

Protein Microarrays in Proteome-wide Applications

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Abstract

Proteomics aims at understanding of protein status under certain physiological or diseased conditions in a large scale. Protein microarray technology has emerged as a promising approach for a wide variety of applications for scientific and clinical research on a whole-proteome scale. Herein, the aim of this review is to summarize the most recent developments in the applications of protein microarrays for biomarker profiling, enzyme substrate profiling, small molecule profiling, protein-protein interaction profiling, and antibody specificity profiling.

Keywords: Protein microarray; Proteome; Biomarker profiling; SELDI-TOF-MS

Introduction

Proteomics aims at understanding of protein status under certain physiological or diseased conditions in a large scale. The study of proteome focuses on the set of proteins encoded by the genome. It covers the whole proteins in a given cell, the protein isoforms and modifications, the protein-protein interactions, the structural description of proteins and their higher-order complexes, involving almost everything 'post-genomic' [1]. Proteomics has showed its promising use obviously, since we can learn how proteins interact with each other and even with non-proteinaceous molecules to control complex processes in cells, tissues and even whole organisms by studying components simultaneously through proteomic technologies [2]. However, it still has to face great challenges with the goals to define the identities, quantities, structures and functions of complete complements of proteins, and to characterize how these properties vary in different cellular contexts [3].

Mass spectrometry (MS) has played a central role in protein detection. Tremendous progress in performance of mass spectrometers has made MS-based methods particularly suitable for high-throughput proteomic profiling [4]. MS-based proteomics has been facilitated by the advances in ionization techniques and mass analyzers. The emerging analytical techniques and methodologies, cysteine mass tagging as an example, have contribute much to MS analysis [5]. Besides, the combination of stable isotope labeling based methods, for example, in isotope coded affinity tag (ICAT), isobaric tags for relative and absolute quantification (iTRAQ) or in stable isotope labeling with amino acids in cell culture (SILAC), with MS analysis has produced a possible measurement with high sensitivity and throughout [4]. Although the ongoing development of MS-based proteomics determined its broad application in scientific and clinical research, the requirement of sophisticated devices brings essential limits [6]. One complement technique, protein microarray, has shown its promising use.

Microarray has been a popular technology in proteomics with the widely use of protein microarrays, which made it possible that the simultaneous analysis of thousands of parameters can be done in a single experiment [7]. Microspots of capture molecules are immobilized in rows and columns onto a solid support and exposed to samples containing the corresponding binding molecules. Readout systems based on fluorescence, chemiluminescence, mass spectrometry, radioactivity or electrochemistry can be used to detect complex formation within each microspot. Such miniaturized and parallelized

binding assays can be highly sensitive, and the extraordinary power of the method is exemplified by array-based gene expression analysis. In these systems, arrays containing immobilized DNA probes are exposed to complementary targets and the degree of hybridization is measured. Recent developments in the field of protein microarrays show applications for studies of enzyme-substrate, DNA-protein and different types of protein-protein interactions.

Protein microarray has rapidly advanced to analyze ever-increasing numbers of proteins in biological samples [8]. Different protein microarrays, including capture arrays, reverse-phase arrays, tissue microarrays, lectin microarrays and cell-free expression microarrays, have emerged, providing information not obtainable by gene arrays [9]. Protein microarrays have demonstrated numerous applications for studying biomarker discovery, protein-protein interaction, protein-ligand profiling, kinase activity and posttranslational modifications of proteins [10]. Nowadays, protein microarrays have been widely applied as a promising proteomic technology with great potential for protein expression profiling, biomarker screen, drug discovery, drug target identification and analysis of signaling pathways in health and disease [11].

Protein Microarrays

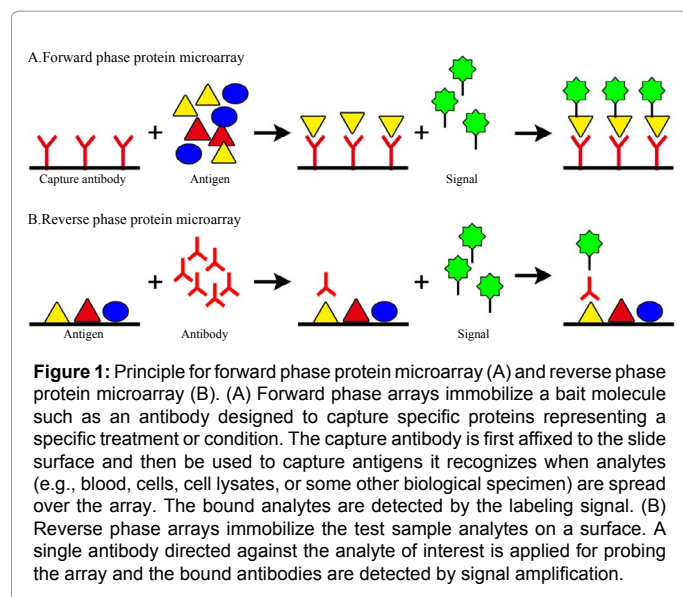
In general, protein microarrays can be classified into two broad categories, forward phase protein microarrays and reverse phase protein microarrays, according to the immobilized molecules (antigen or antibody) (Figure 1) [12]. With so-called forward phase protein microarrays, one protein sample is screened against multiple reagents. The capture reagent, usually an antibody, is first affixed to the slide surface. The immobilized antibody can then be used to capture antigens it recognizes when a test sample is spread over the array [12,13]. The test sample could be blood, cells, cell lysates, or some other biological

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Received February 22, 2014; **Accepted** April 07, 2014; **Published** April 11, 2014

Citation: Chen B, Liu Y, Shen Y, Xia Z, He G, et al. (2014) Protein Microarrays in Proteome-wide Applications. J Proteomics Bioinform S12: 001. doi:[10.4172/jpb.S12-001](https://doi.org/10.4172/jpb.S12-001)

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specimen. The technique offers high specificity, but is time consuming. However, it is limited to analyze the heterogeneous clinical specimens and can suffer from lack of unmatched antibody affinities on a single array [14]. In reverse phase protein microarrays, on the other hand, protein lysates are spotted. The arrays are then probed with antibodies to, for instance, phosphorylated signaling molecules. In this kind of microarray, one antibody probing multiple samples as opposed to one sample for multiple capture reagents [12,15]. With the sensitivity and precision of clinical grades, the reverse phase protein microarray can also reduce the number of antibody probes needed to detect a protein antigen, which could be used to interrogate tissue samples, cells, serum, or body fluids [16].

Some researchers prefer to divide them into functional protein microarrays and protein detecting microarrays according to their applications [11,17]. Functional protein microarrays are composed of arrays containing full-length functional proteins or protein domains [18].

They are used to study and identify new molecular interactions between proteins, small molecules or enzyme substrates. Antibody microarrays are the most common analytical microarray and are well suited for detecting changes of proteins in biological samples with a relatively large dynamic range [11]. They are typically used to profile a complex mixture of proteins in order to measure binding affinities, specificities, and protein expression levels of the proteins in the mixture [19].

In protein microarray experiments, signals can be detected by label-based or label-free strategies. Based on the different detection techniques, protein microarray detection techniques can be also classified as being label-based and label-free [20]. The labelling strategies, such as fluorescent, chemiluminescent and radioactive labelling, have synthetic challenges, multiple label issues and may exhibit interference with the binding site. Therefore, development of sensitive, reliable, high-throughput, label-free detection techniques are now attracting significant attention [21]. Label-free detection techniques monitor biomolecular interactions and simplify the bioassays by eliminating the need for secondary reactants [22]. These techniques include surface plasmon resonance mass spectrometry (SPR-MS) [23], backscattering

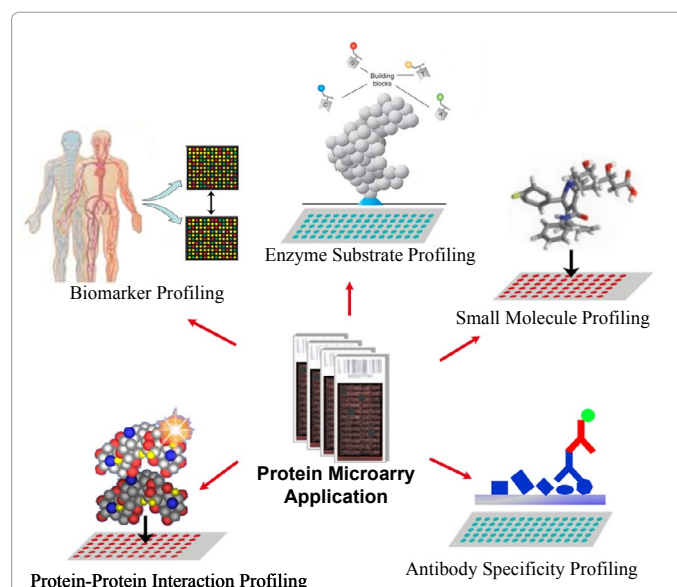
interferometry [24], brewster angle straddle interferometry [25], UV fluorometry [26], surface enhanced laser desorption ionization time-of-flight mass spectrometry (SELDI-TOF-MS) [27], tagged-internal standard assay [28] and spectral-domain optical coherence phase microscopy [29]. The analysis is rapid and simple, requires small sample amount, and can be used for direct detection of analytes bound from complex samples, such as urine, serum, plasma, and cell lysates. Moreover, integration of these techniques in protein microarrays may exhibit significant impact in future and they provide quantitative information for the binding kinetics.

Application of Protein Microarray

Protein microarrays are potentially powerful tools in biochemistry and molecular biology. They provide a powerful tool to profile protein-protein interactions in high-throughput and to quantify the abundances and posttranslational modifications of different kinds of proteins in complex mixtures (Table 1). Their applications for biomarker screening, enzyme substrate profiling, small molecule profiling, discovering protein-protein interactions, and antibody specificity profiling will be summarized as follows (Figure 2).

Biomarker screening

It is critical for early and improved diagnosis and prognosis to profile biomarkers for rapid discovery of disease markers specific to a variety of infectious diseases, cancers and autoimmune diseases [18,30]. Protein microarray has been successfully used for biomarker identification from a number of different diseases including but not limit to colon cancer [31], breast carcinoma [32], prostate cancer [33], bladder cancer [34] and non-small cell lung cancer [35]. For example, it has been used to identify autoantibody signatures in ovarian cancer and 15 proteins have been yielded that were candidates for further



Profiling	Methods	Results	Reference
Biomarkers	Antibody microarray	Profiled MMPs/TIMPs signature in gastric carcinoma	[6]
	Tissue microarray	Identified novel prognostically significant tumor	[29]
	Novel high-density custom protein microarrays (NAPPA)	Detected 119 antigens to autoantibodies in breast cancer	[30]
	ProtoArray Human Protein Microarrays v4.0 & tissue microarray	Identified galectin-8, TARP and TRAP1 for biomarkers in prostate cancer	[31]
	Protein microarray	Profiled biomarkers in bladder cancer	[32]
	Protein microarray	Analysis of antibody responses in non-small cell lung cancer patients and healthy donors	[33]
	ProtoArrays	Discovered 15 potential tumour-associated antigens in ovarian cancer	[34]
	SELDI-TOF-MS protein microarrays	Identified serum biomarkers for lung cancer	[36]
	SELDI-TOF-MS protein microarrays	Identified eIF2B-delta as the potential serum biomarkers for renal cell carcinoma	[37]
	SELDI-TOF-MS protein microarrays & CT scan	Distinguishes renal cell carcinoma from benign renal	[38]
Enzyme substrates	SELDI-TOF MS based ProteinChip arrays	profiling for diagnostic and prognostic bladder cancer biomarkers	[39]
	Protein microarrays	Found 11 known APC substrates polyubiquitinated.	[44]
	Protein microarray	Profiled ubiquitylation, SUMOylation and NEDDylation	[45]
	Yeast Proteome Microarray	Identified and validated the acetylation sites of Pck1p	[46]
Small molecular	Peptide microarray platform	casein kinase I to phosphorylate a subset of the novel motifs	[50]
	Protein microarray	Uncovered that triterpenoids associate with Arp3	[52]
Protein-protein interaction	Protoarray	Analysis of keratan sulfate, chondroitin sulfate A, and hyaluronic acid molecular interactions	[53]
	ImaGenes UniPex colony microarrays	Identified a total of 112 novel NEMO interactors	[54]
Antibody specificity	ProtoArray Human Protein Microarray	Identified GSK3 proteins as possible RKIP binding proteins	[55]
	Protein microarray	Develop a high-affinity synbody that specifically binds AKT1	[62]

Table 1: Overview of protein microarray applications.

study as tumour-associated antigens, 10 of which were reproducible in the cancer set [36].

In another study, a new type of protein microarrays has been explored. It was interfaced with a dual-color fluorescence-based read-out to screen autoantibodies in serum [37]. Molecular designed to contain a five-cysteine tag for immobilization and green fluorescent protein for detection were incorporated as recombinant antigens and they were immobilized on in-house-designed maleimide-incorporated diamond-like carbon substrates. Uniquely, the array was heated subsequently and exposed to a solution of denaturing and reducing agents to denature the arrayed proteins. Successfully, the heating treatment has been proved to improve the subsequent detection. And thus a 4-plex array targeting autoantibodies related to hepatocellular carcinoma has been used in the sera of hepatitis C virus-positive patients as a model system to demonstrate proof-of-concept.

With high specificity effective for high abundance protein, SELDI-TOF-MS protein microarrays have been a high-throughput technique for analysis of complex biological specimens for biomarker profiling. They have been successfully applied to identify serum biomarkers for cancers as a simple, sensitive and highly reproducible method [38-40]. In addition to serum samples, they can be also a suitable approach to identify biomarkers in various biological specimens such as tissues [41] and urine [42].

Enzyme substrate profiling

Identification of protein targets of post-translational modification is an important analytical problem in biological field [43]. Currently, more than 50 protein kinase drug candidates are in clinical trials to treat a number of different diseases. The discovery of compounds that regulate kinase function is very attractive. However, most common used technologies such as mass spectroscopy have some major limitations, including functional redundancy and low throughput [44]. With the

capacity to profile thousands of candidate protein substrates in a single experiment, protein microarrays technology is an excellent method to identify enzyme substrates, especially the profiling of kinases, ubiquitin ligases, and methyltransferases for target discovery and validation. Protein microarrays exposed to cellular extracts could offer a rapid and convenient means of identifying modified proteins. It has been adapted to study numerous modifications including phosphorylation, methylation, ubiquitination, SUMOylation, NEDDylation, and nitrosylation [45].

With this method, extracts that replicate the mitotic checkpoint and anaphase release have been used to identify differentially regulated polyubiquitination [46]. In this study, the complex extracts were incubated under a coverslip on the microarrays and the polyubiquitinated products were detected by specific antibodies. Also by protein microarray, three molecularly complex post-translational modifications (ubiquitylation, SUMOylation, and NEDDylation) have been profiled using purified ligase enzymes and extracts prepared from cultured cell lines and pathological specimens [47], and many nonchromatin substrates of the essential nucleosome acetyltransferase of H4 complex have been identified and validated [48].

As for substrate specificity profiling, peptide microarrays could be an efficient platform, which take peptides as the molecules spotted and immobilized on microarrays [49]. Compared to proteins, peptides tend to be less fragile and delicate and the library fabricating will be much easier [50]. What's more, this strategy could be extended to identification of phosphorylation sites and kinase specificity in vitro. In a study performed with peptide microarray, casein kinase I was evaluated experimentally to phosphorylate a subset of the 299 novel serine/threonine or tyrosine phosphorylation motifs discovered [49], which demonstrate that it is feasible to identify novel phosphorylation motifs through large phosphorylation datasets. Unlike other systems for analyzing protein modification, the enzyme substrate profiling

Products	Companies	Websites
ProtoArray Human Protein Microarrays	Thermo Fisher Scientific Inc.	http://www.lifetechnologies.com
Proteome Profiler Antibody Arrays	R&D Systems, Inc.	http://www.rndsystems.com
Protein Arrays	Cambridge Protein Arrays Ltd.	http://www.cambridgeproteinarrays.com
Protein Arrays	Applied Microarrays, Inc.	http://appliedmicroarrays.com
PAK Protein Array Kits	GE Healthcare Companies	http://www.gelifesciences.com
Antibody microarray kit	Kinexus Bioinformatics Corporation	http://www.kinexus.ca
Products for use with protein microarrays	SurModics, Inc.	http://www.surmodics.com
Microarray tools	Grace Bio-Labs, Inc.	http://www.gracebio.com

Table 2: Summary of commercially available protein microarrays.

application allows researchers to discover and identify new targets in as little as one day. In addition to testing enzyme activity, inhibitors and other compounds can be added to the assay to profile their effect on the enzyme substrate profile. This technique allows researchers to rapidly assess the specificity of drugs on enzymes.

Small molecule profiling

Protein microarrays can also be an ideal drug discovery tool for small molecules. Researchers using the small molecule profiling application on the protein microarrays have been able to identify drug targets and understand their mechanisms of action. For instance, To et al. revealed that in order to inhibit cell migration synthetic triterpenoids target actin related protein 3 and branched actin polymerization by means of protein microarray together with mass spectrography method [51]. Furthermore microarray proteins have also been successfully used to discover the targets of corneal stroma extracellular matrix glycosaminoglycans including keratan sulfate, chondroitin sulfate A, and hyaluronic acid [52].

By detecting the radiolabeled, biotinylated, or fluorescently labeled small molecule against the functional proteins, the possible targets with the protein microarray could be profiled. Thus the binding proteins can be immediately identified by recognizing the proteins on the protein microarray. Besides, the protocol is very straightforward, and can also be adapted to monitor target competition using unlabeled competitor molecules to verify target specificity.

Protein-protein interaction

Comprehensive protein-protein interaction maps promise to reveal many aspects of the complex regulatory network underlying cellular function and would provide a framework to study protein deregulation in complex diseases [53,54]. In order to uncover new protein-protein interactions, protein microarrays can be used to profile the certain purified protein against functional proteins and identify the possible binding partners. By using protein microarrays, Fenner et al. [55] expanded the substantial interactome of the NF- κ B essential modulator. They identified a total of 112 protein interactors, in which more than 30% were kinases, while at least 25% were involved in signal transduction. Another set of protein-protein interaction experiments using proteome microarrays was performed [56]. This study successfully utilized a protein microarray-based screen, with each array containing around 8,000 individual human proteins, to identify the possible Raf kinase inhibitory protein binding proteins.

Antibody specificity profiling

Another important application of protein microarrays has been to determine the specificity of antibodies. Antibodies are the most

widely used protein ligand for all proteins in the human and other proteomes [57,58]. Antibody specificity is a key feature for determining an antibody's value and it is important for research and therapeutic antibody development [59,60]. With these considerations in mind, a new protein binding ligand called a synthetic antibody or synbody has been developed, which is composed of two peptides linked by a scaffold and then the synbody was screened against a library of proteins to discover the target [61]. This method has delivered a high-affinity ligand that specially binds a target protein in a single discovery step. It makes candidate profiling of therapeutic antibodies a rapid way and helps to determine which candidates to move forward earlier.

Conclusion

Protein microarray has been an excellent high-throughput method used to probe proteins for a specific function or biochemistry on a large scale. To have further understanding, several commercial available protein microarrays have been listed in Table 2. They have a main advantage by tracking large numbers of proteins in parallel, which are also rapid, automated, economical, highly sensitive, and saving samples and reagents. By now, it is a promising approach with a wide variety of applications for scientific and clinical research.

Acknowledgements

This work was financially supported by the grants from National Key Basic Research Program of China (2011CB910703, 2013CB911303), the National 863 High Tech Foundation (2014AA020608), National Natural Sciences Foundation of China (30970654, 31071235), grants for New Century Excellent Talents in University (NCET-10-0595) and specialized research fund for the Doctoral Program of Higher Education (20120181110025). The authors gratefully acknowledged the support of Sichuan Province Program (2010JQ0016, 2012SZ0002).

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This article was originally published in a special issue, **Microarray Proteomics** handled by Editor. Dr. Qiangwei Xia, ProQualiX Consultants, Inc., USA

Citation: Chen B, Liu Y, Shen Y, Xia Z, He G, et al. (2014) Protein Microarrays in Proteome-wide Applications. J Proteomics Bioinform S12: 001. doi:[10.4172/jpb.S12-001](https://doi.org/10.4172/jpb.S12-001)