILK	BRD4	TBL3	GNAQ	MAPK14	PRKCA	TLE3
CBLB	EVL	HRMT1L2	PIP5K2B	CLC	GNG2	AXIN1
PHIP	AHSG	INS	CAP1	SLC9A3R1	FYN	ERBB2IP
RGS19	PIK3CG	RGS6	PLCB2	ADAM10	MIF	TGFB1
MYD88	IFITM1	LTB4R2	DGKB	GFRA1	GNAI3	ITGB3
CCNK	PGRMC2	MTSS1	NCSTN	PTK2B	CARKL	DOK1
NCK1	ITGA6	ILKAP	ARL3	ECGF1	12716	TRPV1
CSNK1A1	ITGB7					

To summarize, this T-cell proteomic profiling experiment established an initial AMT tag database with extensive proteome coverage, including low-abundance membrane and signaling proteins. This initial database provides a solid basis for subsequent quantitative proteomic analyses of T-cell samples from severe trauma and burn patients, applying the high-throughput AMT-tag approach.

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Gas-Phase Reactions of Fe-S Clusters

Washington State University Tri-Cities, Richland, Washington
Y Fu, L Wang

Proteomic Analysis of Breast Fluid for Biomarker Discovery

Pacific Northwest National Laboratory, Richland, Washington
RC Zangar

Identification of Post-Translational Modifications and Protein Complexes Under Oxidative Stress Conditions

Pacific Northwest National Laboratory, Richland, Washington
CA Sacksteder, TC Squier

Proteomic Analysis of the Human Dermal Microvascular Endothelial Cell Mitogenic Response

Pacific Northwest National Laboratory, Richland, Washington
BD Thrall

Proteomics of *Shewanella oneidensis* MR-1 Subjected to Solar Ultraviolet Radiation Stress

Michigan State University, East Lansing, Michigan
JM Tiedje

Identification of Protein Components of Vaccinia Virus Particles

National Institutes of Health, Bethesda,, Maryland
B Moss

Identifying Targets for Therapeutic Interventions Using Proteomic Technology

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W.R. Wiley Environmental Molecular Sciences Laboratory, Richland, Washington
HM Mottaz
Oregon Health Sciences University/Oregon Graduate Institute, Beaverton, Oregon
F Heffron, S Wong

Proteomic Characterization of Cerebrospinal Fluid by High-Resolution Liquid Chromatography/Tandem Mass Spectrometry (LC-MS/MS)

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Proteomic Analysis of the *Cyanobacterium synechocystis* sp. PCC 6803
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Washington University, St. Louis, Missouri
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Characterization of the Tumor Cell *Lamellipodia phosphoproteome*
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Pacific Northwest National Laboratory, Richland, Washington
JM Jacobs, DG Camp

Unraveling the Molecular Biology of Host-Pathogen Interactions
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Peptide Observation Model
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Characterization of Neuropeptides Located in Neurosecretory Tissue of *Cancer borealis* and *Cancer productus*
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Protein Complex Identification Using Novel Affinity Resins
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***Shewanella* Strains**
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Reactive Oxygen and Nitrogen Species Produce Dynamic Protein Modifications and Protein Complexes in RAW 264.7 Macrophage
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Advanced Proteomics and Metabolomics Studies of Type-1 Diabetes
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Proteomic Studies of Inflammation and the Host Response to Injury
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A Proteome for Specific Cell Types in *Caenorhabditis elegans*
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Proteomic Characterization of *In Vivo* and *In Vitro* Model Systems of Hepatitis C Virus Infection: Global Quantitative Proteome AM Measurements of Cellular Protein Expression
University of Washington, Seattle, Washington
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Erasmus University Medical Center, Rotterdam, Rotterdam, The Netherlands
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DL Springer

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Comparative Proteomic Analysis of *Desulfovibrio vulgaris*
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Characterization of the Neurite Phosphoproteome
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Determining Proteins Expression in *Ralstonia* Bacteria that Survive in Ultra-Pure Water
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Use of Fourier Transform Ion Cyclotron Resonance (FTICR) Mass Spectrometric Proteomics Analysis for the Identification of Novel Targets in Pain Research
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Hollow Helices as Folding Nanotubes with Tunable Cavity Size
State University of New York at Buffalo, Buffalo, New York
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Determination of the Proteome of Drosophila Lipid Droplets
University of California, Irvine, Irvine, California
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Mechanism of Action of G Protein-Coupled Receptors Studied by Liquid Chromatography-Fourier Transform Ion Cyclotron Resonance (LC-FTICR) Mass Spectrometry
Montana State University, Bozeman, Montana
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Identification of Proteins Differentially Expressed in Response to Infrared
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Proteomic Characterization of *Yersinia pestis*
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Identification and Relative Expression of Membrane Proteins in Breast Cancer Cell Lines

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Biomolecular Mechanisms for Microbe-Fe(III) Oxide Interactions in *Geobacter* Species

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Rhodobacter sphaerodies

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Proteomics of Morphology Determination in a Fungus

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Regulated Proteolysis and Bystander Effects in Radiation

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Determination of the Peptide-Binding Sites on the Bacterial Chaperone Protein SecB
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Mass Spectrometric Analysis of Eukaryotic Proteosome
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Mass Spectrometry Analysis of Nucleotides/Nucleosides
Washington State University Tri-Cities, Richland, Washington
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Identification of the SecB Residue that is Labelled by Methanethiosulfonate
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LL Randall, JM Crane

Protemic Analysis of Naturally Occurring Methane-Oxidizing Archaeal Communities

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A Proteomic Approach to the Study of *Neurospora crassa* Ribosomal Dynamics

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Proteomic Analysis of *Fusarium graminearum* Sexual Development

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Proteomics of Membrane Protein Complexes

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Using Mass Spectrometry-Based Phosphoproteomics and Small Interfering RNA-(SIRNA)-Induced Gene Silencing to Identify New Insulin Receptor-Signaling Components in Cultured Adipocytes

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Proteomic Analysis of *Deinococcus radiodurans* Under Oxidative Stress Irradiation

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Genomes-to-Life Protein Complex Isolation Capability

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Proteomics of Filamentous Fungi

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Sustrate Identification for Ser/Th Protein Phosphatase
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Membrane Proteins of Placental Parasites
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Combined Transcriptome and Proteome Analysis of *Shewanella oneidensis* MR-1 etrA and arcA Mutant Strains
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Proteomic Analysis of *Arthrobacter*

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