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PROTEIN CHIP: AN OVERVIEW

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Abstract

A protein chip is a high-through put method used to track to interaction and activities of protein and to determine their function. This technology have allowed by to analyze the correlation between specific protein with disease. The five major areas where protein chip are being applied are diagnostics proteomics, protein functional analysis, antibody characterization and antibody antigen reaction. protein chips have the potential to function in many other applications including the study of protein-protein, protein-drug interaction, DNA-protein interaction., etc

Keywords: Genomics, Immunoassays, microarray.

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Introduction

In spite of recent advancements in our understanding of molecular biology, in many cases we are unable to implicate specific proteins with a disease. Genomics and microarray technology have allowed us to analyze thousands of mRNAs at one time and determine whether mRNA expression is changed in disease states. However, researchers have long known that the concentration of an mRNA within a cell is poorly correlated with the actual abundance of that protein. This is due to the fact that the rate of degradation of individual mRNAs and proteins differ, post-transcriptional control of protein translation, a number of post-transcriptional modifications of protein, and protein degradation by proteolysis.[1]

Protein chips have the potential to function in many other applications including the study of protein–protein, protein–drug interactions, DNA-protein interactions, protein localization, antigen-antibody interactions, enzyme-substrate, and receptor-ligand interactions all of which may be amendable to array-type high-through put screening.

Two approaches have been used in order to characterize multiple proteins in a biological sample. The first approach is 2-dimensional gel electrophoresis, which has been widely used to separate and visualize up to 2000-10,000 proteins in a single experiment by excision and identification by mass spectrometry (MS). This method is both time consuming and even with MS, only the most abundant proteins can be detected.[3] Also, reproducibility is problematic, even though pre-cast gels and commonly used reagents, protocols, and hardware components have led to improved performance. Due to the limitations of 3D-gel separation technology, increasing attention is focusing on the development of the second approach, the development of protein microarrays as an alternative and complementary approach.[1]

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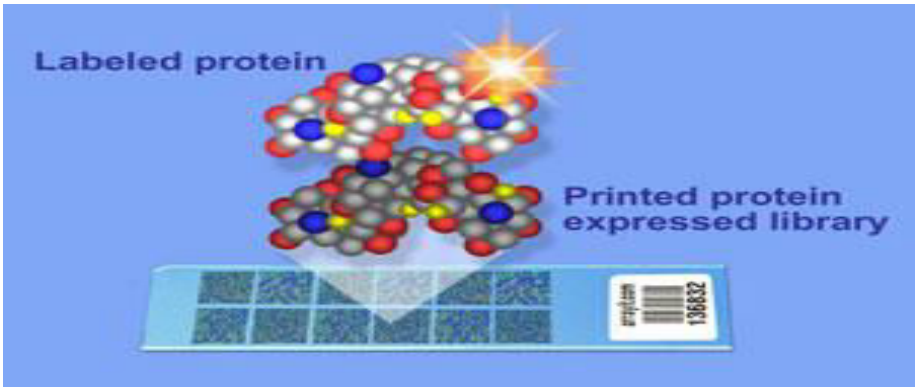


Fig. No. 01: Libraries of express proteins are easily printed into microarrays with the Nano Print Microarrayer. For detection of the printed proteins, samples can be directly labeled or a non-label detection technique can be used. A variety of protein- protein, protein-drug, and protein-small molecule interactions can be implemented. These microarrays can be used to analyze antibody specificity.

Deficiency of Traditional Protein Characterization methods

Before the advent of protein chips, protein measuring and characterization was done using two different methods: 2D gel electrophoresis coupled with mass spectrometry, and liquid chromatography. These methods can separate and visualize a large number of proteins per experiment, however they are time consuming when compared to protein chips. Their process is very low-throughput because of lack of automation. Reproducibility is also a factor because of the large amount of sample handling. A better, more standardized, higher-throughput method needed to be invented for protein measuring and characterization.[2]

Protein Chip Precursors to Modern Day

The equipment and reagents used in an Enzyme-linked Immunosorbent Assay (ELISA), a precursor of protein chips.[1]

Immunoassays, the precursor to protein chips available since the 1980s, exploit the interactions between antibodies and antigens in order to detect their concentrations in biological samples. Their creation, however, is tedious and expensive. As a response to this, researchers at Harvard University combined the technologies of immunoassays and DNA microarrays to develop the protein chip. In their landmark paper, published in 2000, "Printing Proteins as Microarrays for High-Throughput Function Determination," Gavin Mac Beath and Stuart Schreiber described how to create protein chips and demonstrated three types of applications that would benefit from this new technology. The strengths of their approach were the use of readily available materials (i.e. glass slides, poly acrylamide gel), the relative ease of implementation (robotic microarray printers), and compatibility with standard instrumentation.[1]

Within the past five years, many companies, including Biacore, Invitrogen, and Sigma-Aldrich, have begun production of industrial level protein array systems that can be used for drug discovery and basic biological research. Commercial entities have made protein chip research a streamlined and standardized process on the same level as DNA microarrays compared to its inception in 2000.

Academic research plays a huge role in the development and improvement of these technologies. The collaboration of academic research with systems such as the Affymetrix GeneChip and the Human Genome Initiative has allowed for friendly competition, resulting in the advancement of technologies. With more develops come a better understanding and encourages even more research towards these fields.

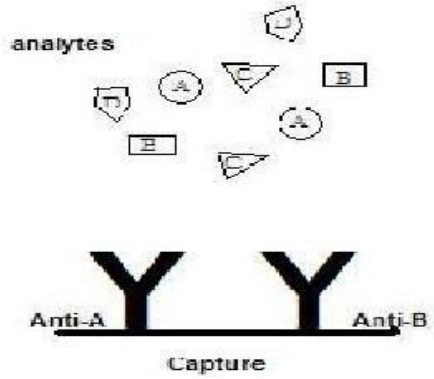
Affymetrix is a company that has been manufactures microarrays, named GeneChip, since 1992. They have 13 locations across the world with

headquarters located in the US (California), UK, Japan, and China.[3]

Types of chips

The two main types of protein chips are analytical and functional. With analytical protein chips, the proteins being studied are in the solution that is washed over the chip. Analytical chips are primarily used to identify the contents of an analyte. With functional protein chips, the proteins being studied are attached to the chip. Functional chips are primarily used to study interactions between the protein of interest and other molecules.[5]

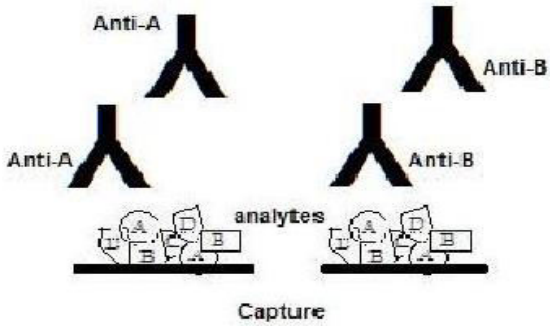
1. Analytical



Example of an analytical protein chip.

Analytical chips are classified according to the capture molecule that is affixed to the chip. The molecule can be very specific as to the types of proteins it binds to. Examples of these specific molecules include antibodies, antigens, enzymatic substrates, nucleotides and other proteins. Analytical chips can also contain molecules that bind to a range of proteins. These molecules are similar to the ones used in liquid chromatography. The techniques include reverse phase, cation exchange and anion exchange.

2. Functional



Example of a functional protein chip.

Unlike analytical chips, there is only one type of functional chip. Functional chips are used to discover additional information and properties about a particular protein. These properties include binding strength, biochemical functions and protein-protein interactions.

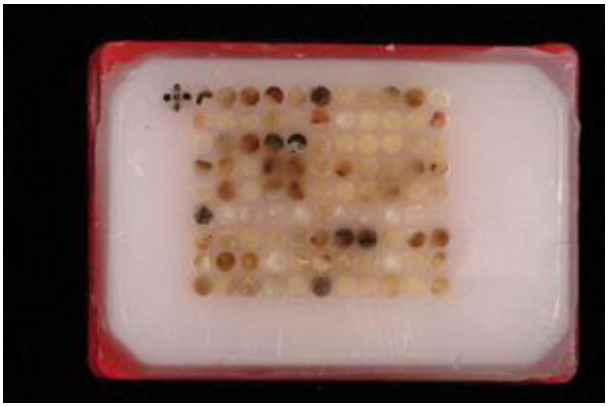
The major methods used to characterize an organism's proteome often result in the denaturing of the sample thus ruling out any functional studies. Current functional analysis methods are mostly *in vivo* techniques which have inherent variabilities. The benefits of functional analysis using these chips is that proteins can be identified and studied *in vitro* while they are still biochemically active and in their multimeric complexed form.

Related chip technology includes main 5 types of chips

1) **Protein chips:** A protein microarray, sometimes referred to as a protein binding microarray, is a piece of glass on which different molecules of protein have been affixed at separate locations in an ordered manner thus forming a microscopic array. These are used to identify protein-protein interactions, to identify the substrates of protein kinases, or to identify the targets of biologically active small molecules. The most common protein microarray is the antibody microarray, where antibodies are spotted onto the protein chip and are used as *capture molecules* to detect proteins from cell lysate solutions.
2) **Antibody chips:** An antibody microarray is a specific form of protein microarrays, a collection of capture Antibodies are spotted and fixed on a solid surface, such as glass, plastic and silicon chip for the purpose of detecting antigens. Antibody microarray is often used for detecting protein expressions from cell lysates in general research and special biomarkers from serum or urine for diagnostic applications.[6]
3) **DNA chips:** A DNA microarray is a high-throughput technology used in molecular biology and in medicine. It consists of an arrayed series of thousands of microscopic spots of DNA oligonucleotides, called features, each containing picomoles of a specific DNA sequence. This can be a short section of a gene or other DNA element that are used as probes to hybridize a cDNA or cRNA sample (called

target) under high-stringency conditions. Probe-target hybridization is usually detected and quantified by fluorescence-based detection of fluorophore-labeled targets to determine relative abundance of nucleic acid sequences in the target.[7] In standard microarrays, the probes are attached to a solid surface by a covalent bond to a chemical matrix (via epoxy-silane, amino-silane, lysine, polyacrylamide or others). The solid surface can be glass or a silicon chip, in which case they are commonly known as *gene chip* or colloquially *Affy chip* when an Affymetrix chip is used. Other microarray platforms, such as Illumina, use microscopic beads, instead of the large solid support. DNA arrays are different from other types of microarray only in that they either measure DNA or use DNA as part of its detection system.[7] DNA microarrays can be used to measure changes in expression levels or to detect single nucleotide polymorphisms (SNPs).

4) **Tissue chips:** Tissue microarrays (also TMAs) consist of paraffin blocks in which up to 1000 separate tissue cores are assembled in array fashion to allow simultaneous histological analysis. In the tissue microarray technique, a hollow needle is used to remove tissue cores as small as 0.6 mm in diameter from regions of interest in paraffin embedded tissues such as clinical biopsies or tumor samples. These tissue cores are then inserted in a recipient paraffin block in a precisely spaced, array pattern. Sections from this block are cut using a microtome, mounted on a microscope slide and then analyzed by any method of standard histological analysis. Each microarray block can be cut into 100 – 500 sections, which can be subjected to independent tests. Testscommonly employed in tissuemicroarray include immunohistochemistry, and fluorescent in situ hybridization. Tissue microarrays are particularly useful in analysis of cancer samples.[8]



A Tissue microarray block

5) **Chemical compound chip:** A Chemical compound microarray is a collection of organic chemical compounds spotted on a solid surface, such as glass and plastic. This microarray format is very similar to DNA microarray, Protein microarray and Antibody microarray. In chemical genetics research, they are routinely used for searching proteins that binds with specific chemical compounds, and in general drug discovery research, they are used for searching potential drugs for therapeutic targets.

6) There are 3 different forms of chemical compound microarrays based on the fabrication method. The first form is to covalently immobilize the organic compounds on the solid surface with diverse linking techniques; this platform is usually called Small Molecule Microarray, which is invented and advanced by Dr. Stuart Schreiber and colleagues. The second form is to spot and dry organic compounds on the solid surface without immobilization, this platform has a commercial name as Micro Arrayed Compound Screening (μARCS), which is developed by scientists in Abbott Laboratories . The last form is to spot organic compounds in a homogenous solution without immobilization and drying effect, this platform is developed by Dr. Scott Diamond and later commercialized as Discovery Dot technology by Reaction Biology Corporation.

Alternative approach to protein chips by forming artificial lipid bilayer membrane

1. What are membrane proteins?

The plasma membrane (cell membrane) of living organisms is made of a double layer of phospholipids that separates inside and outside of

the cell. Plasma membrane is not permeable to most of the water soluble and large molecules, whereas cells must uptake nutrients, regulate ionic concentration, and transducer signals from the environment. Membrane proteins that exist in the plasma membrane (e.g., ion channel, transporter, pump, and receptor) play those extremely important roles in maintaining the homeostasis.

2. Artificial lipid bilayer membrane (Reconstituted membrane or Black lipid membrane)

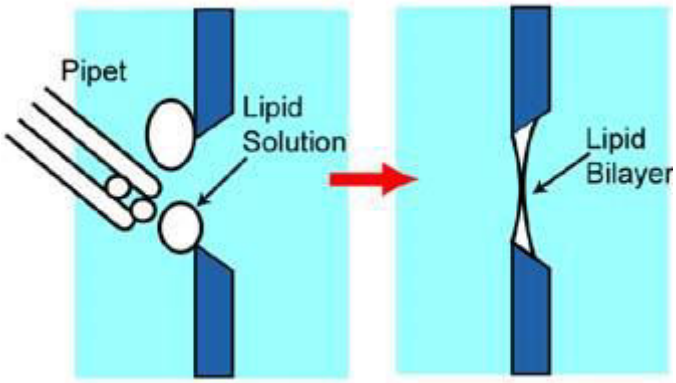


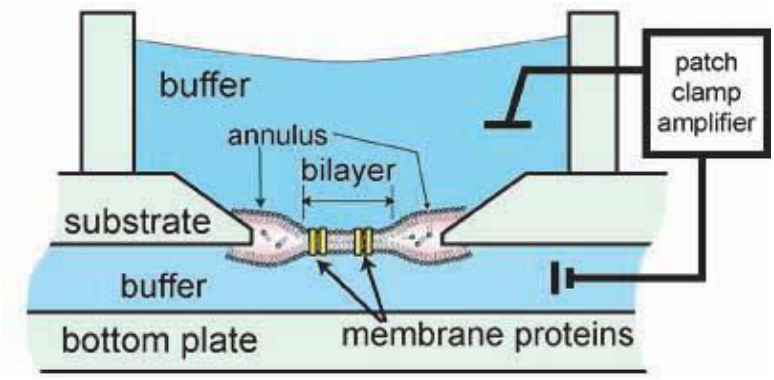
Fig.: A conventional method of forming artificial lipid bilayer membrane (the "painting" method)

Membrane proteins funcion only in the plasma membrane. Thus, unlike water soluble proteins (e.g., proteins that exist in blood or cytosol), it is very difficult and tricky to perform functional analyses. Scientists in biology use culture cells, in which target membrane proteins are expressed by genetic engineering, to investigate reactions of proteins to drugs. However, sometimes it is difficult to attribute the cause of reactions to the specific protein, since there are a large number of different membrane proteins on the living cell.

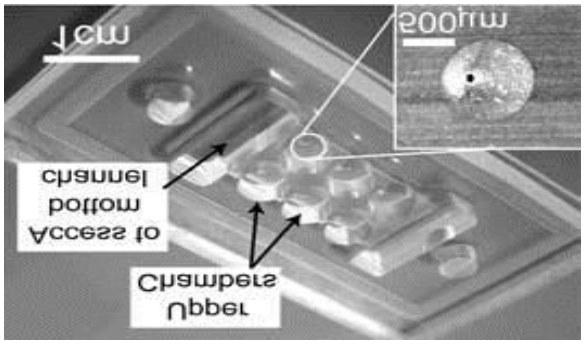
There is an alternative approach to investigate membrane protein functions. In this method, a target membrane protein is incorporated in a lipid membrane that is made of purified or synthesized lipids. In this artificial cell membrane system we can achieve information only from proteins in interest. In conventional methods of forming the artificial membrane, phospholipids dissolved in non-polar solvent (mainly n-decane) are painted over a tiny aperture (diameter = 0.1-1mm) opened in a teflon sheet (figure below). As the layer of lipid solution spanning over the aperture thins down, a lipid bilayer forms spontaneously. In past studies the opening and closing of single ion channels such as K^+ channel and Ca^{2+} channel, whichare reconstituted in the artificial lipid bilayer, were electrically measured. Although this is a powerful tool in membrane biology, it has not been widely used due to the poor reproducibility in membrane forming. The planar membrane is extremely fragile because the membrane is as thin as 5-10nm.

3. The " Membrane Protein Chip"

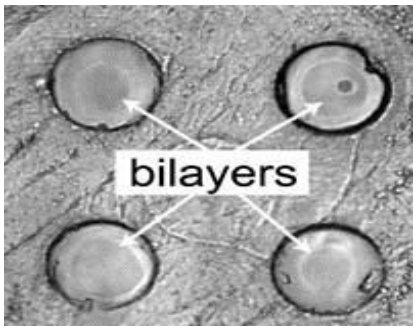
micro fluidic chip technology" to efficiently reconstitute the abovementioned artificial planar lipid bilayer. Micro fluidic chips allow to control the tiny amount of liquid, which increases the reproducibility. Moreover, with microfabrication technology we can make apertures of micro-metre size accuracy, realizing formation of stable and uniform lipid bilayers. We fabricated a micro fluidic chip with PMMA plastic as shown in the figure below. Micro fluidic channel, wells, and apertures (D=100um) are all integrated in the chip. By simply flowing the lipid solution and buffer alternately in the micro-channel, lipid bilayer membranes form spontaneously. Success rate of bilayer forming was improved up to 90% in contrast to less than 10% in conventional methods. Therefore, we succeeded in forming multiple lipid membranes simultaneously in one chip (figure). We also measured activities (opening and closing) of single ion channel peptides incorporated in the membrane.



Membrane protein array chip



Prototype plastic chip



Four planar bilayers simultaneously formed in a chip

Analysis of protein chips

Within the realm of protein chip analysis, there are several hurdles presently being cleared by scientists experimenting at the fringe of this technology. Several of the main challenges facing scientists include: dynamic protein concentrations, almost overwhelming numbers of unique proteins, and creation of specific probes for each protein. Dynamic protein concentration refers to the reality that the exact number of any given protein within a cell can be drastically different than the exact number of another protein within the same cell. In fact, there are current estimates that place the difference in protein numbers between those expressed at the lowest levels and those expressed at the highest levels at an order of magnitude of roughly 10^{12}. In other words, a million million fold difference can exists between the rarest and the most abundant proteins.

To further complicate the issue of dynamic protein concentrations, the exact number of any given protein will fluctuate based on which type of cell is being considered. Of course, there are some proteins, known as housekeeping proteins, that are highly expressed in every cell. These proteins are needed for the basic structure and functions that keep the cell alive. Housekeeping proteins aside, cells of different tissues will often have varying protein concentration profiles. For example, proteins that are expressed at high concentrations in heart tissue will most likely be expressed at different concentrations in liver tissue. Therefore,

methods that detect a protein in one cell type may not work for a different cell type.

Unfortunately, scientists are often interested in those proteins that are rare and present in low concentrations. One method devised in order to overcome this limitation has been the removal of abundant proteins. Usually, there are a very small number of proteins that are present in copious quantities. Through the removal of these proteins, scientists can reduce the order of magnitude differences between the lowest and highest expressed proteins. However, protein purification can be a very laborious and time consuming process and many scientists recognize that it may be prudent to run several protein chip experiments, each analyzing a different range of protein concentrations.

Another massive challenge exists in determining a specific probe for each protein. Currently, one attempt to solve this problem is in the designing of specific antibodies. In the past, antibodies have been created through the immunization of animals. This method, however, is not high-throughput enough to be considered effient for protein chip experimental design. Instead, methods are being developed in order to create antibody libraries. A few examples from are listed below.

- Phage antibody display
- Ribosome display
- SELEX (Systomatic Evolution of Ligands by Exponential Enrichment)
- mRNA display

Antibody approaches can be narrowed somewhat by creating antibodies that bind specific protein groups and not specific proteins. An alternative to antibody usage exists in aptamers, or short oligonucleotides which are much easier to create, but can be difficult to select for a specific proteins.

In order to analyze the results of the protein chip it is necessary to determine the types or numbers of proteins that were bound to the capture agents (for analytical chips) or the number of interactions the proteins had (for functional chips). Analysis of protein chips can be broken into two categories: Labeled and Label-Free. Analysis of protein chips can be a difficult and tricky process. Many intuitive methods have been developed, but each has its own limitations. Short descriptions of these methods and their limitations exists below.

- Labeled

This type of analysis involves labeling either with radioactivity or fluorescence. The label can be attached directly to the protein or capture agent (such as an enzyme or substrate) and detected immediately. The label can also be attached to a different molecule (such as an antibody, antigen or substrate) that is washed over the chip as part of a second step. This prevents the label from altering the conformation of the proteins being studied. However there are several issues that arise with labeled protein chips. Although methods exist to prevent a label from altering protein conformation, a probability of interference with protein interactions still exists. Label influence on binding properites, along with a knack for having a lack of reproducability provide for a couple of significant issues which can be seen as sufficient reasoning for using a label-free approach.

Common Detection Methods

ELISA (Enzyme-Linked Immunosorbent Assay)

ELISA assays are useful tool in the detection of antigens or proteins. A specific antibody is used to target the desired antigen or protein. The complex that forms from the antibody-antigen binding is bound by another antibody which recognizes such complexes. The latter antibody is attached to an enzyme thus 'enzyme-linked' is part of the acronym, ELISA. The binding of the antigen will usually trigger a reaction that can be observed and qualified or quantified.

Sandwich Immunoassay

Sandwich immunoassays, a version of an ELISA assay, use fluorescently labeled antibodies for the probe and laser scanning for collecting the data.

Software

Software packages used for analysis of labeled protein chips include

- Protein Microarray Analysis Tool (ProMAT) is a freely available software package used to evaluate the intensity of the spots. ProMAT was developed at Pacific Northwest National Laboratory.
- Zepto VIEW PRO is a commercially available software package from Zeptosens that allows quantification of spot intensity and is used with their protein chips.

Label-Free

This type of analysis takes advantage of the properties of the proteins and includes mass spectrometry, surface plasmon resonance (SPR) and atomic force microscopy (AFM).

ProMAT

Protein Microarray Analysis Tool

The Protein Microarray Analysis Tool (ProMAT) is a data analysis application for protein microarray data. The software was developed for use with ELISA microarray experimental data by the Statistics Group and the Cell Biology and Biochemistry Group at Pacific Northwest National Laboratory.

ProMAT takes microarray data (the output of microarray image analysis) where some arrays are treated with standards of known antigen concentration and some arrays are treated with samples of unknown concentration. ProMAT fits standard curves to the standards data to relate spot fluorescence to concentration. ProMAT also calculates and outputs confidence bounds on these standard curves. The tool then uses the standard curves to predict antigen concentrations for the unknown samples, along with prediction intervals.[15]

ProMAT also produces diagnostic plots to aid the user in determining where there may be problems with the data. The figure above shows one such diagnostic plot. The lower right panel shows an example standard curve (in black) with its prediction intervals (in blue) plotted with the standard data. The gray region is the region of the curve where concentration predictions are the "best." The histogram in the lower left panel shows the sample spot values. The upper panel gives the coefficient of variation of the standard curve. The plot quickly shows the user how well the standard curve fits the data, how wide the prediction intervals are, and how well the range of the sample data matches up to the range of the standard curve. This type of plot is produced for each antigen and is displayed in an HTML interface for easy viewing.

Protein Chip Analyzer

Application of protein chip

Proteomics:

Protein chip technologies will provide a powerful, high-throughput and versatile tool for the genome-scale analysis of gene function .Enzyme activity, protein–protein and protein–nucleic-acid interactions, and small-molecule drug interactions may all be analyzed directly on the protein level. Arrays may be engineered to address protein identification, quantitation, and affinity studies. A profiling array may quantitative levels of specific proteins on a global scale allowing for a comparison of normal and disease states. An affinity array may analyze the interactions of peptides, proteins, oligonucleotides, sugars, lipids, or small molecules and chemicals with immobilized proteins such as receptors, enzymes, or antibodies .Currently, the rate-limiting step is the production of large numbers of proteins. The ability to automate protein production and proteins fused to high-affinity tags will greatly expedite protein-chip development. High-density chips containing large sets of proteins or even entire proteomes will allow the high-throughput analysis of biochemical activities, protein–protein interactions and post-translational modifications, such as phosphorylation, dephosphorylation, protein methylation, and ubiquitination.[7]

The ultimate goal of proteomics is to the study biochemical activities of every protein encoded by an organism or proteome. A landmark study conducted prepared the first proteome chip by cloning ~94% (>5800 of 6200) of the yeast open reading frames in a yeast expression vector which expressed the proteins as N-terminal GST-His x6

double tagged fusions. A high-throughput yeast protein purification method was developed to individually purify proteins. 80% of yeast proteins were full length and of sufficient quantity to be detectable by most assay types.[11] The proteins were then purified using the GST tags and were then attached to Ni-NTA-coated glass slides using the HisX6 tags. In addition to identifying known interactions, 33 novel binding proteins were detected. 150 novel lipid-binding proteins were also identified. This study demonstrated that an entire proteome can be immobilized on a glass surface to directly screen for interactions with proteins and small molecules. The coupling of mass-spectrometry and protein chips will have wide applications in identifying players in protein–protein interactions, and also in drug discovery. Proteins and small-molecule ligands bound to proteins immobilized on chip can be identified using matrix-assisted laser desorption/ionisation time of flight (MADLI-TOF) mass spectroscopy. Microwell formats are particularly suited for this purpose.[7] Thus, molecules and proteins that specifically bind to many different proteins can be identified and this information can be used to deduce molecular networks and pathways.

One area that will require technological improvements is the analysis of membrane proteins. A large amount of proteins are likely to be membrane-bound, since as many as one third of all yeast proteins are membrane proteins or secreted proteins. Due to the fact that many of these proteins are active when in membranes, it therefore may be necessary to purify or reconstitute them with associated lipids. However, this may not be so difficult. One group was able to immobilize biotinylated membranes that contain the G-protein-coupled receptor rhodopsin on a gold-coated glass surface, and establish a functional assay for that protein. Similar procedures may make it possible to analyze membrane proteins in a chip format.[7]

Diagnostics:

- Another area which will benefit from protein areas is diagnostics. Highly parallel analysis on arrays will allow determination of disease markers (e.g tumour markers) in extracts with only a minimum of biopsy (sample) material, creating new possibilities for monitoring disease (cancer) treatment and therapy.
- In the area of disease diagnosis, the types of leukemias are differentiated by particular subsets of the 247 cluster of differentiation (CD) antigens on the plasma membrane. Diagnosis currently involves a combination of morphology, immunophenotype cytochemistry, and karyotype. Flow cytometry is constrained in analyzing up to 3 CDs in any one assay but using an antibody array, 50 or more CD antigens on leukocytes or leukemia cells can be analyzed. Results compare well with those from flow cytometry.[11]
- This allows for extensive immunophenotyping, and the intact cells that bind to the arrayed antibodies can be further characterized.
- Diagnostic chips based on dielectrophoresis technology have been proposed. Dielectrophoresis is particle motion caused by polarization effects in non-uniform electric fields. The chip can, in principle, separate bacteria, fetal cells, or cancer cells from blood so infections and diseases can be diagnosed in minutes. It is capable of single particle detection but at least 10 are preferred for better statistics. Organisms can also be tagged with antibodies coated on polystyrene beads. Their application is also expected to extend beyond the medical field into food and water safety testing.
- Another chip, the T-sensor, can perform analyte separation and detection from complex solutions. Only minute sample amounts are required in both immunoassay and kinetic assay formats. Several clinical parameters can be tested such as blood pH and oxygen level, and detection methods range from fluorescence and light absorption to voltammetry.
- Lastly, the DIA/PRO, BioChip eliminates the need to centrifuge samples. Blood, urine, and feces require no prior sample preparation and can be tested directly. The BioChip can perform single or multiple assays and is then inserted into an electronic reader for full quantitative analysis in minutes. The chip can run diagnostic tests for Alzheimer's disease (apo E4), many cancers bacterial and viral infections, heart attack, and stroke. They also have tests for food and water safety.

Nanotechnology

- Nanotechnology extends the limits of molecular diagnostics to the nanoscale. Nanotechnology-on-a-chip is one more

dimension of microfluidic/lab-on-a-chip technology. Biological tests measuring the presence or activity of selected substances become quicker, more sensitive and more flexible when certain nanoscale particles are put to work as tags or labels. Magnetic nanoparticles, bound to a suitable antibody, are used to label specific molecules, structures or microorganisms. Magnetic immunoassay techniques have been developed in which the magnetic field generated by the magnetically labeled targets is detected directly with a sensitive magnetometer. Gold nanoparticles tagged with short segments of DNA can be used for detection of genetic sequence in a sample. Multicolor optical coding forbiological assays has been achieved by embedding different-sized quantum dots into polymeric microbeads. Nanopore technology for analysis of nucleic acids converts strings of nucleotides directly into electronic signatures. DNA nanomachines can function as biomolecular detectors for homogeneous assays. Nanobarcodes, submicrometer metallic barcodes with striping patterns prepared by sequential electrochemical deposition of metal, show differential reflectivity of adjacent stripes enabling identification of the striping patterns by conventional light microscopy. All this has applications in population diagnostics and in point-of-care hand–held devices.

Medical research

The application of protein chips, or more broadly, biochips is accelerating medical research and providing information that was inaccessible prior to the use of this technology. The use of tissue arrays and layered expression scanning has provided researchers and clinicians with a molecular profile for each cell type. On the one hand, this has immediate implications for disease monitoring and molecular profiling for drug discovery. On the other, it paves the way for a better understanding of human biology. A true example where arrays are used in medical research is the study of electrophysiological parameters that are influenced by neurotoxicity in the brain. In the future, this research can help in understanding how electrophysiological activity is developed or organized in the brain. It can also lead to screening methods that detect toxic effects that . Rat lead to neuro denegeneration hippocampal and corticostriatal brain slice cultures are grown on perforated silicon in microelectrode arrays. The neurons in culture developed normally and showed the susceptibility to the neurotoxin trimethyltin (TMT) and the excitotoxin NMDA (N- methyl-D-aspartate) as would neurons in vivo. Wider use of biochips in medical research can be expected as the chip technology becomes more accessible either through literature or commercial tools.

Identification of novel therapeutic compounds:

The use of protein arrays to identify and validate novel therapeutics necessitates the development of assays. For example, besides identifying relevant enzyme-substrate pairs, new kinase inhibitors can be discovered using protein chips. Some commercial entities intend to sell arrays containing whole protein families for use in drug lead optimization. Before actual optimization studies begin, the viability of a compound as a drug lead, is determined from its binding kinetics to a specific protein drug target. SPR-BIA technology can be used for this purpose. A commercial collaboration intends to carry out SPR-BIA technology in an array format for the next step, characterizing lead compounds via ADMET (Absorption, Distribution, Metabolism, Excretion and Toxicity) assays. All this, however, is in the near future, at the very least.

Identification of protein function:

The impetus for molecular profiling is the identification of disease related protein and ultimately, the discovery of novel drug targets and prognostic and diagnostic markers. One possible strategy is to have organ and disease-specific protein arrays. However, a disease-associated protein does not automatically imply an appropriate drug target or biomarker. Since molecular profiling catalogs the up- and down-regulation of proteins the functional relevance for any one of these over or under-expressed proteins can and should be determined or verified by another means. The probability of functional relevance increases if this protein's participation in a signaling cascade is established. The definitive criterion for function should then be the demonstration of direct causative role of the protein in the prevailing condition or disease. Various types of functional studies have been carried out on protein arrays. Live cell arrays are appropriate for large-scale characterization of protein function. On the other hand, kinase chips are specifically used to determine function in the

context of cell signaling, to evaluate substrate selectivity, to identify physiologically relevant enzyme-substrate pairs, and to discover novel inhibitors. One kinase chip indirectly addresses function of 119 yeast protein kinases with a wide variety of substrates. MacBeath and Schreiber described the first attempt to develop microarrays to study protein function. Their major efforts involved immobilizing functionally active, folded proteins and detecting the interactions. They alluded to the hope of assigning function at a broader level, running miniaturized assays in parallel. Whether it is necessary to express and purify thousands of proteins or to carry out functional assays in specific cellular systems or with cell lysates remains to be seen.

Problems & their solutions

Antibody Microarray Limitations:

Availability of antibodies

Another challenge to antibody arrays is to obtain antibodies against the ≥ 100,000 proteins that comprise the human proteome. There are currently a very small fraction of antibodies available of the proteome. In addition, the specificity of many of these antibodies is poorly documented and additional antibodies may be required to permit the detection of post-translational modifications. The detection of proteins by antibody–antigen interactions is also characterized by a broad range of specificity and affinity. Also, it is difficult to obtain a large set of highly specific antibody molecules. Therefore, most antibody arrays are limited in their use and contain a few well-defined capture agents directed at a particular class of protein markers to not act similar glycosylation.[18]

Other Problems Facing Antibody Microarrays and Possible Solutions

Classical strategies of antibody generation by animal immunization seem to be impractical. Recombinant antibody display libraries are more promising. Recently, many recombinant methods of generating antibodies have been investigated. These include phage antibody-display, ribosome display, SELEX (systematic evolution of ligands by exponential enrichment), mRNA display, and affibody display, all of which have been developed to advance the production of antibodies and/or antibody mimics. These methods all involve the construction of large repertoires of viable regions with potential binding activity, which are then selected by multiple rounds of affinity purifications. Candidate clones can be further selected using maturation selection which improves binding affinities. However, an ideal selection system, which is fast, robust, sensitive, low cost, automated, and minimized, is yet to be fully developed. These smaller antibody fragments usually have decreased cross-reactivity and similar properties upon attachment.

Also because recombinant antibodies have a potentially high affinity, high specificity, and their smaller molecular weight, which is usually about 30 kDa while non-recombinant antibodies are about 150 kDa, dense and oriented attachment to support surfaces is facilitated. Antibodies are not the only molecules that may bind proteins. Aptamers are short oligonucleotids that can bind and crosslink covalent target proteins. This facilitates higher stringency wash conditions which promotes detection of specific binding. Furthermore, these molecules are easily selected, arrayed, and synthesized. Purified protein target is a requirement for their use however, and aptamers can exhibit biased binding as RNAs tend to be highly negatively charged. A group (Somalogic) recently used immobilized anti-human immunodeficiency virus-gp120 aptamer to detect subnanomolar concentrations of target protein in 5% human serum.

Capture molecules & their limitations

The most common form of analytical protein arrays are antibody microarrays in which antibodies (or antibody mimics) that bind specific antigens are arrayed on a glass slide at high density. A lysate is passed over the array and the bound antigen is detected after washing. The biggest challenge with these methods is producing reagents that identify the protein of interest and with high enough specificity in a high-throughput fashion. Although antibodies are the traditional reagent of choice for detecting proteins in complex mixtures, polyclonal sera are often not specific and are expensive to produce. Also, the conventional hybridoma method of producing highly specific monoclonal antibodies is time-consuming, laborious and costly.

Several studies using antibodies have recently been conducted despite the obstacles in obtaining specific antibodies. In one of the largest studies to date, Sreekumar et al. spotted 146 distinct antibodies on glass to monitor the alternations of

protein quantity in LoVo colon carcinoma cells. Their results revealed radiation-induced up-regulation of many interesting proteins, including p53, DNA fragmentation factor 40 and 45, tumour necrosis factor-related ligand, as well as down-regulated proteins.

To date, most antibody microarrays were produced with several dozen or a few hundred commercially available poly- or mono-clonal antibodies. Although tens of thousands of antibodies are commercially available, this number is insufficient because for most proteins there are no available antibodies. The fact that many antibodies areglycosylated and contain large protein-based supporting structures means that they often cross-react with more than one target protein. This can contribute to a large number of false positives. Thus another problem has been obtaining high-specificity antibodies.

One of the greatest problem with antibody arrays is specificity. Proteins are often present in a very large dynamic range; thus, reagents that might have high affinity for one protein, but are low affinity for another will still exhibit detection of the lower affinity protein if it is much more prevalent. One group investigated the ability of 115 well-characterized antibody–antigen pairs to react in high-density microarrays on modified glass slides. 30% of the pairs showed the expected linear relationships, indicating that a fraction of the antibodies were suitable for quantitative analysis. Many groups have been using sandwich assays to avoid this problem. A sandwich assay is performed by spotting the first antibody on the array and then detecting the using a second antibody that recognizes a different part of the proteins. This approach dramatically increases the specificity of the antigen detection, but required that a least two high-quality antibodies exist for each antigen to be detected.

Future directions & conclusions

Future goals:

Membrane proteins are very important in pharmaceutics, since more than 60% of drugs is designed to target them. Our technology is suitable for drug screening for ion channels. In pharmaceutical industries, a large number of chemical compounds are being screened using the "patch clamping[*1]" method. Although it is very sensitive and reliable technique, it is in turn a laborious process and takes for a long time period.[19]

(*1)A technique in electrophysiology to measure the trans-membrane ionic current. A fine glass pipette (tip diameter typically 1-10um) is pressed against a cell membrane and suction is applied to the inside of the pipette to form electrically "tight" seal. Single ion channel activities can be detected. Developed by E. Neher and B. Sakmann in 1970s who received the Nobel Prize in physiology or medicine in 1991.

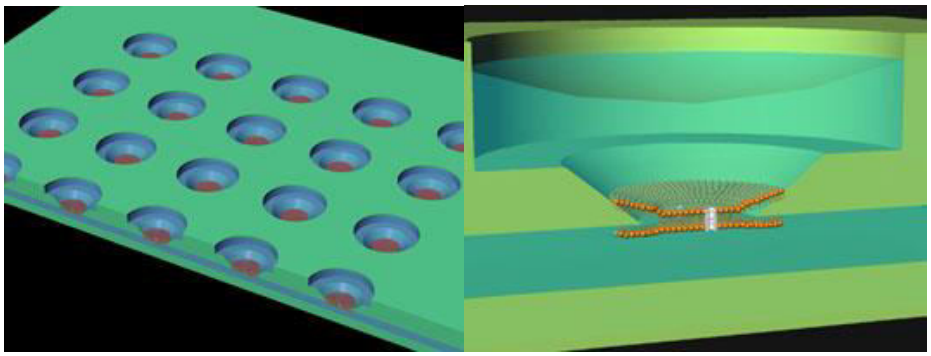


Fig. Conceptual image of the membrane protein array chip

In the membrane protein array chip, we reconstitute lipid bilayer membranes in an array (matrix) as an alnalogy to DNA or protein array chip. We expect to realize rapid and efficient (high-throughput) system that consumes much less amount of protein and chemical samples. Another application will be, by using ligand-dependent ion channels, an ultra-sensitive chemical biosensor**.** We are also developing a planar bilayer system to measure transport of non-ionic molecules due to the transporter proteins.

- **In general future conclusions:**

Although substantial investments have been made by a large number of companies, protein chips

have yet to reach the mainstream of research. Manufacturers have found that proteins are much more complex than nucleic acids, and the perceived market demands have not matched those initially anticipated.

Future developments of protein arrays lie in many areas of chip production including the area of protein attachment. Although most groups are currently adhering proteins to the microarray surface in a random fashion, it is technically feasible to attach proteins through affinity tags. Affinity attachment is likely to result in a higher proportion of active protein and homogeneous attachment of all immobilized proteins. Detection methods are another important consideration, with requirements of sensitivity, accuracy and quantitation over a wide range. The design of arrays will be influenced by the readout systems which might need to be improved/replaced to take advantage of future advances. Standardization is another issue common to all high throughput technologies: the existence and development of many alternative formats and conditions inevitably leads to problems in comparison of results. Standards for protein arrays and a framework for their implementation will need to be established at an international level.

Currently there is little indication of the relative costs the technologies which are offered commercially. Although diagnostics would drive the technology, it is not clear whether companies will be particularly eager to develop cheaper miniaturized alternatives. Proteins carry out most of the functions encoded by our genes, and protein array studies will lead to new advances and discoveries in molecular biology and drug design.

Although a wide variety of technologies have been created for protein arrays, selection must occur to standardize and employ a few of the techniques in order to allow comparison and reproducibility of results, which is critical for the proper advancement of research. Bioinformatics and genomics have paved the way for anew wave of proteomic advancements. Using high-throughput methods such as protein chips, researchers may finally be able to look at the big picture instead of focusing on one or two genes at a time. Furthermore, even if the costs of a protein chip are in the thousands of dollars, the price of one antibody is still in the hundreds. Moreover, analysis of thousands of proteins will take days instead of years or decades. The availability of the full human-genome sequence will push the application of protein-chip technology to analysis of the whole human proteome, and allow proteomics to fall into stride with the huge achievements of genomics.

Manufacturing companies

Selected companies developing microarray technology

COMPANY	CHIP MATERIAL	CAPTURE AGENTS	SIGNAL DETECTION TEHCHNOLOGY
Ciphergen Biosystems (Fremont, CA, USA)	Aluminum coated silicon wafer	Metal affinity, charged or hydrophobic chromatographic surface, antibodies	SELDI and time-of-flight MS
Lumicyte (Fremont, CA)	Silicon wafer	Chemical, biochemical or biological affinity surface	SELDI and time-of-flight MS
Biacore (Uppsala, Sweden)	Glass slide coated with layer of gold, dextran hydrogel surface	NHS/EDC activated surface, antibodies, streptavidin	Prism based SPR technology
HTS Biosystems (Hopkinton, MA, USA)	Plastic with fine grating molded on surface, coated with a thin layer of gold	Antibodies and antibody fragments	Grating-coupled SPR technology

Large Scale Biology (Vacaville, CA, USA)	Plastic	Antibodies	Fluorescence
Biosite Diagnostics (San Diego, CA, USA)	Plastic	Antibodies	Fluorescence
Zyomyx (Hayward,CA, USA)	Silicon	Antibodies and antibody fragments	Fluorescence
Phylos (Lexington,MA,USA)	N/A	Fibronectin based polypeptide scaffold molecules	Fluorescence
Somalogic (Boulder,CO,USA)	N/A	Aptamers	Fluorescence
Akceli (Cambridge,MA,USA)	Glass slide	cDNA expressed by cultured human embryonic kidney cells	Fluorescence
Protometrix (Guilford, CT,USA)	Glass slide	Ni2 + coated surface for capture of His-tagged proteins	Fluorescence
Molecular Staging Inc.	Glass slide	Antibodies & rolling circle amplification	RCAT-based amplification of associated DNA tag
BD Biosciences Clonthch (Palo Alto, CA)	Glass slide	Antibodies	Fluorescence
Interactiva Biotechnology (Ulm, Germany)	Long chain alkylthiols on a gold surface, biotinylated surface	Streptavidin mediated capture of biotinylated biomolecules	Fluorescence

Conclusion

Although substantial investments have been made by a large number of companies, protein chips have yet to reach the mainstream of research. Manufacturers have found that proteins are much more complex than nucleic acids, and the perceived market demands have not matched those initially anticipated.

Future developments of protein arrays lie in many areas of chip production including the area of protein attachment. Although most groups are currently adhering proteins to the microarray surface in a random fashion, it is technically feasible to attach proteins through affinity tags.

Currently there is little indication of the relative costs the technologies which are offered commercially. Although diagnostics would drive the technology, it is not clear whether companies will be particularly eager to develop cheaper miniaturized alternatives. Proteins carry out most of the functions encoded by our genes, and protein array studies will lead to new advances and discoveries in molecular biology and drug design. [19]

Using high-throughput methods such as protein chips, researchers may finally be able to look at the big picture instead of focusing on one or two genes at a time. Furthermore, even if the costs of a protein chip are in the thousands of dollars, the price of one antibody is still in the hundreds. Moreover, analysis of thousands of proteins will take days instead of years or decades. The availability of the full human-genome sequence will push the application of protein-chip technology to analysis of the whole human proteome, and allow proteomics to fall into stride with the huge achievements of genomics.[19]

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