Protein biochips for differential profiling

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Progress has been made in utilizing ProteinChip® technology to profile and compare protein expression in normal and diseased states, particularly in the areas of cancer, infectious disease and toxicology. The past year has also seen the development of several novel chip types designed to analyze proteins in a fashion analogous to the array-based format of DNA microarrays. Some of these platforms may be used for differential profiling.

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Abbreviations

ACPm	acyl carrier protein
INH	isoniazide
LCM	laser capture microdissection
MALDI	matrix-assisted laser desorption/ionization
PSA	prostate-specific antigen
PSMA	prostate-specific membrane antigen
SELDI	surface-enhanced laser desorption/ionization
TOF	time-of-flight

Introduction

Virtually all strategies for studying cellular function require the comparison of control states with perturbed states. At the heart of all such strategies is the requirement for an assay that sensitively and specifically measures differences in gene or protein expression or function. In the genomic and proteomic era, these assays no longer limit themselves to individual or small sets of genes or proteins; rather, they seek to examine the full complement of protein expression in a cell under a given set of physiological conditions. Because this represents a daunting task, protein differential display techniques that compare protein profiles between control and experimental populations have become increasingly useful. In these systems, expression of proteins common to both groups is ignored and emphasis is placed upon identifying and quantifying those proteins whose expression level is either upregulated or downregulated. These proteins become potential biomarkers representative of a given metabolic disease, drug reaction, neoplasm or microbial infection and are thus diagnostic or possible therapeutic targets.

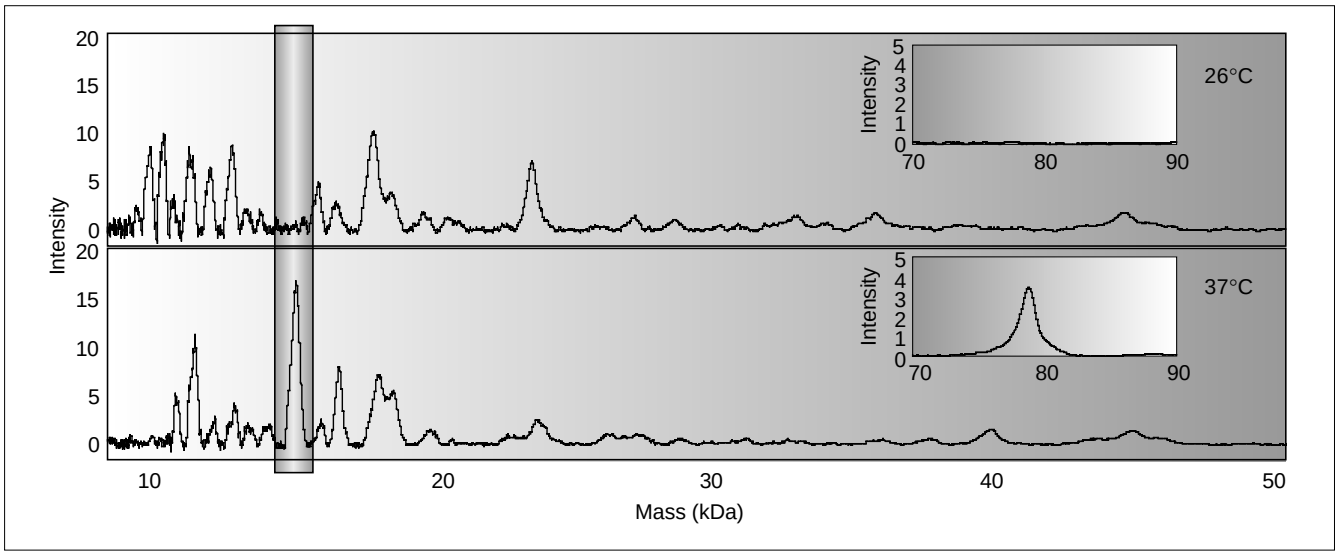
Chip-based protein differential display systems have, for several reasons, been significantly more difficult to develop than their counterparts that examine differential gene expression. First, whereas the reverse-transcriptase polymerase chain reaction (RT-PCR) permits the amplification of mRNA, there is no analogous method to amplify protein expression. Second, for most functional assays (e.g. interaction studies), the proteins must be immobilized on the surface of the chip such that they retain their native conformation and also such that their active site(s) are exposed rather than buried. Additionally, the heterogeneity of the biophysical properties of proteins makes it virtually impossible for any single surface chemistry to bind the full complement of proteins present in a cell, particularly if one attempts to maintain the proteins in their active state. Finally, the capacity of the chip must be sufficient to allow as complete a representation of the proteome to be visualized as possible; abundant proteins (metabolic and cytoskeletal) overwhelm the detection of less abundant proteins such as signaling molecules and receptors, which are generally of more therapeutic interest. Strategies used to circumvent these problems are discussed by Weinberger *et al.* [1].

In this review, we focus on a subset of chip-based assays that may be used to compare protein expression in normal and perturbed states. The most advanced commercially available system for this purpose is the ProteinChip® proteomics platform, and so most of the review will be devoted to describing applications of this system. The review will also describe briefly some of the more novel and unique chip-based assays that have been described in the literature. Because of space limitations, we cannot cover all chip-based proteomics formats. Another commonly used chip technique, surface plasmon resonance (SPR), is described in greater detail by Scheller *et al.* in this issue (pp 35–40) and by Rich and Myszka [2] in a recent issue of this journal. We refer the reader to reviews by Figeys [3] and Guetens *et al.* [4] for discussions of microfluidic protein biochips, and peptide arrays are reviewed by Schneider-Mergener and colleagues in this issue (pp 59–64).

ProteinChip® proteomics

Perhaps the most established chip-based proteomics profiling platform is the incorporation of ProteinChip® technology with mass spectrometry, as commercially available from Ciphergen Biosystems, Inc. (reviewed in [5•]). At the heart of this technology are the ProteinChip® arrays, which have varying chromatographic properties, for example anion exchange, cation exchange, metal affinity and reverse phase. A complex mixture of proteins, as from cells or body fluids, can be reduced to sets of proteins with common properties by binding the sample to chips with differing surface chemistries in parallel and in series. After the chips are washed to remove unbound proteins, the bound proteins are

Figure 1



Protein profiling of *Yersinia pestis* cultured at its two physiological temperatures. 10 μg of crude cytosolic extracts of *Y. pestis* grown at 26°C versus 37°C were analyzed on a strong anion exchange chip (SAX-2). Proteins expressed only at 37°C, selected for purification and identification, were the 14.9 kDa (boxed) and 78.8 kDa (inset) proteins. The 14.9 kDa protein was identified as antigen 4 and the 78.8 kDa protein as the catalase/peroxidase KatY protein. Adapted from [6] with permission.

read in a time-of-flight mass spectrometer (TOF MS). The resulting spectra give a multidimensional binding picture on the basis of different types of interaction. This process, known as surface-enhanced laser desorption/ionization (SELDI), has several advantages over matrix-assisted laser desorption/ionization (MALDI). As in MALDI, the sample is admixed with a small acidic molecule (the matrix) that crystallizes around the sample. In SELDI, however, the ProteinChip® array acts as a surface to which the sample binds uniformly and the matrix is placed on the chip only after the proteins are bound to the chip. Consequently, the spectra obtained are more uniform and reproducible as compared with MALDI-obtained spectra. This difference enables relative protein quantitation with SELDI that is not possible with MALDI. Although SELDI has numerous applications, we focus in this review on one specific application, protein profiling, and refer the reader to the review by Merchant and Weinberger [5•] for descriptions of some of its other applications.

Protein profiling or protein differential display studies have been widely used in the area of disease research, as comparison of lysates from normal versus diseased cells can reveal the expression of important marker proteins. Lysates from disease and control samples are processed on the same types of chip surfaces and the chips are read under the same data collection conditions. Subsequent peak comparison allows the identification of differences. Once a peak of interest has been detected, the analyte can be enriched or purified for further analysis. This is possible by washing the chip with varying stringencies of pH, salt or organic solvent, depending on the type of chip surface chemistry. Once a peak is sufficiently purified, on-chip digestion with proteolytic enzymes followed by analysis of the peptide patterns can yield important identification information. At the current stage of the technology, protein identification applications are still somewhat limited by the mass accuracy of the ProteinChip® reader and it is therefore important to use careful calibration techniques with known peptides of comparable molecular weight to obtain reliable peptide maps. Even with a mass accuracy in the milliDalton range, the proteomic researcher is cautioned that the protein chest of nature contains enough redundancy and sequence conservation to make wrong identification calls on the basis of peptide masses. The combination of profiling using ProteinChip® arrays with powerful fragmentation and sequencing capabilities can enable the researcher to obtain high-resolution data and novel protein information directly from biological samples.

As an example of profiling followed by protein identification in the realm of infectious disease, the ProteinChip® technology has been used to compare the pattern of protein expression in two physiological states of *Yersinia pestis*, the causative agent of the plague. Because it exists in two carriers, the flea and the rodent, *Y. pestis* has evolved to express different sets of proteins at the different temperatures encountered in the carrier organisms (26°C and 37°C). Thulasiraman *et al.* [6] have used ProteinChip® technology to identify two proteins whose expression is regulated by temperature. Lysates of *Y. pestis* grown at the two different temperatures were fractionated on strong anion exchange and immobilized metal-affinity chromatography chips, and the protein expression profiles were

compared (Figure 1). Two of the proteins seen to be upregulated at 37°C were purified and identified as the catalase/peroxidase KatY protein (78.8 kDa) and antigen 4 (14.9 kDa) via mass spectrometric analysis of tryptic peptide fingerprints.

ProteinChip® technology is also useful in linking gene-array expression data with protein discovery and, unlike its gene-based counterpart, can be used to examine post-translational modification of proteins. This approach was applied to verify upregulation of the acyl carrier protein (ACPm) in *Mycobacterium tuberculosis* treated with isoniazide (INH), an antifungal agent known to disrupt both micolic acid and cell wall synthesis. Transcription-array studies of INH-treated *M. tuberculosis* indicated a 4.3-fold increase in ACPm mRNA levels after a six hour exposure to INH. Lysates of control and INH-treated *M. tuberculosis* cultures were examined using anion exchange ProteinChip® arrays and SELDI-TOF MS, followed by analysis of an on-chip protein digest of a 13,217 Da protein using a laser desorption/ionization triple quadrupole TOF MS capable of MS/MS operation [7]. This protein, which was determined to be ACPm conjugated with its cofactor (phosphopantetheine) and a C_{26} fatty acid, appeared to be downregulated on the basis of ProteinChip® array data. However, closer examination of the ProteinChip® array data revealed that peaks found at 12,943.9, 12,997.7 and 13,025.0 Da in the INH-exposed lysates were in fact ACPm–phosphopantetheine conjugated with C_6, C_{10} and C_{12} fatty acids, respectively. In this manner, the presence of INH disrupted proper cell wall synthesis, reducing the abundance of the ACPm–C_{26} conjugate while elevating levels of the smaller fatty acid varieties to a point where total ACPm abundance was upregulated when compared with the control group.

Cancer research in particular has embraced platforms that enable differential protein profiling, as the identification of upregulated or downregulated proteins suggests the presence of a tumor marker that might be used for diagnosis, prognosis monitoring of disease progression or therapeutic success and/or as a therapeutic target. Cancer specimens can be compared with normal specimens, or cancerous regions of a biopsy or specimen can be directly compared with non-cancerous regions of the same specimen. Specific regions of a specimen can be selected for analysis using a technique called laser capture microdissection (LCM) [8]. Petricoin and colleagues [9] have used LCM in conjunction with ProteinChip® proteomics to study protein expression profiles in patient-matched normal colon, cancerous and metastatic samples with the generation of distinct protein profiles for each group. In particular, the authors used this approach to study normal prostate, prostatic intraepithelial neoplasia (widely considered to be a precursor lesion to invasive carcinoma) and prostate cancer, and they showed that the cancer specimens demonstrated downregulation of a 28 kDa protein. A similar study was performed on head and neck cancers [10].

Although SELDI studies are routinely performed on tissue samples, isolation of markers from body fluids, particularly serum or urine, may prove more valuable for diagnostic purposes. Wright *et al.* [11] have taken the approach of studying both tissue and body fluids in the search for biomarkers of prostate cancer. In this study, the authors showed that ProteinChip® proteomics could be used to identify known prostate cancer-associated biomarkers (prostate-specific antigen [PSA], prostatic acid phosphatase, prostate-specific membrane antigen [PMSA] and prostate-specific peptide) in cell lysates of LCM-captured prostatic cells and body fluids from prostate cancer patients, as well as to discover several potential biomarkers, including a 33 kDa and an 18 kDa protein found to be upregulated in prostate cancer cells. When the authors pre-fractionated the samples by size exclusion chromatography followed by ionic exchange chromatography before binding to the ProteinChip® array, they could detect over 300 protein peaks. On the basis of this approach, over 30 proteins were detected as being either over-expressed or under-expressed in specimens from prostate cancer patients [12]. Although no single protein was found to distinguish prostate cancer from the non-cancer groups, a combination of multiple protein peaks was capable of discriminating prostate cancer from a normal age-matched healthy male population. These studies were performed on a variety of body fluids, including serum, urine and seminal plasma.

The ProteinChip® platform used to identify biomarkers can also be used to develop a rapid, sensitive and high-throughput multi-marker assay. The premise of this approach is first to establish composite fingerprint profiles of both disease and non-disease states from a series of training samples, and then to use these composite profiles to make a diagnosis on actual unknown patient samples. In this case it is not essential to identify the proteins to make a diagnosis. Moreover, by utilizing a group of biomarkers one is not constrained by the sensitivity and specificity of any single biomarker, which may be relatively low. For example, by evaluating multiple urinary proteins by SELDI, the detection rate for low stage/grade bladder cancer increased to greater than 75%, compared with 30% by conventional urine cytology [13]. Another potential clinical application of the SELDI system is to develop immunoassays by immobilizing antibody to a specific biomarker on the ProteinChip® array surface. Wright and co-workers [14,15] have successfully used this approach to measure free PSA and complexed PSA, and PSMA in serum and seminal plasma. Interestingly, this platform was successful for quantitation of PSMA in serum where other immunoassay formats had failed. Also of interest was the detection of other possible PSMA isoforms that appear to be differentially expressed in normal, benign and malignant prostate samples, and that would not have been detected with other assay formats. By adding a variety of antibodies to a single array, it is possible to develop a multiplex assay to measure simultaneously eight or more biomarkers using a single ProteinChip® array.

New chip platforms for protein expression analysis

In recent months, several important papers have described the development of novel chip techniques, all of which employ the same concept as DNA arrays — to place thousands of addressable spots in a small grid. Emili and Cagney [16] propose segregating such arrays into nonliving and living arrays. Nonliving arrays consist of grids of proteins or peptides and are assayed biochemically, whereas living arrays consist of grids of organisms, typically bacteria or yeast, and are assayed metabolically. The first large-scale array of proteins was developed as a 'living array' to study two-hybrid interactions — yeast colonies expressing specific combinations of proteins were spotted in known positions and their phenotype (growth on selective media) was assayed [17]. Such a platform could be adapted for profiling analysis by constructing numerous types of selective media and replica plating grids of yeast onto each substrate. Extension of such a principle to the study of mammalian cells (e.g. tumor cell lines) will prove more technically challenging.

Another example of a living array is the antibody array recently described by de Wildt *et al.* [18••]. The arrays created by these authors contain a collection of 18,342 bacterial clones, each expressing a different single-chain antibody. These are spotted onto a 22×22 cm filter in duplicate, or duplicate filters might be used. It may be possible to use this technique in the future to randomly generate antibodies, to create arrays of these randomly generated antibodies, to incubate lysates of relevant tissue or even body fluids with the filters and to identify upregulated or downregulated proteins. The proteins of interest can then be enriched and identified using, for example, MALDI-MS.

Recently, MacBeath and Schreiber [19•] have described a novel protein microarray that utilizes glass slides coated with an aldehyde-containing silane reagent. The aldehydes react with primary amines (contained primarily in lysines and at the amino terminus) exposed on the protein's surface, leading to the binding of proteins in many different conformations on the chip. In a small-scale test of this chip, protein–protein interaction studies were performed — baits were printed onto the slide and probed with fluorescently labeled partners. In a larger-scale test, a slide was printed containing 10,799 spots of protein G and one spot of specific bait (FKBP12–rapamycin-binding protein, FRP) and was probed with a mixture of BODIPY–FL–IgG and Cy5–FKBP. All of the protein G spots were bound by the BODIPY reagent whereas the single spot of FRP was detected by the FKBP. The challenge is to extend this system to permit the analysis of large sets of unique proteins.

A distinct type of protein array that has been utilized for differential protein expression analysis is the tissue microarray [20]. This type of array consists of cylinders of tissue 0.6 mm in diameter punched out from paraffin blocks. Grids of these cylinders are constructed by aligning cylinders from a number of samples (e.g. several thousand) into a recipient paraffin block, and serial sections are cut. Each section can then be analyzed by fluorescent *in situ* hybridization or immunohistochemistry. Although this technique obviously relies on choosing specific antigens for visualization, it does allow the concurrent examination of potentially thousands of specimens across a gradient of severity (e.g. benign, *in situ* carcinoma, invasive carcinoma and metastatic carcinoma). This technique has been used to examine potential tumor markers in bladder cancer, renal cell carcinoma and prostate cancer [21–23] and combined with cDNA microarray analysis to study glioblastomas [24].

Conclusions

The ProteinChip® proteomics platform provides an outstanding system for chip-based analysis of differential protein expression, given its low sample requirements and high-throughput format. Moreover, once a specific biomarker is found, the same platform may be used for diagnosis. This is particularly useful if a panel of biomarkers is more sensitive or specific than a single biomarker. Because this platform does not rely on protein conformation for detection, it has a great advantage over new chip platforms designed to bind recombinant proteins in an array-based manner analogous to DNA microarrays. In their current state, these systems suffer from a number of requirements: the identity of the protein placed at each position must be known, the recombinant protein must be soluble, the protein must be folded such that an appropriate domain for detection is displayed on the chip surface and a detection method must be either available or developed. To be useful for general protein profiling, future generations of these techniques will require adaptability in design and detection. It is likely that the next iteration of microarray-based protein chips will be best suited for the study of specific families of proteins, as family members share a common domain to which a detecting moiety can be synthesized. Enzymes make particularly good profiling targets, as the detection of their activity is a natural assay (as an example, see Update). Future generations of these techniques will likely rely on the expression of fusion proteins that incorporate one or more domains that can be used simultaneously to increase the solubility of the protein, orient the protein on the chip surface and act as a detection substrate (e.g. change in fluorescence wavelength when a ligand is bound). Molecular breeding will almost certainly play a role in the development of such domains, while advances in surface chemistry will be needed to increase the capacity of chip surfaces. Finally, improvements in the sensitivity and sequencing capabilities of mass spectrometry instrumentation will enable identification of the proteins that are upregulated or downregulated.

Update

Recently, a chip designed to assay kinase activity was described [25•]. Because the entire yeast genome has been

sequenced, these researchers could clone and express glutathione-S-transferase (GST) fusions of almost all the yeast kinases. The kinase-assay chip was constructed by first creating a mold over which a liquid silicone elastomer was poured. This was allowed to harden, was peeled away and was then placed on a glass slide. This array, which measured 28 mm × 14 mm, contained wells 1.4 mm in diameter and 300 μm deep. The potential substrates were covalently attached to the walls of the wells and incubated with recombinant kinase in the presence of radiolabeled ATP. After the kinase reaction was completed, the wells were washed and the chip exposed to either X-ray film or a phosphoimager. In this manner, each kinase could be tested against each of the different substrates. It is easy to envision modifications of this system to study other enzymatic activities such as GTP cleavage or protein lipidation, as well as to serve as a platform for studying the affects of drug candidates on such enzymatic activities.

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