

Proteomics: a technology-driven and technology-limited discovery science

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An emerging field for the analysis of biological systems is the study of the complete protein complement of the genome, the 'proteome'. There are several complementary tools available for proteome analysis including 2D protein electrophoresis and mass spectrometry. Emerging technologies for proteome analysis include spotted-array-based methods and microfluidic devices. Taken together, these technologies provide a wealth of information that is useful in discovery-based science. However, there are some key limitations of these approaches and new technology is required to be able to fully integrate proteomic information with information obtained about DNA sequence, mRNA profiles and metabolite concentrations into effective models of biological systems.

The past few decades have seen tremendous advances in technology for the study and analysis of biological systems. One of the key technologies that have been developed is instrumentation that permits the faithful amplification of nucleic acid molecules by PCR. This technique permits the study, manipulation and sequencing of very small amounts of material. Arguably, even more essential advances in technology for life science research have been motivated by the Human Genome Project. The desire to obtain a complete sequence map for several different organisms has helped to fuel the development of new technologies for automated high-throughput DNA sequencers and related bioinformatics strategies. The early investment in technology development by the Human Genome Project has enabled this effort to proceed in a timely manner.

More recently, the development of array-based methods for the analysis of genome-wide gene expression at the mRNA level has helped to generate a more integrated view of the relationship between the genome, gene expression and phenotypes. These chip-based experiments (either spotted arrays¹ or commercially available high-density arrays²) permit the measurement, simultaneously and semiquantitatively, of changes in gene expression between two different biological states. Interpretation of the data from these experiments relies on statisticians, bioinformaticists and engineers, and an understanding of the connection between changes in the mRNA expression level and phenotype is often complicated, at best. One reason for this complexity is that these experiments do not include information about protein expression. The microarray-based methods are critically important for a detailed understanding of the regulation of biological systems; however, such methods provide no information about post-transcriptional control of gene

expression, changes in protein expression levels, changes in protein synthesis and degradation rates or protein post-translational modifications. Indeed, recent experimental evidence suggests that there is no obvious correlation between mRNA expression levels and protein expression levels either in human liver cells³ or in yeast⁴. Furthermore, it has been recently proven mathematically that a detailed understanding of the control of gene networks (even simple networks) requires information on both mRNA and protein expression levels⁵. Ideally, one would integrate experimental information obtained from a combination of genome-wide monitoring tools (DNA, mRNA, protein, metabolites, etc.) to develop a detailed understanding of the basis for various phenotypes (Fig. 1). Although the newly available technologies to measure and study DNA and mRNA have recently driven life science research, the technology to study the protein complement of the genome, or proteome⁶, is more limiting at this time.

2D electrophoresis and mass spectrometry

The most widely used tool for proteome analysis has been available for more than 25 years⁷. This technique, called 2D protein electrophoresis (2DE), resolves complex mixtures of proteins first by isoelectric point and then by size. Such an analysis begins with the solubilization of proteins from samples of interest using nonionic and zwitterionic detergents. Sample preparation is followed by isoelectric focusing in one of several commercially available instruments⁸ using carrier ampholytes and/or immobilized pH gradients⁹ to form the pH gradient. The isoelectric focusing is typically performed in an anticonvective medium such as a low concentration polyacrylamide gel. Prefractionation of complex mixtures using preparative-scale isoelectric focusing significantly improves final resolution¹⁰. After an equilibration of the proteins in sodium dodecyl sulfate (SDS) and other reducing agents, the resulting focused bands are separated according to size using traditional polyacrylamide gel electrophoresis¹¹. This separation can be tuned using different concentrations of polyacrylamide or using gradient concentration gels. Depending on the particular application, proteins are detected using Coomassie blue stain, silver stain¹², fluorescent dyes¹³, or radiolabels, and the images can be acquired into a computer using a laser densitometer, fluorescence imager or other device. The resulting gel

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Fig. 1. Technology permits the molecular analysis of biological systems at several levels. Information obtained from each of these technologies must be integrated to establish a deep understanding of the relationship between genotype and any particular phenotype.

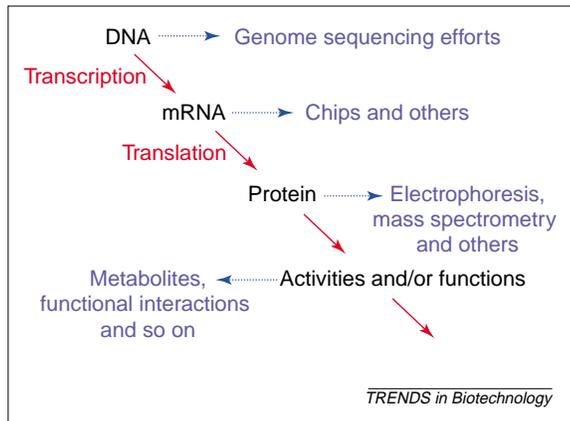


image patterns (Fig. 2, for example) from different samples can be compared either manually or using commercially available software packages to identify protein spots that are qualitatively different or that are up- or down-regulated. Examples of such software packages are Melanie 3 produced by GeneBio (Geneva, Switzerland), PDQuest produced by Bio-Rad (Hercules, CA, USA) and Imagemaster produced by Amersham Pharmacia Biotech (Piscataway, NJ, USA).

Relatively poor control over protein detection chemistry coupled with sequence specificity of most, if not all, detection strategies yields a technique that is not amenable to quantitation. Although large

increases in spot staining intensity correlate reasonably well with increases in the quantity of protein present in a given spot, more subtle changes are difficult to detect unless radiolabel approaches are used¹⁴. In this regard, the technology for measuring quantitative changes is more limiting than the bioinformatic software tools available for gel analysis. The utility of this approach is also limited by the inability to reliably monitor proteins that are present in very low abundance in a sample, as well as proteins that are very hydrophobic or that are very acidic or basic¹⁵. Low copy number proteins might represent key regulatory molecules within cells or signaling molecules in tissues and organs, and the inability to measure changes in expression levels of these important proteins is an important consideration¹⁶. Hydrophobic proteins that are present in membranes might have key roles in communicating extracellular information to the inside of cells.

The above general approach will permit the generation of a fingerprint or barcode of the proteome of a particular biological system. A comparison of two or more such fingerprints might help to identify differences in protein expression that result in particular phenotypes. This comparative information is useful to help classify samples or tissues into different groups. However, the 2DE proteomics approach provides an opportunity to relate changes of interest to information concerning the underlying gene. Such a connection can be made if enough of the protein spot of interest can be purified and if further microchemical analysis of the purified protein (such as direct amino acid sequencing, amino acid analysis and mass spectrometry [MS]) can be performed. In this manner, the use of the 2DE approach as a discovery-based tool reaches its potential when a fully sequenced genome is available. One of the key reasons that 2DE has become an essential technique in proteome analysis is because it is useful both as an analytical tool and as a micropreparative purification tool.

The need to characterize spots and the limited sensitivity of automated amino acid sequencers¹⁷ has fostered an increasing use of MS for protein characterization. The two most commonly used approaches for spot characterization involve peptide mass mapping and tandem MS of a proteolytic digest of a 2DE spot. Matrix-assisted laser-desorption/ionization time-of-flight (MALDI-TOF) instruments are relatively simple to use, have high mass accuracy and are reasonably tolerant of contaminants and solvents. The masses of the resulting peptides from a proteolytic digest can be accurately and quickly measured using MALDI-TOF MS. These masses can be compared with *in silico* digests of protein databases or six-frame translations of nucleic acid databases to help characterize the spot. Alternatively, a tandem MS experiment using an ion trap mass spectrometer or a triple quadrupole mass

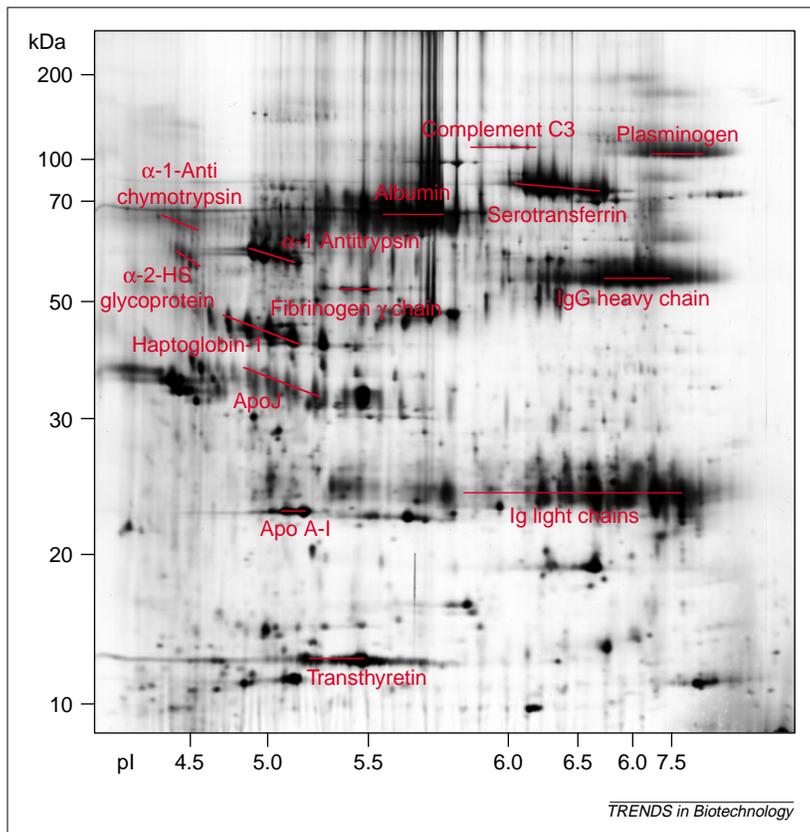


Fig. 2. Example of a silver-stained 2D protein electrophoresis gel. Proteins from human cerebrospinal fluid are separated by isoelectric point and by size, resulting in a fingerprint of protein expression. Abbreviation: Ig, immunoglobulin.

spectrometer can be performed. In a tandem MS experiment, peptide mixtures are studied in an initial MS scan and particular peptides can be fragmented during a second step to generate amino acid sequence information. The sequence information is derived by an attempt to match mass spectra from fragmentation patterns that correspond to individual amino acid residues with *in silico* spectra obtained from databases or by matching amino acid sequence information with available databases. For a detailed discussion of MS applied to proteome analysis, there are some recent outstanding reviews^{18,19}.

Emerging technologies for proteome analysis

Chip-based methods for proteome analysis

There is a variety of new methods available for proteome analyses that take advantage of materials processing and manufacturing expertise available in the semiconductor industry. In particular, improved knowledge about how to manipulate both the physical and chemical surface properties of silicon-based materials and the promise of fast, reliable and economical manufacturing of chip-based devices and arrays has encouraged the development of such tools and methods. These emerging technologies generally fall into two categories: (1) spotted array-based tools; and (2) microfluidic-based tools.

In array-based methods, small spots of proteins are immobilized onto silicon-based substrates (typically glass). Such an array can then be used to screen complex protein mixtures for particular binding affinities or other interactions. These arrays potentially address several of the concerns about the existing 2DE-MS approach: the arrays can be produced for relatively little cost, provide consistently reliable and rapid results and are simple to use. Using existing technology²⁰, it is possible to array nearly the entire complement of proteins produced from a library of cDNA clones onto a surface and then to probe for small molecule interactions, antibody–antigen specificity or to identify unique proteins from a mixture using fluorescence detection.

Antibodies can be arrayed onto silicon substrates using bacteria that express recombinant antibodies²¹. These arrays can be probed for specific antibody–antigen binding interactions using a filter-based enzyme-linked immunosorbent assay (ELISA) technique. Almost 20 000 antibody clones can be screened using this system and this approach has demonstrated relatively high sensitivity when compared with screens of phage libraries²¹. As a complement to the antibody arrays, others²² have developed transfer stamps with blunt end tips that measure 250 μm . Using these tips, spotting robots are able to deposit 5 nl drops of protein solution onto small (25 \times 75 mm) polyvinylidene-difluoride (PVDF) membranes. These arrays can be used to probe antibody–antigen specificity to levels of 10 fmol antigen μl^{-1} above background as determined using chemiluminescence. Another group at

Stanford University (CA, USA) uses printed protein arrays to measure protein–protein interactions based on a fluorescence assay²³.

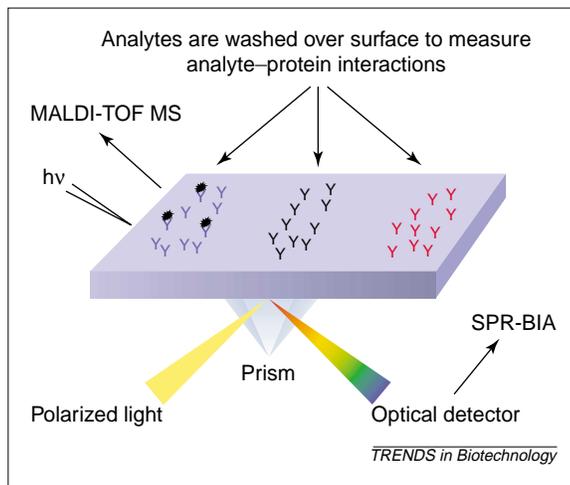
Spotted arrays can also be used to probe protein–small molecule interactions. Immobilized proteins are patterned²⁴ onto a microscope slide using high-precision contact printing to deliver small quantities of protein to an aldehyde-coated glass surface. Using this system, spot densities of >1600 spots cm^{-2} can be achieved with spot diameters of 150–200 μm . This technology has been applied to the identification of protein kinase substrates and for screening protein–protein interactions.

Ciphergen Biosystems (Fremont, CA, USA) has a commercially available device that combines chip-based techniques with MS. Surface-enhanced laser desorption and ionization (SELDI) technology used in the ProteinChip System²⁵ permits the selective capture of proteins from biological samples. Protein mixtures can be incubated with a variety of available chips that probe Lewis acid/base interactions or hydrophobic, electrostatic and coordinate covalent bonding. The surfaces of these chips are precoated with chromatographic affinity surfaces, or the user can covalently couple enzymes, antibodies, receptors, DNA or other small molecules, which are used to probe the sample mixture. Surface-enhanced affinity capture, the most promising version of SELDI technology, uses probe surfaces to extract, structurally modify or amplify a particular protein. After the addition of a matrix solution to enhance laser energy transfer and sample ionization, samples are analyzed using TOF-MS. Advantages of this approach include a reduced amount of sample preparation before MS and the ability to capture trace amounts of proteins directly from biological fluids. Also, probes provide information on hydrophobicity, total charge, isoelectric point, phosphorylation, glycosylation and primary structure²⁶. Recently, ProteinChip Arrays have been used to identify disease markers²⁷. For example, prostate-specific membrane antigen, a 100 kDa transmembrane glycoprotein thought to indicate prostate cancer tumor progression, can be detected in blood sera and quantified based on normalized peak via ProteinChip technology. ProteinChip assays for other cancers are also in development²⁸.

Nelson and colleagues²⁹ combined surface plasmon resonance–biomolecular interaction analysis (SPR-BIA) with MALDI-TOF analysis (BIA-MS analysis). This technique (Fig. 3) uses an immobilized receptor to monitor biomolecular interactions and the proteins are subject to TOF analysis for characterization. This approach has detection limits below 20 fmol and a mass range of up to 150 kDa. One application of this technology involves the isolation and detection of tagged polypeptides retrieved from the expression system on a BIA chip.

Another important field of technology is the development of microfluidic devices for the analysis of

Fig. 3. Schematic depiction of the SPR-BIA analysis of tagged proteins (adapted from Nelson and co-workers²⁹). Digested and tagged proteins or other analytes are collected and affinity-retrieved on a sensor chip through a bound receptor. Surface plasmon resonance is performed to measure quantity and specificity and MALDI-TOF MS is performed to confirm the identity of the affinity-retained analyte. Abbreviations: hv, MALDI laser; MALDI-TOF MS, matrix-assisted laser-desorption/ionization time-of-flight mass spectrometry; SPR-BIA, surface plasmon resonance – biomolecular interaction analysis.



proteins. Miniaturization reduces the amount of analyte required and potentially provides low-cost disposable chips for a variety of applications thus addressing some of the concerns about limited sensitivity of the 2DE approach. Pinto *et al.*³⁰ report the use of a novel interface for the use of microfabricated fluidic systems with electrospray ionization (ESI) MS. Liu³¹ and Gottschlich³² both report lab-on-a-chip techniques for separating and detecting protein mixtures. Gottschlich and co-workers have integrated a microreactor, injector and electrophoretic separator and a second reactor for derivatization on a monolithic substrate followed by fluorescence detection. Liu integrates capillary electrophoresis, postcolumn labeling and fluorescence detection on a microfabricated system. Others at the University of Michigan have developed integrated microfluidics systems capable of accurately metering nanoliter drops (0.5 – 125 nl) in channels using a combination of hydrophobic surface modification and air pressure³³. Wang and colleagues³⁴ describe a microfluidic device that integrates an electrospray ionization source for MS with a protein digestion bed, a capillary electrophoresis channel and an injector on a monolithic substrate. The protein digestion bed incorporates trypsin immobilized onto microbeads to permit faster digestion and to eliminate autodigestion products that might hinder sample characterization. Such a device can rapidly digest, separate and characterize proteins. Although these devices have not been applied to proteome analysis *per se*, they hold tremendous promise to address several key limitations in this approach. Additional information about miniaturization can be found in a recent review³⁵.

Isotope coded affinity tags

One of the key concerns with existing proteome analysis tools is the relatively poor quantitation. In this regard, the available microarray tools for mRNA profiling offer some advantage over protein expression profiling tools. To address this concern, Gygi and co-workers³⁶ developed small molecule reagents, 'isotope coded affinity tags', which can be

used to more accurately quantitate changes in protein expression between two different biological samples. These reagents have a biotin section that can be used to affinity-purify labeled proteins and peptides, a linker that can incorporate stable isotopes and a reactive group with specificity toward thiol groups. Using the heavy and light versions of this molecule, which differ by eight mass units, the cysteine-containing proteins and peptides from two different biological samples can be differentially labeled. The labeled proteins and peptides can be affinity-purified and studied using liquid chromatography MS, which will measure the relative abundance of the peptide from the two different biological samples but will also provide protein characterization via tandem mass spectrometry. The approach is somewhat analogous to one used to profile differences in mRNA concentration on microarrays in that samples from two different states are differentially labeled and analyzed together. Although there are still some issues related to the analysis of very low abundance proteins, as well as large proteins, this technology offers a major improvement in the ability to quantitate protein expression and the method is effective at measuring changes in gene expression from yeast grown on galactose or ethanol.

The 'molecular scanner'

One concern with the standard 2DE-MS approach to proteome analysis is the limited throughput for 2DE. Although a tremendous amount of information from any particular sample is acquired at once, the number of samples that can be analyzed in parallel is limited. This bottleneck results from the technical complexity of performing 2DE experiments and the need to individually purify and analyze spots using MS. Recently, Hochstrasser and colleagues^{37,38} started to address some of the concerns by automating parts of this procedure. The 'molecular scanner' is a device that simultaneously digests and electrotransfers proteins and peptides onto a PVDF membrane. The membrane is then directly scanned using MALDI-TOF MS to generate peptide mass fingerprints and these data are used to create a fully annotated 2DE map of the initial proteome mixture. Such a device automates several key steps in the 2DE-MS approach to proteome analysis, such as the need to isolate individual spots and perform a series of proteolytic digests on each purified spot. One possible limitation with this device is the reliance on 2DE technology, which does not resolve hydrophobic proteins effectively as discussed previously. Nevertheless, this instrument has demonstrated the ability to generate proteome information from both human plasma and *Escherichia coli*.

Conclusions

The paradigm of proteome analysis has many applications in biotechnology including: (1) the

Table 1. General overview of technologies discussed in this article

	Advantages	Disadvantages
Existing tools		
2DE	High resolution and generally available	Technically challenging, poor resolution of certain classes of proteins
MS approaches	Yields structural information	Relatively high capital costs
Emerging tools		
Spotted arrays	Simple, reproducible, fast and can use for protein-protein interactions	Not yet ubiquitous and limited ability to study certain classes of proteins depending on specific technology
Microfluidic-based devices for sample preparation and analysis	Potentially improved sensitivity and improved speed of analysis	Not widely available for protein analysis
Isotope coded affinity tags	Relative quantitation of protein expression	Some classes of proteins not resolved, requires access to a mass spectrometer
Molecular scanner	Couples 2DE imaging and MALDI-MS into a single instrument	Not commercially available and relies on 2DE

discovery of biochemical pathways that can result in the identification of targets for therapies; (2) the monitoring of manufacturing processes for quality control; (3) the development of new processes for the manufacture of biological materials; and (4) the development of diagnostic tests for clinical disease and for the efficacy of treatment strategies.

Furthermore, data from a variety of the aforementioned tools and others might be necessary to help to answer particular questions. For a more detailed discussion of specific application of proteomics technology, the reader is referred to some recent review articles^{39–41}.

Currently, there is a variety of tools available for proteome analysis, only some of which have been discussed in this review (see Table 1). Tools such as 2DE and MS have provided the community with a tremendous amount of new data, which can be integrated with large datasets from mRNA profiling experiments and other essential analytical tools. In a sense, current life science research is technology-driven and newly available tools have helped to evolve such efforts from hypothesis-driven studies to discovery-driven studies. These tools have enabled

new questions to be asked and new paradigms in biology to be considered⁴². However, some of these tools have important limitations or are not generally accessible to the community at large either because of cost or because the technology is still in development. For example, there is still no effective means to quantitatively monitor changes in all the membrane proteins and soluble proteins at the same time. In a sense, current life-science research is still technology-limited; new tools and methods must be developed to fully probe the biochemistry that underlies various phenotypes and cell states. For a deep understanding of the relevant biology, this information must be integrated with data from various 'levels' including DNA sequence, mRNA profiles, protein expression and metabolite concentrations as well as information about dynamic spatio-temporal changes in these molecules. Ultimately, this data will lead to useful predictive mathematical descriptions of biological systems, which will permit improved design of experiments and help to inspire a deeper understanding of the genotype-phenotype relationship.

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Biomaterials integrated with electronic elements: en route to bioelectronics

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Bioelectronics is a progressing interdisciplinary research field that involves the integration of biomaterials with electronic transducers, such as electrodes, field-effect-transistors or piezoelectric crystals. Surface engineering of biomaterials, such as enzymes, antigen-antibodies or DNA on the electronic supports, controls the electrical properties of the biomaterial-transducer interface and enables the electronic transduction of biorecognition events, or biocatalyzed transformation, on the transducers. Bioelectronic sensing devices, biosensors of tailored sensitivities and specificities, are being developed.

The electronic communication between the biomaterials and the respective transducers is the essence of tailored bioelectronic devices^{1–4}. As shown in Fig. 1, the electronic communication can proceed in two directions: (1) the biological events, such as recognition or catalysis, which occur on the transducer might be reflected by an electronic signal leading to the formation of electronic biosensors; or (2) electronic signals, such as potential, might

activate biological functions, such as biocatalysis, thus enabling the development of electrically-driven biotransformations.

Biomaterials and electronic transducers are foreign components in respect of one to the other, leading to a lack of electronic coupling or communication between them; for example, the lack of electrical communication between redox enzymes and electrode supports. The electron transfer theory^{5,6} implies that the electron transfer rate-constant between an electron acceptor and a donor pair is given as follows:

$$k_{et} \propto \exp[-\beta(d - d_0)] \exp[-(\Delta G^0 + \lambda)^2 / 4RT\lambda] \quad (1)$$

In Eqn 1, d and d_0 are the actual distance and the Van-der Waals distances, respectively, that separate the donor-acceptor pair, ΔG^0 and λ correspond to the free energy change and the reorganization

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