

Developments and Applications of Functional Protein Microarrays

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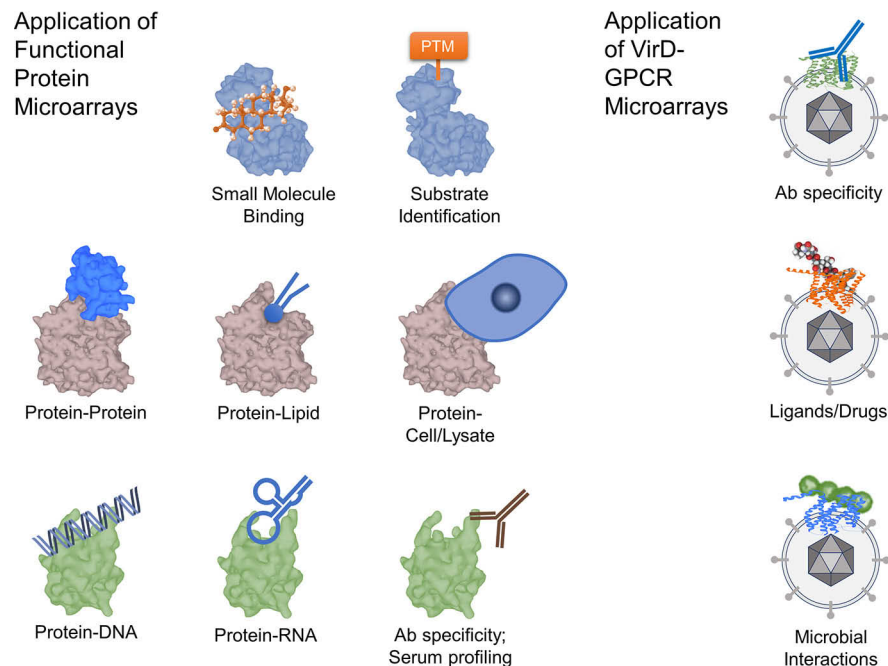
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In Brief

Functional protein microarray is a crucial tool in the study of proteins in native, unbiased, and high-throughput manner. There is a wide variety of applications, including the study of proteome-wide molecular interactions, analysis of post-translational modifications, identification of novel drug targets, and examination of pathogen-host interactions. Functional protein microarray is also useful in profiling antibody specificity, as well as in the discovery of novel biomarkers, especially for autoimmune diseases, infectious diseases, and cancers. Recently, the virion display method has been applied to produce functional GPCR array for various research and pharmaceutical applications.

Graphical Abstract

Application of Functional Protein Microarrays



Application of VirD-GPCR Microarrays

Highlights

- Summarize the development of functional protein microarray.
- Application of functional proteome microarray in basic research.
- Application of functional proteome microarray in translational research.
- Fabrication of functional membrane protein array using virion display method.



Developments and Applications of Functional Protein Microarrays*

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Protein microarrays are crucial tools in the study of proteins in an unbiased, high-throughput manner, as they allow for characterization of up to thousands of individually purified proteins in parallel. The adaptability of this technology has enabled its use in a wide variety of applications, including the study of proteome-wide molecular interactions, analysis of post-translational modifications, identification of novel drug targets, and examination of pathogen-host interactions. In addition, the technology has also been shown to be useful in profiling antibody specificity, as well as in the discovery of novel biomarkers, especially for autoimmune diseases and cancers. In this review, we will summarize the developments that have been made in protein microarray technology in both in basic and translational research over the past decade. We will also introduce a novel membrane protein array, the GPCR-VirD array, and discuss the future directions of functional protein microarrays. *Molecular & Cellular Proteomics* 19: 916–927, 2020. DOI: 10.1074/mcp.R120.001936.

Proteins are diverse biomolecules with a wide variety of structures and functions, and as such, it is a challenge to study them in a high-throughput fashion. There are three major types of protein microarrays: functional, analytical, and reverse phase. Functional protein microarrays are constructed with proteins purified/synthesized in a high-throughput fashion, enabling hundreds, and even thousands of different proteins to be probed for their biochemical properties in parallel. Analytical protein microarrays use affinity reagents that are immobilized on the array to detect or quantify complex biological samples. Finally, reverse phase protein microarrays utilize complex biological samples immobilized on the array and use affinity reagents for detection (1). In this short review, we focus on functional protein microarrays, summarize the recent developments in functional microarray technology, and discuss potential future applications.

Compared with other methods, such as mass spectrometry, functional protein microarrays are more capable of de-

tecting weak interactions, more flexible with low abundance proteins, and more amenable to analyzing crude samples such as serum. However, there are still some limitations for protein microarrays. The binding events observed during microarray experiments may not reflect the binding events that occur in the context of a cellular environment. Also, most of the detection methods involve labels and thus require proper controls (2). To date, many different types of functional protein microarrays have been developed in terms of differences in proteome coverage, protein lengths, and production pipelines. Some notable examples of the different categories of protein microarrays include purified proteome microarrays for full-length proteins, purified protein family microarrays for different protein categories, purified protein domain microarrays for user-defined domains/epitopes, and cell-free protein/peptide microarrays for *in vitro* translation from cDNA or *in vitro* synthesis. In Table I, we summarize the current developments in functional protein microarrays and divide them into these four categories. We also introduce a new concept, a membrane protein microarray (*i.e.* VirD¹ array), and discuss the possible future directions of VirD array technology.

Development of the Functional Protein Microarray—The proteome is the entire set of proteins that can be expressed by a genome. The development of a purified proteome microarray usually requires assembly of a genome-wide collection of open reading frames (ORFs) cloned into an expression vector, expression of the encoded proteins in cells, individual protein purification in a high-throughput fashion, and immobilization of the proteins on a microarray. Advances in purified proteome microarrays for model organisms, such as *S. cerevisiae*, *E. coli*, humans, and *Arabidopsis thaliana*, have propelled functional and biochemical studies of proteins to a proteomic level. The first of its kind is the *S. cerevisiae* (budding yeast) proteome array, developed by the Snyder group in 2001 and containing 5,800 full length yeast proteins (3). Currently, there are many purified proteome microarrays covering a wide variety of model systems, including coronaviruses (4), flaviviruses (5), human herpesviruses (6), *M. tuberculosis* (7),

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TABLE I
Summary of high-content functional protein microarrays

Organism/Protein classification	Protein No.	Coverage	Expression system	Refs.
Purified proteome microarray				
<i>Homo sapiens</i> ^a	21,000	81%	<i>S. cerevisiae</i>	(11, 12)
<i>S. cerevisiae</i>	5800	80%	<i>S. cerevisiae</i>	(3)
<i>E. coli</i> K12	4256	90%	<i>E. coli</i>	(8)
<i>Arabidopsis thaliana</i>	15,000	56%	<i>N. benthamiana</i>	(9, 10)
<i>M. tuberculosis</i>	4262	95%	<i>S. cerevisiae</i>	(7)
Coronavirus	82	75%	<i>S. cerevisiae</i>	(4)
Epstein-Barr virus	60	66%	<i>S. cerevisiae</i>	(6)
Zika and dengue viruses	48	86%	<i>S. cerevisiae</i>	(5)
Herpes simplex virus-1/2	72	50%	<i>E. coli</i>	(94)
Purified protein family microarray				
G protein-coupled receptors	315	84%	Mammalian cell lines	(14)
Membrane and secrete proteins ^b	505/1121	<19%	<i>E. coli</i>	(15)
Influenza (HA antigens)	283	NA	Baculovirus or human cell	(16, 17)
HIV (gp120 and gp140)	10	NA	Mammalian or insect cell	(18)
Purified protein domain microarray				
Protein domains	~400	NA	<i>E. coli</i>	(20, 111)
Protein epitope signature tags	21,120	NA	<i>E. coli</i>	(21, 22)
Consensus sequence ^c	44	NA	<i>E. coli</i>	(23)
Cell-free protein/peptide microarray				
Various pathogen antigens ^d	100–7500	10–90%	<i>In vitro</i> expression	(24, 25)
Nucleic acid programmable	~10,000	NA	<i>In vitro</i> expression	(26–28)

^aCommercialized with the trademark HuProt™ by CDI Laboratories and expanded to >21,000 proteins in version 4.

^bThe authors select proteins for expression based on a secretion signal peptide or at least one transmembrane domain. They express 505 proteins in full-length and 1121 protein fragments.

^cDesigned 44 consensus coding sequences from 3,604 different dengue strains.

^dPathogens included: *Borrelia burgdorferi*, *Coxiella burnetii*, *Burkholderia pseudomallei*, *Schistosoma japonicum*, *Chlamydia trachomatis*, *Bartonella henselae*, *Brucella melitensis*, *Hookworm Necator americanus*, *Leptospira interrogans*, *Plasmodium vivax*, *Schistosoma mansoni*, *Francisella tularensis*, *Toxoplasma gondii*, *Cytauxzoon felis*, *Plasmodium falciparum*, *Candida albicans*, *Mycobacterium tuberculosis*, *Salmonella enterica Typhi*, Human papillomaviruses, and herpes simplex viruses 1&2.

E. coli K12 (8), *S. cerevisiae* (3), *Arabidopsis thaliana* (9, 10), and humans (11, 12). Because of the coverage of ORF collections and the efficiency of protein expression/purification, the proteome coverage on such arrays ranges from 56% to 95% (Table I). The choice of protein expression system greatly influences post-translational modifications and can affect the success rate of protein purification. For example, because of a lack of eukaryotic posttranslational modifications and chaperones, proteins encoded by *C. elegans* were poorly expressed in *E. coli*, with an expression rate of 48%. Of this 48%, only 15% were soluble (13). Therefore, homologous expression systems are generally preferred to obtain the highest protein activity and expression efficiency. The *S. cerevisiae*, *E. coli*, and *Arabidopsis thaliana* proteome arrays are three of the best examples for use as homologues expression systems. In some cases, especially with mammalian cells, it is difficult and expensive to transfect cells, and thus one can use an alternative expression system, such as budding yeast, to accommodate protein production pipelines. Indeed, the hu-

man proteome microarray (i.e. HuProt) is one of the best examples to use a heterologous expression system, as it exhibits the most comprehensive human proteome collection purified from yeast (81% proteome coverage). Another commercial human proteome microarray, called ProtoArray, contained >9,000 human proteins purified from insect cells (43% proteome coverage), but was discontinued in 2018.

A protein family microarray is designed to interrogate specialized groups of proteins for their biochemical functions. Today, there are many different protein family microarrays, each used for different purposes. For example, one can utilize a G-protein coupled receptor (GPCR) array for pharmaceutical applications (14), a membrane/secreted protein array for profiling autoantibodies (15), a hemagglutinin antigen array for investigating influenza vaccines (16, 17), and a gp120/140 array from HIV for analyzing immune responses (18). Because most protein family microarrays have a relatively small number of proteins, the expression system can be tailored for desired qualities and quantities. For example, the GPCR array is developed using Virion Display (VirD) technology (19) to maintain the seven transmembrane structure and to obtain the best GPCR expression in several mammalian cell lines, including Vero, HEL, HeLa, and 293T cells (14).

¹ The abbreviations used are: VirD, Virion Display; PrEST, Protein Epitope Signature Tag; IVTT, *in vitro* transcription and translation; NAPPA, Nucleic Acid Programmable Protein Array; PTM, post-translational modifications; HBMEC, human brain microvascular endothelial cells.

Alternatively, protein domain microarrays can be designed to analyze certain regions, domains, or epitopes within the proteins. These arrays often involve the careful design of desired gene sequences before entering the protein production pipeline. Protein domain arrays, Protein Epitope Signature Tag (PrEST) arrays, and consensus sequence protein arrays are the three best examples of this sort. The protein domain arrays reported by Jones *et al.* contain all the human Src homology 2 and phosphotyrosine binding domains to profile the interaction networks for tyrosine phosphorylation on ErbB receptors (20). PrEST arrays contain the unique signature in the human proteome developed by the Human Protein Atlas Consortium for identifying multiple sclerosis autoantibodies (21) or for validating antibody specificity (22). In a consensus sequence protein array, Qi *et al.* summarize 44 consensus serotype sequences out of 3604 different dengue strains and construct a protein array accordingly for dengue serotyping (23). Overall, both purified proteome, protein family, and protein domain arrays have a wide variety of applications in basic and translational research, as well as pharmaceutical industry.

The cell-free protein/peptide microarray is designed to display a short peptide or full-length protein using a cell-free system. Cell-free expression is designed to bypass the expensive and often tedious work of cell-based protein production. To construct protein assays with an *in vitro* expression, many expression systems, including expression lysate from *E. coli*, insect cells, wheat germs, and human cells, are commercially available. For instance, the Felgner Lab established various pathogen arrays ranging from viruses to bacteria and yeasts by using an *in vitro* transcription and translation (IVTT) system adopted from *E. coli* (Table I and footnote) (24, 25). On the other hand, the LaBaer group utilized a DNA array, dubbed as the Nucleic Acid Programmable Protein Array (NAPPA), to construct human proteome arrays using *in vitro* transcription/translation system (26–28). Because cell-free expression lacks regulated protein folding, segregated cellular compartments, and coordinated post-translational modifications (PTMs), the protein functions are not guaranteed (27). The IVTT system also suffers from a lower yield of larger proteins (e.g. >50 kDa), potential contamination by other proteins presented in the lysates, and low array density (e.g. ~2,000 features per array) (27). Nevertheless, protein arrays produced by cell-free expression are quite useful to analyze immune responses (24–26).

Application of Yeast Proteome Microarrays in Basic Research—Functional protein microarrays, especially purified proteome microarrays, are useful for profiling proteome-wide molecular interactions and allow for a comprehensive, unbiased screening. In basic research, researchers have been using functional protein microarrays to study protein-protein interactions, protein-lipid interactions, protein-cell/lysates, protein-DNA interactions, protein-RNA interactions, small molecule binding, and PTMs, such as glycosylation, ubi-

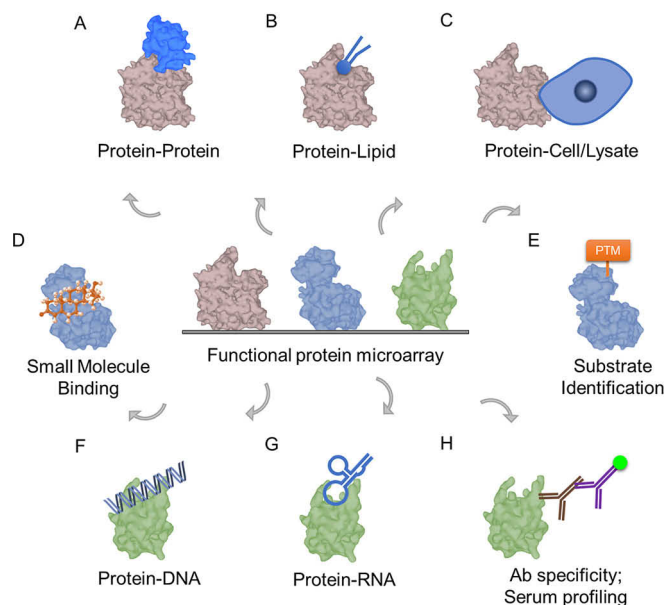


Fig. 1. Application of Functional Protein Microarray. Applications of functional protein microarray for interrogating protein-binding property include *A*, Protein-protein interactions; *B*, Protein-lipid interactions; *C*, Protein-cell/lysate interactions; *D*, Small molecule binding; *E*, Enzyme-substrate relationships; *F*, Protein-DNA interactions; *G*, Protein-RNA interactions; *H*, Antibody specificity/Serum profiling. PTM = post-translational modification.

quitylation, SUMOylation, acetylation, phosphorylation, and methylation (Fig. 1A–1G). In Table II, we summarize representative studies based on the research applications illustrated in Fig. 1. Here, we review research studies based on the proteomes immobilized on microarrays.

Zhu *et al.* constructed the very first proteome microarray, the yeast proteome microarray, and utilized it to investigate protein-protein interactions and protein-lipid interactions. The array was probed with biotinylated calmodulin and 33 new calmodulin binding proteins with new common motifs were identified (3). In the same study, the yeast proteome array was probed with fluorescently labeled liposomes carrying various phosphatidyl-inositides and more than 150 phospholipid binding proteins were identified (3). Huang *et al.* used the same microarray to identify binding proteins for two small molecule inhibitors of rapamycin, SMIR3 and SMIR4, and identified 8 and 30 protein targets, respectively. Most target proteins were involved in $PI_{3,4}P_2$ signaling (29). Hall *et al.* used the yeast proteome microarray to profile DNA binding proteins and revealed a mitochondrial enzyme, Arg5,6, can regulate both nuclear and mitochondrial gene expression (30). Similarly, Zhu *et al.* used the yeast proteome microarray to profile RNA hairpin binding proteins and identified two proteins: Pus4 and App1. Their antiviral activity against the spread of brome mosaic virus was demonstrated in tobacco (31). The Zhu lab further demonstrated the utility of proteome arrays by performing covalent enzymatic reactions on the arrays. They were the first to establish the protein acetylation reactions

TABLE II
Representative studies using functional protein microarrays

Classification/Research	Tools	Major findings	Refs.
Protein-Protein			
Calmodulin	Yeast proteome array	Identified 30 new targets	(3)
4 antimicrobial peptides	<i>E. coli</i> proteome array	Identified many intracellular targets	(35)
2-oxohistidine peptide	<i>E. coli</i> proteome array	Identified 9 redox targets	(112)
NS5A	HuProt	Identified 90 targets and validated Pim1	(44)
PknG	HuProt	Identified 125 targets and validated CypA	(47)
	MTB proteome array	Identified 59 targets	(7)
ROP18	HuProt	Identified 68 targets and validated 4 bindings	(46)
SidM, LidA, and AnkX	Human NAPPA	Identified 18, 20, and 8 host targets	(48, 49)
61 ErbB peptides	SH2 PBD array	Profiled interaction networks	(20)
Protein-Lipid			
5 Phospholipids	Yeast proteome array	Identified 150 targets	(3)
Protein-Cell/Lysate			
HBMEC	<i>E. coli</i> proteome array	Identified 23 targets and validated YojI	(38)
Macrophage lysate	MTB proteome array	Identified 26 targets	(113)
Small Molecule Binding			
2 inhibitors of rapamycin	<i>E. coli</i> proteome array	Identified 39 targets and validated Tep1p and Nir1p	(29)
Arsenic	HuProt	Identified 360 targets and validated hexokinase	(53)
6-O-angeloylplenolin	HuProt	Identified 99 targets and validated STAT3	(54)
Cyclic di-GMP	MTB proteome array	Identified 30 targets	(7)
	<i>E. coli</i> proteome array	Identified 8 targets and validated CobB	(37)
Substrate Identification			
Six SUMO E3 ligases	HuProt	Identified 250 substrates and validated PYK2	(114)
289 kinases	HuProt ver. I	Constructed a high resolution kinase-substrate network	(115)
Four herpesvirus kinases	HuProt	Identified a conserved host pathway for viral replication	(116)
ppGalNAc-Ts	HuProt	Identified 128 common substrates for glycosylation	(51)
VopS and IbpAFic2	Human NAPPA	Identified 21 AMPylation substrates	(52)
87 yeast kinases	Yeast proteome array	Constructed a kinase-substrate network	(117)
Ubiquitin E3 Rsp5	Yeast proteome array	Identified 84 substrates and validated Rnr2	(33)
NuA4	Yeast proteome array	Discovered two yeast ageing pathways involving Pck1p and Sip2	(32, 118)
Tyrosine sulfation	<i>E. coli</i> proteome array	Identified 875 substrates	(40)
11 MTB kinases	MTB proteome array	Identified 1,027 interaction network	(58)
Protein-DNA			
Yeast genomic DNA	Yeast proteome array	Identified 200 targets and validated Arg5,6	(30)
Mismatch and abasic site	<i>E. coli</i> proteome array	Validated YbaZ and YbcN	(8)
Promoter DNA of fimS	<i>E. coli</i> proteome array	Identified 19 targets and validated Spr	(34)
460 DNA motifs	4,191 human array	Discovered many unconventional DNA-binding proteins and showed Erk2 as a transcriptional repressor	(12)
Protein-RNA			
BMV viral RNA	Yeast proteome array	Identified and validated Pus4 and App1's role in preventing viral spreading in tobacco	(31)
13 lncRNAs	HuProt	Found many unconventional RNA-binding proteins and validated IDH1	(42)
miR-122	HuProt	Identified 40 targets and validated hnRNP K	(43)
Antibody specificity			
mAbs against TFs	HuProt	Demonstrated the use of HuProt for specificity test of mAbs	(57)
400 Abs against SH2	SH2 PrESTs array	Verified Abs specificity	(111)

mAbs = monoclonal antibodies.

using the yeast NuA4 complex, and two parallel signaling pathways in yeast aging were discovered (32). It has also been applied to determine the substrates of a HECT domain ubiquitin E3 ligase Rsp5 (33). These studies demonstrate

the usefulness of the yeast proteome microarray in basic research.

Application of E. coli Proteome Microarrays in Basic Research—Chen *et al.* established a purified *E. coli* proteome

microarray in 2008, comprising of 4256 unique proteins and applied it to identify potential new players in the DNA damage response. The *E. coli* proteome microarray was probed with several short DNA probes containing mismatched base pairs or abasic sites, and two DNA repair proteins were identified: YbaZ and YbcN (8). In another study the same array was used to detect DNA binding proteins to the promoter of type 1 fimbriae and identified Spr as a phase switch for type 1 fimbria expression (34). Ho *et al.* probed several antimicrobial peptides using the *E. coli* proteome array and identified many intracellular targets. Among the four antimicrobial peptides, they identified some shared and unique targets and suggested a synergistic effect on LfcinB and Bac7, as well as LfcinB and PR-39 (35). Hsiao *et al.* probed the *E. coli* proteome array with four glycosaminoglycans that are common on host cells and identified a hundred protein targets. They further validated ycbS as a bacterial factor for cell entry (36). Xu *et al.* probed the *E. coli* proteome array with an important bacterial second messenger, cyclic di-GMP, and identified CobB as a strong binder. Because CobB is a deacetylation enzyme, they subsequently found that cyclic di-GMP inhibits the enzymatic activity and forms a novel feedback loop to the cyclic di-GMP production (37). Feng *et al.* used *E. coli* proteome microarray to investigate protein-cell interactions by probing the human brain microvascular endothelial cells (HBMEC) on the array. They identified 23 target proteins and validated Yojl as a protein for *E. coli* invasion. Moreover, they purified Yojl, probed using HuProt, and further identified interferon-alpha receptor as a host receptor for Yojl (38). Besides various binding assays, the *E. coli* proteome microarray has also been applied to identify substrates, including substrates of glycoproteins (39), tyrosine sulfation (40), and ClpYQ protease (41). As demonstrated by these representative works, the *E. coli* proteome microarray is widely used to study bacterial physiology as well as host-microbial interactions.

Application of Human Proteome Microarrays in Basic Research—The human proteome microarray is the most widely used array in basic research, translational research, and in the pharmaceutical industry. There are three popular human proteome microarrays: HuProt, ProtoArray, and NAPPA. HuProt contains ~21,000 individual purified human proteins in full-length, which is by far the most comprehensive human proteome collection. ProtoArray contained ~9000 human proteins purified from insect cells, but was discontinued commercially in 2018. NAPPA is an *in vitro* expression system that has been applied to express 10,000 human proteins.

The HuProt array was not made overnight. In its early stages, it contained 4191 unique human proteins, mostly transcription factors and co-factors. Hu *et al.* performed a large scale DNA-binding assay with 460 DNA motifs on this array and found 17,718 protein-DNA interactions. Not only were numerous known protein-DNA interactions recovered, but they also found many unconventional DNA-binding proteins,

including a mitogen-activated protein kinase (MAPK), Erk2. In-depth mutagenesis studies and cell-based assays demonstrated that Erk2 acts as a transcriptional repressor in the regulation of interferon-gamma signaling (12). In 2012, the Zhu lab published the construction of HuProt version I, which contained 16,368 individual purified human proteins in full-length and demonstrated that it could serve as a useful tool to identify highly specific monoclonal antibodies (11). The work laid the foundation for the NIH-funded Protein Capture Reagents Program (PCRP; <https://commonfund.nih.gov/proteincapture>).

The birth of HuProt arrays expanded researchers' arsenal for interrogation of a great fraction of the entire human proteome for specific biochemical properties. For example, Liu *et al.* profiled the binding specificities of 13 long noncoding RNAs (lncRNAs) on HuProt to determine potential players in lncRNA-mediated biological processes. Ultimately, 671 lncRNA-binding proteins were found, 525 of which lacked any known RNA-binding domains. A novel RNA binding protein, IDH1, was further validated in cells and shown to bind thousands of RNA transcripts (42). Similarly, Fan *et al.* probed HuProt with miR-122 and identified 40 target proteins. Because miR-122 is required for hepatitis C virus (HCV) replication, they further validated the target hnRNP K as a repressor for HCV replication (43). Therefore, the human proteome microarray is a valuable tool to study the complex regulatory networks of protein-DNA and -RNA interactions (Fig. 1F and 1G).

The human proteome microarray is also useful for the analysis of protein-protein interactions, especially for determining players involved in pathogen-host interactions (Fig. 1A). Park *et al.* probed the nonstructural 5A protein from HCV on ProtoArray and identified 90 proteins. They further validated one of these proteins, Pim1, as a factor involved in HCV cell entry (44). Yoon *et al.* constructed a Zika virus-host protein-protein interaction network using a similar approach and compared its dengue virus counterparts to determine Zika virus-specific interactions (45). Further orthogonal large-scale screenings allowed them to pinpoint drug targets in the host involved in Zika virus replication. Yang *et al.* investigated the binding events of *T. gondii* virulence factor ROP18 using HuProt and identified 68 targets. They subsequently validated the crucial role of ROP18 on p53, p38, UBE2N, and SMAD1 through phosphorylation-dependent degradation (46). Wu *et al.* investigated the binding events of PknG, an important kinase in *M. tuberculosis* (MTB), using HuProt and identified 128 binding proteins. They further validated that one of these binding proteins, CypA, is degraded upon phosphorylation and subsequently inhibits inflammatory responses (47). Using human NAPPA, Yu *et al.* identified 18, 20, and 8 host proteins that interact with *L. pneumophila* effectors SidM, LidA, and AnkX, respectively (48, 49).

Human proteome microarrays have also been widely used to study PTMs (Fig. 1E). Song *et al.* established methods to

detect global tyrosine phosphorylation, lysine acetylation, ubiquitylation, and SUMOylation on HuProt. The HuProt arrays were incubated with cell lysates diluted in different PTM reaction buffers to perform covalent protein modifications, and the modified proteins on the array were visualized using the corresponding PTM antibodies. Among the complex regulation of PTMs in cancers, they validated the hyperactivities of PTK2 and PTK2B kinases in ovarian cancer (50). Xu *et al.* surveyed the substrate for ppGalNAc-Ts using HuProt and identified 128 common substrates for O-GalNAc glycosylation (51). Yu *et al.* used human NAPPA to identify the 20 and 21 AMPylation substrates for VopS and IbpAFic2, respectively (52). Overall, the human proteome microarray serves as an unbiased platform for studying many kinds of binding events and enzyme-substrate relationships.

The two major pharmaceutical applications of the human proteome microarray are drug target identification (Fig. 1D) and specificity tests for monoclonal antibodies (mAbs) (Fig. 1H). HuProt was used to identify the targets of arsenic, a cancer drug, and 360 potential binders were identified. Hexokinase was validated to bind arsenic, and this binding event was further shown to result in the inhibition of glycolysis (53). With a similar strategy, Cheng *et al.* screened the targets of 6-O-angeloylplenolin, a drug that induces cell cycle arrest, and identified 99 proteins. The proteins Skp1 and STAT3 were further validated to show involvement in cell cycle arrest (54). Because the mAb-based biologicals are one of the fastest growing therapeutic modalities, quality control is extremely important. Many commercial mAbs, however, exhibit poor quality and have wasted \$350 million annually in the United States alone (55). HuProt arrays are an ideal platform to screen mAbs for mono-specificity (56). As such, Venkataraman *et al.* established a production pipeline for the mAbs against transcription factors and adapted HuProt as a primary validation tool for specificity tests (57). Of the 5882 mAbs tested on HuProt arrays, 2000 passed the specificity tests, 1462 of which eventually passed the secondary cell-based validation for their ability to perform Western blot analysis and/or immunoprecipitation.

Proteome microarrays other than those already mentioned can also be quite useful in basic research, such as the Arabidopsis proteome array (9) and the MTB proteome array (7) to name a few. Popescu *et al.* established the Arabidopsis proteome microarray and profiled the binding of calmodulin and calmodulin-like proteins (9). Deng *et al.* developed the MTB proteome microarray and used it to identify the binding partners of PknG and the protein interactions of second messenger cyclic di-GMP (7). Wu *et al.* probed 11 serine/threonine protein kinases on the MTB proteome array and identified 492 binding proteins with 1027 network interactions (58).

Application of Functional Protein Microarrays in Translational Research—Serological biomarkers are valuable tools for diagnosis, prognosis and companion diagnosis in vari-

ous autoimmune diseases, cancers, and infectious diseases (59, 60). One of the early applications of functional protein microarrays was to discover new serological biomarkers for autoimmune diseases because they can serve as antigen surveying platforms to detect subtle changes in antibody composition. In a dysregulated immune system, the antibodies that are generated by humoral immunity and react with self-antigens are referred to as autoantibodies (AABs). When a functional protein array covers most of the human proteome (e.g. HuProt), a specific AAB signature can be readily detected by probing the array with a diluted patient serum/plasma sample. When this approach is used to profile AAB signatures for a large cohort, subsequent statistical analysis can reveal potential biomarkers associated with a disease of interest (Table III). This approach has three major advantages. First, patient samples are easy to obtain and store because they are mostly in the forms of serum, plasma or body fluid. Second, detection of AABs on a human proteome array is very sensitive and quantitative, only requiring several microliters of samples. Finally, the presence of AABs is detectable before symptoms can be identified, making early diagnosis possible.

Human proteome microarrays have been used to identify diagnostic AABs for more than 13 autoimmune diseases, including autoimmune hepatitis (15, 61), ankylosing spondylitis (62), multiple sclerosis (21, 63), type 1 diabetes (64), Alzheimer's disease (65), rheumatoid arthritis (66), Sjögren's syndrome in saliva (67), primary biliary cirrhosis (68), amyotrophic lateral sclerosis (69), male subfertility (70), juvenile idiopathic arthritis (71), Behcet's disease (72), and sarcoidosis (73). For biomarker identification, it is necessary to include the most comprehensive human proteome collection for unbiased screening, and to validate candidate biomarkers using additional cohort to avoid overfitting. These requirements often result in a high price tag for biomarker research. Song *et al.* developed a strategy to overcome this issue by dividing the process into two phases. In phase I, also known as the biomarker discovery or screening step, they used the HuProt array to survey AABs in a smaller cohort of sera from 22 autoimmune hepatitis (AIH) patients and 30 healthy controls. In this phase, they narrowed down thousands of human proteins to 11 candidate autoantigens. In phase II, also known as the biomarker verification or validation step, they fabricated a focused antigen array with the 11 candidate antigens to survey AABs in a much larger cohort composed of sera from 44 AIH patients, 50 healthy controls, and 184 patients suffering from other autoimmune diseases as a disease comparison group. With this two-phase strategy, they identified and validated three new antigens, RPS20, Alba-like, and dUTPase as highly specific biomarkers for AIH (61).

In translational cancer research, it is important to identify early diagnosis markers to allow for earlier treatment and intervention. Human proteome arrays are widely used to profile the AABs in 10 cancer types, including ovarian cancer (74, 75), glioma (76), lung cancer (77), gastric cancer (78), bladder

TABLE III
Functional protein microarrays for biomarker identification

Diseases/Classifications	Tools	Major findings	Refs.
Autoimmune diseases			
Autoimmune hepatitis	Purified 5,011 human array	Validate 3 AAbs	(61)
	Purified 1,626 human array	Validate 6 AAbs	(15)
Ankylosing spondylitis	NAPPA 3,498 human	Identify a set of AAbs	(62)
Multiple sclerosis	11,520 PrESTs array	Validate 51 AAbs	(21)
	ProtoArray	Validate CSF AAbs against RBPJ	(63)
Type 1 diabetes	NAPPA 10,000 human	Validate 5 AAbs	(64)
Alzheimer's disease	ProtoArray	Validate 10 AAbs	(65)
Rheumatoid arthritis	ProtoArray	Validate 2 AAbs	(66)
Sjögren's syndrome	ProtoArray	Validate 4 saliva AAbs	(67)
Primary biliary cirrhosis	HuProt	Validate 6 AAbs	(68)
Amyotrophic lateral sclerosis	ProtoArray	Validate 20 AAbs	(69)
Male subfertility	ProtoArray	Validate AAbs against TGM4 in prostate	(70)
Juvenile idiopathic arthritis	NAPPA 768 human	Identify 18 AAbs	(71)
Behcet's disease	HuProt	Validate AAbs against CTD1	(72)
Sarcoidosis	3,072 PrESTs array	Validate 4 AAbs	(73)
Cancers			
Ovarian cancer	Purified 5,005 human array	Validate 4 AAbs	(74)
	NAPPA 5,177 tumor antigens	Validate 3 AAbs	(75)
Glioma	HuProt	Identify a set of AAbs	(76)
Lung cancer	HuProt	Validate 3 AAbs	(77)
Gastric cancer	HuProt	Validate 4 AAbs	(78)
Bladder cancer	ProtoArray	Validate 2 AAbs	(79)
Prostate cancer	Purified 123 antigen	Validate 3 AAbs	(80)
Colon cancer	ProtoArray	Validate 3 AAbs	(81)
Breast cancer	NAPPA 4,988 tumor antigens	Validate 28 AAbs	(82)
Myelodysplastic syndromes	HuProt	Validate 3 AAbs	(83)
Meningiomas	HuProt	Identify a set of AAbs	(84)
Infectious diseases			
Coronaviruses	Coronaviruses proteome array	Identify a set of Abs	(4)
Flaviviruses	Zika/Dengue proteome array	Validate a set of Abs	(5)
<i>M. tuberculosis</i>	Purified MTB proteome array	Identify 14 Abs	(7)
	NAPPA 4,045 MTB	Identify 8 Abs	(87)
Varicella zoster virus	NAPPA 69 VZV	Identify 19 Abs	(86)
<i>P. aeruginosa</i>	NAPPA 262 <i>P. aeruginosa</i>	Identify 12 Abs	(85)
Herpes simplex virus	HSV-1&2 proteome array	Validate 2 Abs	(94)
<i>L. interrogans</i>	IVTT 3,359 <i>L. interrogans</i> array	Identify 191 Abs	(88)
<i>S. Typhi</i>	IVTT 2,724 <i>S. Typhi</i> array	Identify 93 Abs	(89)
<i>B. melitensis</i>	IVTT 3,046 <i>B. melitensis</i> array	Identify 33 Abs	(90)
Human papillomavirus	IVTT 104 HPV array	Identify E7 Ab in cancer	(91)
<i>C. albicans</i>	IVTT 451 <i>C. albicans</i> array	Identify 13 Abs	(92)
<i>F. Tularensis</i>	IVTT 1,741 <i>F. Tularensis</i> array	Identify 15 Abs	(93)
Other diseases			
Asthma	ProtoArray	Validate 4 AAbs	(95)
Kawasaki disease	<i>E. coli</i> proteome array	Validate a set of AAbs	(96)
Preeclampsia	<i>E. coli</i> proteome array	Validate 5 AAbs	(97)
Bipolar disorder	<i>E. coli</i> proteome array	Validate 6 AAbs	(98)
Parkinson's disease	ProtoArray	Validate 10 AAbs	(99)
Osteoarthritis	3,840 PrESTs array	Validate a set of AAbs	(100)
Chronic renal disease	ProtoArray	Validate 4 AAbs	(101)
Inflammatory bowel disease	ProtoArray	Validate AAbs against FAM84A	(102)
	<i>E. coli</i> proteome array	Identify a set of AAbs	(103)
Meniere's disease	ProtoArray	Identify 18 AAbs	(104)
Chronic humoral rejection	ProtoArray	No common AAbs	(119)

AAbs = autoantibodies. AAbs if not specified, they are from blood. IVTT = *in vitro* transcription and translation.

cancer (79), prostate cancer (80), colon cancer (81), breast cancer (82), myelodysplastic syndromes (83), and meningiomas (84). Orenes-Pinero *et al.* performed serum profiling on

ProtoArray and identified 171 autoantigens related to bladder cancer. They validated selected candidates by using a cancer tissue array and confirmed that dynamin is not only an au-

toantigen biomarker, but it is also associated with poor survival (79).

Regarding infectious disease, the purpose of using protein microarrays is quite different from autoimmune diseases or cancer because the serum antibodies in infectious diseases are a part of the normal immune response. Ways in which the protein microarray can be used to study infectious disease include serotyping, identifying markers for prognosis, and identifying immunogenic proteins for vaccine development. To serve these purposes, the protein array must be tailored according to the pathogens being studied. NAPPA techniques have been applied to vaccine development by profiling serum antibodies against *P. aeruginosa* in a varicella-zoster virus proteome array (85, 86). Because *in vitro* expression arrays are more flexible, most of the pathogen-protein arrays are built with either IVTT or NAPPA. Such arrays include MTB (87), varicella zoster virus (86), *P. aeruginosa* (85), *L. interrogans* (88), *S. Typhi* (89), *B. melitensis* (90), human papillomavirus (91), *C. albicans* (92), and *F. Tularensis* (93). A few pathogen-protein arrays are purified from yeast, including MTB (7), flaviviruses (5), and herpes simplex virus (94).

Other diseases with altered immune responses can also be examined using protein microarrays in order to identify AAbs relevant to disease. To date, there are nine inflammatory diseases with biomarkers that have been discovered using protein microarrays, including asthma (95), Kawasaki disease (96), preeclampsia (97), bipolar disorder (98), Parkinson's disease (99), osteoarthritis (100), chronic renal disease (101), inflammatory bowel disease (102, 103), and Meniere's disease (104).

GPCR-VirD Microarray—GPCRs form the largest transmembrane protein family in humans, consisting of seven transmembrane domains. This complex structure allows GPCRs to bind to a variety of ligands, ranging from protons, ATP, amino acids, peptides, proteins, and to many other unidentified ligands. To date, ~40% of the FDA-approved drugs target GPCRs. Because the lipid bilayer is required to maintain the conformation of GPCRs, purification attempts often disrupt the GPCR conformation. To overcome this hurdle, Hu *et al.* developed VirD technology by replacing a viral envelope gene in herpes simplex virus-1 (HSV-1) with an ORF encoding a human transmembrane protein. The production of this recombinant virus from mammalian cells allowed the human receptor to be embedded in the viral envelope with correct conformation and function (19). More importantly, these recombinant viruses were arrayed on a glass slide to facilitate high-throughput screenings. Syu *et al.* expended the VirD technology to cover most of the non-odorant GPCRs (*e.g.* 315) for further biochemical interrogation (14). We demonstrated that the GPCR-VirD array is useful to profile specificity of mAbs (Fig. 2A). Among the 20 commercial mAbs tested, only 10 mAbs were determined to be ultra-specific. The rest either failed to show specificity entirely, or at least had several off-targets. Interestingly, all four mAbs with reported neutral-

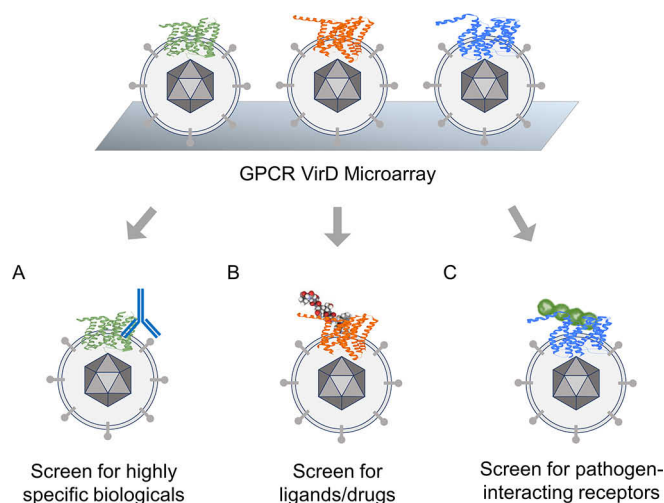


FIG. 2. Application of GPCR-VirD Display (VirD) Microarray. 315 non-odorant GPCRs are displayed on the HSV-1 envelope to maintain the native conformation and form the world's largest functional GPCR-VirD array. The GPCR-VirD array is useful to screen for highly specific biologicals (A), ligands (B), small molecule drugs (B), and pathogen receptors (C).

ization activity were shown to be ultra-specific on the GPCR-VirD array. Next, we performed specificity tests with known ligands (Fig. 2B) and revealed several off-targets for a peptide hormone, somatostatin-14. Two selected off-targets along with the canonical GPCR were validated with virion nano-oscillators for real-time and label-free detection (105) and showed significant binding affinities. Lastly, we probed the GPCR-VirD array with a clinical strain involved in neonatal meningitis (Group B *Streptococcus* K79) and identified five potential GPCR targets (Fig. 2C). CysLTR1 was further validated *in vitro* and *in vivo* as a host receptor for K79 invasion. We believe that the VirD array is a robust platform to profile many kinds of membrane protein interactions.

Future Directions—Membrane proteins are one of the most important protein categories, as they play important roles in many biological processes, such as signal transduction, cell recognition, cell-cell communication, transport, and anchorage, to name a few. It is highly desirable to develop a high-content and high-throughput platform for functional membrane proteins to enable meaningful screening for ligands, biologicals and small molecule drugs. To date, many methods have been developed to maintain the native conformation of membrane proteins, including nanodiscs (106), macrodiscs (107), Salipro nanoparticales (108), virus-like particles (109), and VirD (14, 19). Unlike VirD, the other methods are not easy to scale up for multiplexed, highly parallel screening while maintaining the flexibility of massive production of the reagents from various mammalian cell lines. When the VirD array is coupled with nano-oscillator technology (105), the entire membrane protein family can be screened simultaneously with candidate drugs or biologicals in a label-free, real time fashion, and binding specificity and kinetics can be ob-

tained in a single experiment. We envision that VirD array technology can expand to all kind of membrane protein families and holds promise for discovering biologicals, drugs, and receptor interactions. Besides VirD tailored for membrane proteins, all other human proteins need a proper expression system for the best folding and PTMs. For this reason, it would be desirable to use a mammalian expression system. In combination with transfection, transformation, and CRISPR knock-in technologies (110), it is possible to generate a human proteome microarray from human cells and accelerate research, potentially leading to the discovery of novel drugs or biologicals.

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