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Top-Down, Bottom-Up

The Merging of Two High-Performance Technologies

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Bio-Rad Laboratories, Inc. introduced the ProteinChip® SELDI system in 2006 to provide researchers engaged in biomarker discovery with a high-throughput, high-sensitivity approach to protein expression profiling.

The ProteinChip system incorporates chip-based applications with top-down mass spectrometry (MS) for protein profiling, an area of research that has typically relied on more conventional approaches such as 2-D gel electrophoresis. Since its introduction, the ProteinChip system has been used by researchers in many fields of study to discover biomarker candidates from a variety of sample types.

Until now, researchers have had to choose between such top-down intact protein profiling methods and digest-based bottom-up methods utilizing high-performance MS for biomarker discovery. Bio-Rad has recently partnered with Bruker Daltonics to combine the benefits of the SELDI chromatographic retention sample preparation technology with the high-performance mass spectrometers also used for bottom-up proteomic analyses, providing researchers with both discovery methods on one platform.

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Current State of Protein Profiling

In the past decade, interest in protein profiling has grown exponentially (Table 1). Protein profiling technologies are evolving as rapidly as the field is expanding. The main goal is discovery of protein biomarkers (based on determination of differences in their expression levels) that can be used for disease diagnosis, disease prognosis, and prediction of drug response (either positive or adverse) based on patient-specific or disease-specific protein profiles.

The single biggest challenge facing researchers hoping to achieve these objectives is the complexity of the proteome — it is estimated that approximately 30,000 genes code for up to 30 times as many proteins, with concentration ranges varying by 10–12 orders of magnitude. Furthermore, there is a small number of high-abundance proteins relative to the many low-abundance — and often more biologically relevant — proteins. Unlike the genome in which genetic information is fairly static, protein characteristics are constantly in flux and affected by changing environments. Therefore, tools developed to help advance proteomics research must provide researchers with a way to wade through immense amounts of information to achieve results that are meaningful.

There are currently two major types of approaches to protein biomarker discovery (Figure 1): the analysis of intact proteins (top-down proteomics) and the analysis of peptide mixtures from digested proteins (bottom-up proteomics). In the past, researchers have been limited by protein biomarker discovery systems that have required a choice between top-down and bottom-up methods. This decision has meant choosing between throughput (top-down systems) and resolution (bottom-up systems).

Although the literature suggests exponential growth in research interest in protein profiling, to date very few biomarkers discovered by MS have been approved by the U.S. Food and Drug Administration. One approach alone (for example, the widely adopted bottom-up methodology) may not provide sufficient information or include enough samples to provide results that survive the validation process. The result of a joint product development and co-marketing agreement involving Bio-Rad's SELDI technology and Bruker Daltonics mass spectrometers, the Lucid Proteomics System™ now offers researchers the opportunity to utilize both bottom-up and top-down methodologies in one system — merging the benefits of both approaches to MS-based protein profiling.

Table 1. "Protein profiling" in the literature.

Year	Number of Articles Published*	Year	Number of Articles Published*
1998	54	2004	5,626
1999	229	2005	7,120
2000	1,041	2006	12,290
2001	1,880	2007	12,240
2002	2,663	2008	15,132
2003	3,738		

* Results returned from a search of "protein profiling" in journal articles listed in the PubMed database (**www.ncbi.nlm.nih.gov/pubmed/**).

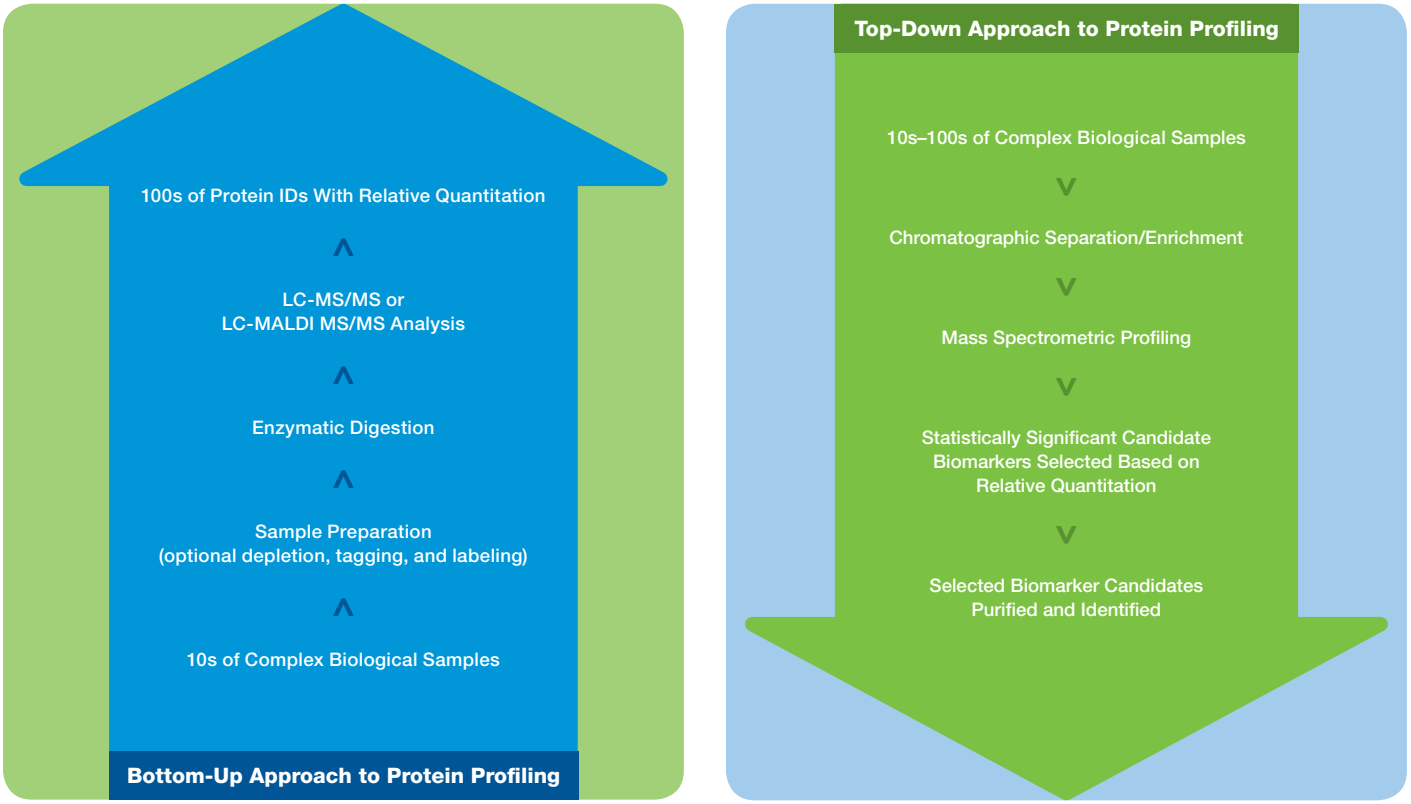


Fig 1. General bottom-up (left) and top-down (right) proteomics profiling workflows. The primary characteristics of bottom-up approaches are enzymatic digestion of a small set of biological samples, liquid chromatography–based high-resolution peptide separation, and MS/MS analysis for both relative quantitation and identification. The primary characteristics of top-down profiling approaches are direct analyses of statistically meaningful numbers of biological samples to determine differences in relative expression levels of undigested native proteins, followed by identification of small numbers of selected biomarker candidates.

The Bottom-Up Approach

The bottom-up approach to protein profiling (proteolytic digestion of proteins prior to MS analysis) has been widely adopted in modern MS-based proteomics research. Known as "shotgun proteomics," the bottom-up proteomic approach involves direct digestion of a biological sample using a proteolytic enzyme (such as trypsin) that cleaves at well-defined sites to create a complex peptide mixture. The digested samples are then analyzed on platforms that include liquid chromatography and tandem mass spectrometry (LC-MS/MS or LC-MALDI MS/MS). Differential expression using the bottom-up approach often involves labeling the sample with isobaric tags prior to digestion. All methods using the bottom-up approach require the use of high-resolution, high-performance instrumentation (Han et al. 2008).

Because the bottom-up approach involves exhaustive analysis of samples, analytical systems are low throughput by requirement, and basic biological questions are usually addressed using a small number of samples from simple model systems. For example, an oncology study might compare differential protein expression between paired cell lines (cancer vs. noncancer or invasive vs. noninvasive cancer) or focus on a single cell line with samples taken at a few time points (before and after drug treatment using different treatment doses, or after treatment with different drug candidates).

The main advantage of the bottom-up approach is the ability to achieve high-resolution separations. Other strengths include a comprehensive coverage of proteins with the workflow producing protein identifications and relative expression for hundreds of proteins within a small number of samples (for example, two to eight samples). Because the method is widely used and accepted, a variety of sophisticated technologies from a number of manufacturers have been developed to aid researchers using this approach. Bruker's versatile mass spectrometers offer a complete solution for the bottom-up workflow with instruments that perform sample preparation, liquid handling, liquid chromatography, and spotting followed by MALDI TOF/TOF analysis — all on one platform.

Although this method has proven successful for the profiling and identification of proteins, researchers continue to face challenges when screening for biomarkers, in particular for candidates below 30 kD. Smaller proteins and peptides have fewer proteolytic cleavage sites and often do not generate enough peptides for confident identification. Information is therefore potentially lost for natively occurring small peptides and biologically generated protein cleavages as well as posttranslational modifications (PTMs).

The Top-Down Approach

In the field of proteomics, the term "top-down" describes two different techniques. Top-down as applied to protein identification, also known as "top-down sequencing," is so named because of its similarity to DNA sequencing methods and is typically conducted on highly purified protein preparations. Top-down as applied to protein profiling, also known as "top-down proteomics," involves separating intact proteins from complex biological samples using traditional separation techniques such as liquid chromatography or 2-D gel electrophoresis, followed by differential expression analysis using spectrum analysis or gel imaging platforms. Spots or fractions that are predicted to contain biomarkers are identified using MS. The top-down methods discussed in this article focus on proteomic profiling of intact proteins.

The strength of top-down approaches lies in direct detection of the native molecular mass of biological protein species. Mass information is retained for natively occurring small peptides, biologically generated protein cleavages, and PTMs — all of which are postulated to be relevant in many diseases and other biological processes. Other major advantages of top-down strategies are simplified sample preparation and elimination of the time-consuming protein digestion required for bottom-up methods.

Unlike bottom-up methods in which biomarker discovery is driven from more specific and limited sample sets, the starting point for top-down proteomics can be hundreds of different complex biological samples. Scientists using top-down approaches are generally interested in addressing clinical questions requiring larger numbers of samples, for example, biomarker discovery using body fluids (plasma, serum, cerebrospinal fluid, urine) from humans or from animal models.

SELDI-TOF MS is a widely used top-down biomarker discovery method that combines the selectivity of chromatography with the sensitivity of mass spectrometry. In the ProteinChip SELDI system, complex samples are applied to chromatographic arrays for separation based on physicochemical interactions. The chromatographic surfaces reduce the sample complexity and facilitate washing to remove salts and detergents that interfere with MS-based detection, thereby significantly increasing the number of detected protein species. The array footprint is compatible with liquid handling robotics systems, facilitating high-throughput analysis. High throughput is particularly important for clinical biomarker studies, which generally require large patient cohorts to compensate for patient-to-patient variability and generate results with sufficient statistical power to accurately assess the predictive value of a potential biomarker. Until now, the major challenge for this approach was the requirement for off-line enrichment and purification of the selected biomarker candidates followed by MS/MS identification using a different MS platform.

Introducing the Lucid Proteomics System

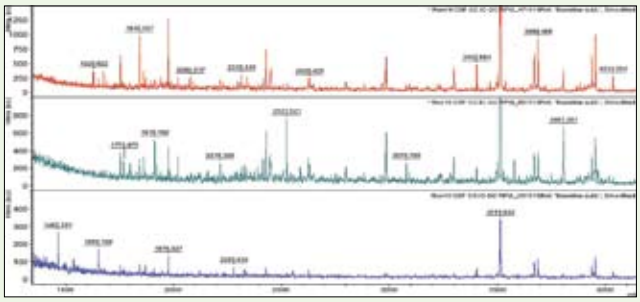
Bio-Rad has partnered with Bruker Daltonics, a leading manufacturer of MS instruments and accessories for life science, pharmaceutical, biochemical, and chemical research, to bring all the advantages of Bio-Rad's ProteinChip SELDI technology to Bruker's high-resolution mass spectrometers. This collaboration enables both bottom-up and top-down approaches on the same MALDI TOF/TOF systems, as well as a complete SELDI-based biomarker discovery solution that includes both protein profiling and, for the first time in a SELDI-based workflow, protein identification of biomarker candidates, either by direct on-chip TOF/TOF analysis (suitable for small peptides) or by enrichment, purification, and digestion followed by TOF/TOF analysis (for larger proteins) (Figure 2).

Bruker's MALDI TOF and TOF/TOF systems offer reliable and detailed protein characterization and identification, high-resolution MALDI imaging, and LC-based bottom-up biomarker discovery. The ultrafleXtreme mass spectrometer, Bruker's most advanced MALDI TOF/TOF system, offers high efficiency and sensitivity and delivers MS/MS spectra with nominal mass resolution for peptides. Typically, full MS/MS data sets can be acquired from low femtomole levels of peptides within seconds. The unique modular design of Bruker's mass spectrometers enables versatile instrument configurations including linear-only mode for screening applications, reflectron mode for improved resolution, and TOF/TOF technology for identification. With the introduction of the Lucid Proteomics System, SELDI-based biomarker discovery leverages the flexibility and versatility of Bruker's MALDI TOF/TOF systems for top-down proteomic biomarker discovery.

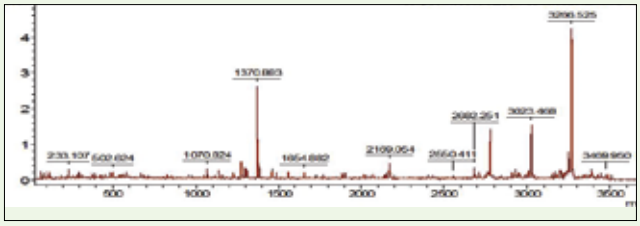
Bio-Rad has developed a complete line of Lucid Proteomics System products, including profiling, identification, and system check kits, that bring ProteinChip SELDI capabilities to the Bruker ultrafleXtreme mass spectrometers as well as specially configured autoflex and ultraflex MS instruments. Bruker's MALDI TOF/TOF users are now able to profile native peptides and low-mass proteins (<30 kD) in a large number of samples. Bio-Rad's portfolio of array chemistries provides researchers with an easy and robust method for biomarker discovery that combines on-chip chromatographic enrichment for simplifying complex protein mixtures with rapid, label-free analysis of large numbers of biological samples. The top-down method preserves information about PTMs or truncations and facilitates subsequent purification and identification of candidate markers.

The Lucid Proteomics System combines and refines the benefits of ProteinChip SELDI technology with Bruker's high-resolution mass spectrometers for increased peak counts, better peak resolution, improved quantitation, and facilitated identification, thereby increasing opportunities to discover biomarkers important for disease diagnosis, disease prognosis, monitoring disease progression, and determining drug response (positive or adverse).

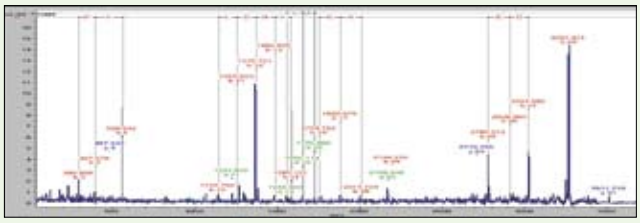
Case Study: Identification of a Neuropeptide From Cerebrospinal Fluid Using the Lucid Proteomics System



Human cerebrospinal fluid (CSF) enriched using reverse-phase chromatography beads and eluted with 30% acetonitrile, 0.5% TFA was profiled on three different ProteinChip array surfaces (cation exchange, metal affinity, and anion exchange) followed by addition of 25% CHCA as the matrix. The arrays were analyzed using a Bruker ultraflex III MALDI TOF/TOF system in linear MS mode. For biomarker discovery, profiling on multiple surfaces captures different subsets of proteins within complex samples and increases the potential of finding peaks that are differentially expressed between study groups.



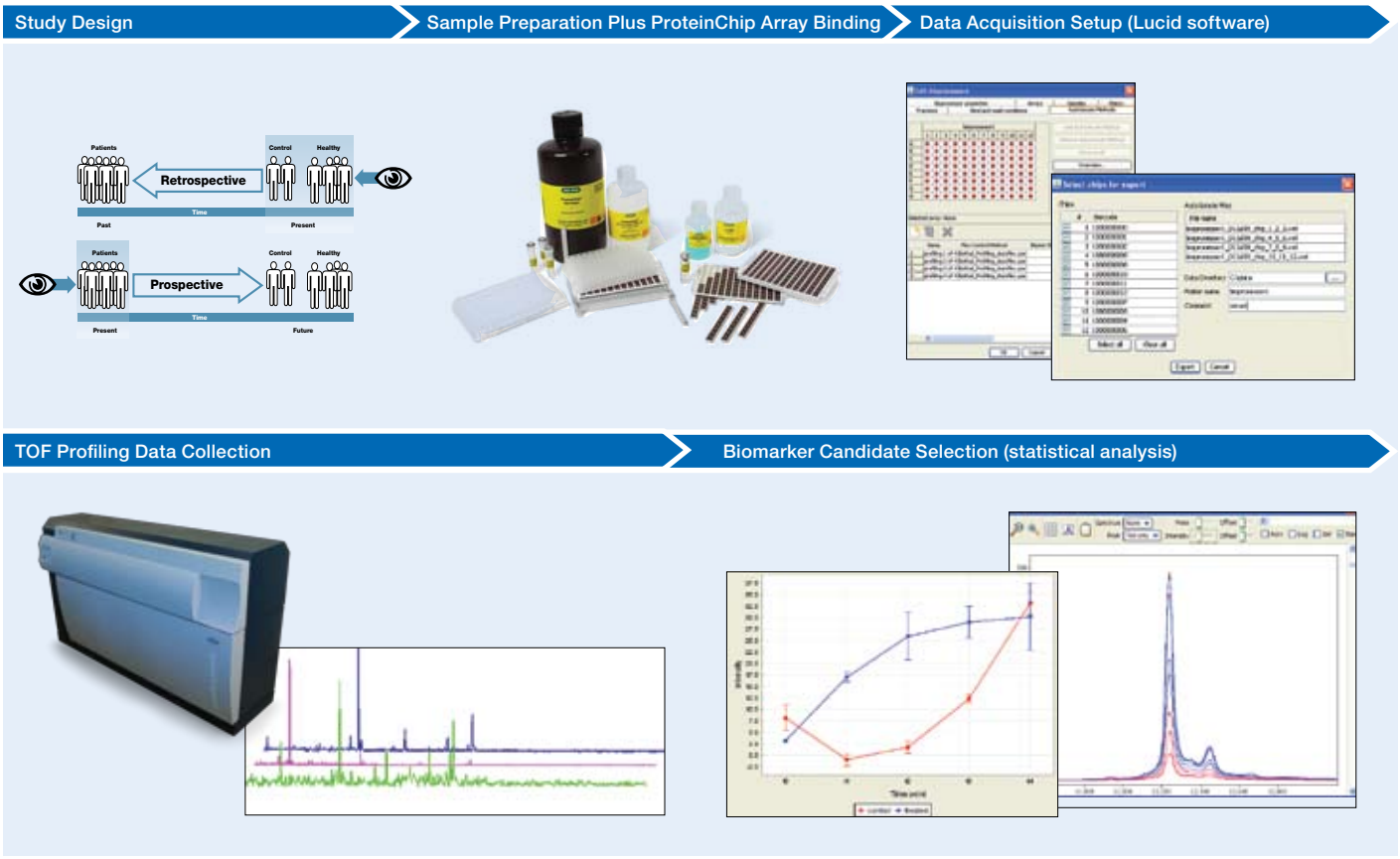
The 3,511 Da peptide from this CSF profiling study was directly identified from the CM10 cation exchange ProteinChip array surface by MALDI TOF/TOF analysis using the same Bruker ultraflex III system.



The protein was identified as a fragment of neuroendocrine protein 7B2, corresponding to amino acids 182–212.

Neuroendocrine protein 7B2 (also known as secretogranin V) is known to be posttranslationally cleaved into N-terminal (27–176) and C-terminal (200–212) peptides. Neuropeptide 7B2 has been shown to be a good marker of neuroendocrine tumors (Mbikay et al. 2001), and increased levels of the C-terminal fragment (7B2CT) have been detected in amyotrophic lateral sclerosis and frontotemporal dementia (Ranganathan et al. 2005). The fragment found in this study does not correspond to any of the predicted peptides, and its presence would have been missed in a digestion-based (bottom-up) discovery approach. Top-down profiling of biological samples provides valuable information about a biological system's true proteomic state, and direct, on-chip capture and identification of peptides can accelerate biomarker-driven functional studies.

A. Lucid™ Profiling



B. Lucid ID

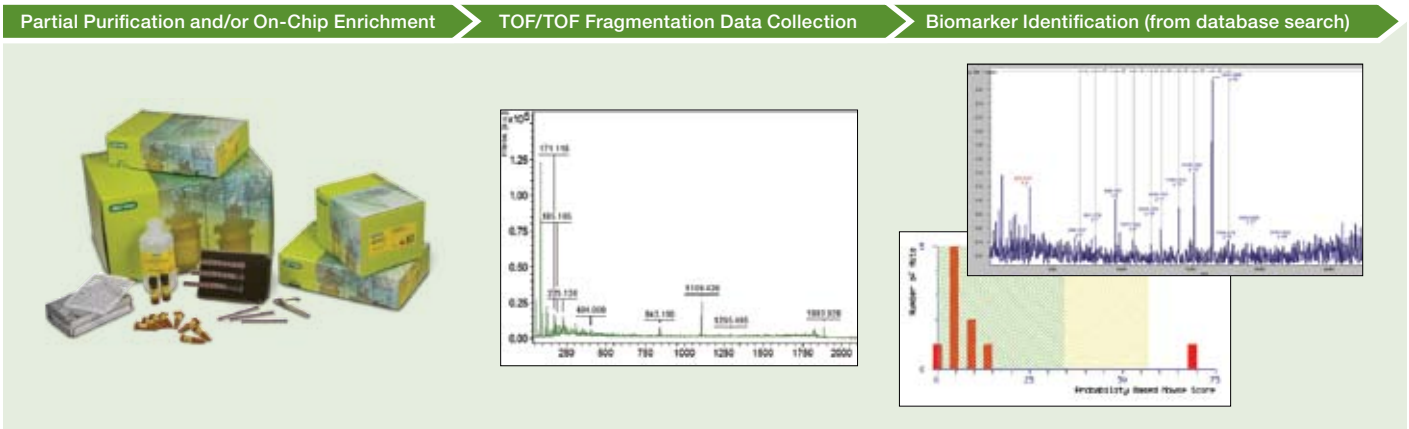


Fig 2. Lucid Proteomics System protein profiling and identification workflows. A, the Lucid Proteomics System protein profiling workflow. Proper study design, including appropriate definition of the clinical questions and selection of patients and controls, improves success rates of biomarker discovery studies. Sample preparation, including optional prefractionation, combined with binding to ProteinChip arrays, enhances visualization of low-abundance proteins and improves reproducibility. The Virtual Notebook feature of Lucid Proteomics System software is utilized to program data acquisition for large numbers of samples and to store patient information critical during subsequent data analysis. Acquisition of top-down protein profiles takes full advantage of Bruker Daltonics MALDI TOF/TOF systems to collect MS data. Statistical analysis of peak intensities from profiling data is performed to select robust biomarker candidates. **B**, the Lucid Proteomics System protein identification workflow. Biomarker candidates are identified directly on-chip (smaller peptides) or after enrichment and proteolytic digestion (proteins). MS/MS peptide fragmentation data is collected using a Bruker Daltonics MALDI TOF/TOF MS system and analyzed using database search engines to obtain high-confidence protein identifications.

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A Researcher's Perspective on the Lucid Proteomics System

"Collaboration among scientists leads to more productive research; no scientist can become an expert in all facets of a multidisciplinary project," says Dr John Whitin, researcher with the Cohen Lab in the Department of Pediatrics at Stanford University's School of Medicine. "In a similar manner, I believe that collaborations on technology by vendors such as Bio-Rad and Bruker will aid investigators in their proteomic research." Such was Whitin's response when asked how the partnership between Bio-Rad and Bruker might help him in his proteomics research.

Whitin's laboratory is currently working on finding biomarkers in diseases of children for which there is a significant diagnostic dilemma, or where a different approach to research might improve the understanding of mechanisms of disease. "An example of the former," says Whitin, "is Kawasaki disease, an illness characterized by coronary vasculitis that is the leading cause of acquired heart defects in children. An example of the latter is the study of plasma biomarkers that correlate with premature birth. In this case, we are studying plasma in a mouse model of premature labor and birth."

In most studies, Whitin and colleagues prefer to work with plasma rather than serum samples, though urine and CSF samples have been used to study certain diseases. "Plasma is not absolutely superior to serum, but we are particularly interested in novel truncated forms of biomarkers and wish to avoid as much proteolysis as possible," explains Whitin.

For all sample types processed, his group most often performs discovery studies as a top-down strategy. However, they have also been working on a better method for purifying phosphopeptides for subsequent analysis, and these studies follow the bottom-up approach.

Because the group's background is in traditional biochemistry and not mass spectrometry, the first-generation ProteinChip SELDI system enabled them to pursue top-down strategies for biomarker discovery. Whitin describes the ProteinChip SELDI instrument — and the whole SELDI system — as "relatively easy for us to master."

Whitin's group is self-characterized by a constant quest for techniques and strategies that will lead to answers for questions posed in their research. "For example," says Whitin, "we look for techniques that add value to the study of large peptides/small proteins. Sometimes the biomarkers discovered on the SELDI platform are easy to identify, but sometimes peptides between 3 and about 7 kD are difficult to purify in sufficient quantities to be visualized on SDS-PAGE." The group is therefore intrigued by the potential of the Lucid Proteomics System to define a biomarker on a ProteinChip array, and then use the same system for direct identification — essentially providing the final piece to the puzzle of the SELDI protein profiling workflow.

Whitin concludes by saying, "It will also be interesting to perform discovery studies on the smaller peptidome of various biological fluids, for example, urine. Our current methods are really optimized for peptides/proteins larger than approximately 3 kD, and we would love to be able to extend our range to peptides smaller than 3 kD."

Conclusions

Though interest in protein profiling for biomarker discovery continues to grow, current findings indicate that a single-method research approach does not foster rapid advances. A collaboration between Bio-Rad Laboratories and Bruker Daltonics has resulted in the Lucid Proteomics System, which enables both top-down and bottom-up proteomics approaches on one platform for maximum coverage of the proteome — allowing greater flexibility with experimental design and accelerating biomarker research programs.

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