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Protein Arrays

Methods and Protocols

Edited by

Eric T. Fung, MD, PhD

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METHODS IN MOLECULAR BIOLOGY™

Protein Arrays

Methods and Protocols

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Eric T. Fung, MD, PhD

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Preface

Protein Arrays: Methods and Protocols is an introduction to protein array technology and its application to the multiplexed detection of proteins. Although protein array technology has some roots in gene array technology, it can only be described as a distant relative. Unlike DNA, with its established rules of base pairing, and therefore predictable biochemical behavior, proteins are rich with diversity. Proteins can be large or small, compact or extended, basic or acidic, hydrophobic or hydrophilic, and so on. Just as importantly, their behavior is determined by the environment in which they reside, and so the composition of the buffer in which experiments are performed has a dramatic impact on the outcome of the experiment.

Thus, if the goal is to simultaneously measure the expression of a large number of proteins, these variables must be addressed. Not to be deterred, scientists have created a variety of solutions to successfully detect and characterize multiple proteins simultaneously. It is the intent of this volume to introduce to the reader a set of technological solutions to the diversity problem as well as to provide the reader with some examples of practical applications of these technologies.

Like the gene array, the protein array requires an immobilized capture reagent (such as antibody, aptamer, or chromatographic substrate) to which the experimental sample is bound. Following a wash step to remove nonspecific bound materials, the specifically bound material is visualized using, for example, fluorescence or mass spectrometry. The first task in designing a protein array is to create and immobilize the capture substrate, which often is a collection of a large number of distinct probes, and this task is described in Chapters 1–10. Although antibodies are the most typically used affinity capture reagents, other capture reagents include biotin (Chapters 7 and 8), chemical reagents (Chapters 9 and 10), and chromatographic substrates (Chapter 22). Once the affinity capture reagent is chosen, the arrays themselves must be constructed. Though some of the above-mentioned chapters describe methods to construct these arrays, Chapters 11–14 describe other methods. The remaining chapters describe more specific applications of protein arrays. These include identifying proteins that bind to specific protein domains (Chapters 15 and 16), analyzing protein kinase activity (Chapters 17 and 18), assessing protein families (Chapters 19–21), and probing serum for diagnostic information (Chapters 22 and 23).

In reality, this overview is too simplistic, since most of the chapters provide unique methodologies for each of the steps of protein array construction and use. Indeed, this is the richness and reward of participating in an emerging field, where creative approaches have been taken to solve difficult problems. Each contributor to *Protein Arrays: Methods and Protocols* has provided unique insight into the task of studying proteins in a high-throughput manner. This is the new protein chemistry, and it is hoped that readers of our book will learn from these insights and, more importantly, create novel solutions of their own that may appear in future editions.

I thank the authors of the chapters first for their willingness to share their insight and experiences and second for their patience with me. I also would like to express my gratitude to John Walker for his expert editorial opinion and to the staff of Humana Press for taking the final steps to publication. Finally, many thanks to Ka'imilani Alvarado and Amy Jacobs for their outstanding administrative assistance.

Eric T. Fung, *MD, PhD*

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Contents

Preface v
Contributors ix

1 Protein Arrays From cDNA Expression Libraries
Hendrik Weiner, Thomas Faupel, and Konrad Büssow ***1***

2 Protein Expression Arrays for Proteomics
Michele Gilbert, Todd C. Edwards, and Joanna S. Albala ***15***

3 Generation of Protein *In Situ* Arrays by DiscernArray™ Technology
Mingyue He ***25***

4 Multiplexed Protein Analysis Using Spotted Antibody Microarrays
Brian B. Haab and Heping Zhou ***33***

5 Target-Assisted Iterative Screening of Phage Surface Display cDNA Libraries
Alexei Kurakin, Susan Wu, and Dale E. Bredesen ***47***

6 Design, Construction, and Use of Tissue Microarrays
Stephen M. Hewitt ***61***

7 A Streptavidin–Biotin-Based Microarray Platform for Immunoassays
Petra Pavlickova and Hubert Hug ***73***

8 Site-Specific Immobilization of Biotinylated Proteins for Protein Microarray Analysis
Rina Y. P. Lue, Grace Y. J. Chen, Qing Zhu, Marie-Laure Lesaicherre, and Shao Q. Yao ***85***

9 Processing of Photoaptamer Microarrays
Helen Petach, Rachel Ostroff, Chad Greef, and Gregory M. Husar ***101***

10 Use of a Small Molecule-Based Affinity System for the Preparation of Protein Microarrays
Karin A. Hughes ***111***

11 Evaluation of Antibodies and Microarray Coatings As a Prerequisite for the Generation of Optimized Antibody Microarrays
Philipp Angenendt and Jörn Glökler ***123***

12 Printing Functional Protein Microarrays Using Piezoelectric Capillaries
James B. Delehanty ***135***

13 The Use of Precision Glass Syringes and a Noncontact Microsolenoid Dispenser for the Production of High-Throughout Low-Density Arrays
Arezou Azarani ***145***

14 A Protein Microarray ELISA for Screening Biological Fluids
Susan M. Varnum, Ronald L. Woodbury, and Richard C. Zangar ***161***

15 Protein-Domain Microarrays
Alexsandra Espejo and Mark T. Bedford ***173***

16 SH3 Domain Protein-Binding Arrays
Sangpen Chamnongpol and Xianqiang Li ***183***

17 Site-Specific Peptide Immobilization Strategies for the Rapid Detection of Kinase Activity on Microarrays
Mahesh Uttamchandani, Grace Y. J. Chen, Marie-Laure Lesaicherre, Shao Q. Yao ***191***

18 Simultaneous Monitoring of Multiple Kinase Activities by SELDI-TOF Mass Spectrometry
Vanitha Thulasiraman, Zheng Wang, Anjali Katrekar, Lee Lomas, and Tai-Tung Yip ***205***

19 Cytokine Protein Arrays
Ruo-Pan Huang ***215***

20 Fabrication and Application of G Protein-Coupled Receptor Microarrays
Ye Fang, Brian Webb, Yulong Hong, Ann Ferrie, Fang Lai, Anthony G. Frutos, and Joydeep Lahiri ***233***

21 ProteinChip® Array-Based Amyloid β Assays
Lisa E. Bradbury, James F. LeBlanc, and Diane B. McCarthy ***245***

22 Serum Protein-Expression Profiling Using the ProteinChip® Biomarker System
Kate Gilbert, Sharel Figueredo, Xiao-Ying Meng, Christine Yip, and Eric T. Fung ***259***

23 Protein Arrays for Serodiagnosis of Disease
Tito Bacarese-Hamilton, Andrea Ardizzoni, Julian Gray, and Andrea Crisanti ***271***

Index ***285***

1

Protein Arrays From cDNA Expression Libraries

Hendrik Weiner, Thomas Faupel, and Konrad Büssow

Summary

This chapter describes the production of a cDNA expression library from human fetal brain, the construction of a high-density protein array from such a library, and two applications to screen the array for binding proteins. After producing the library and decollating the expression clones, one can pick thousands of expression clones with a laboratory robot and can deposit them into microtiter plates in an ordered manner. Such ordered clone libraries are the starting material for the construction of a high-density protein array. This array is constructed by spotting the expression clones onto a protein-binding membrane. Following cell growth and induction of protein expression on the membrane, the cell spots are lysed and their recombinant protein immobilized on the membrane. The so-constructed array carries thousands of proteins without the need to clone, express, and spot individual proteins. Such arrays allow one to screen for numerous protein functions in a high-throughput manner.

Key Words:

Protein array; cDNA expression library; high-density spotting; clone array; protein antigen; protein function; protein–protein interaction; posttranslational modification; high-throughput screening.

1. Introduction

Arrays of complementary DNA (cDNA) expression libraries carry thousands of proteins without the need to clone, express, and spot individual proteins *(1)*. These arrays are practical formats to screen en masse for a given protein function, that is, to identify protein antigens *(1,2)*, including autoantigens *(3)*, binding proteins *(4)*, and substrates for arginine methyltransferases *(5)*. Although not yet demonstrated, the arrays may also permit studies on posttranslational modifications other than protein methylation, that is, to find substrates for certain protein kinases.

The protein arrays described here are made using cDNA libraries that are constructed in expression vectors. With the help of a laboratory robot, one can pick thousands of library clones and can deposit them into microtiter plates in an ordered

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manner. Such ordered clone libraries are the starting material for the construction of high-density DNA or protein arrays that require additional robotics *(1,6,7)*. The arrays are constructed by spotting thousands of bacterial clones onto a protein-binding filter membrane. On cell growth and induction of protein expression on the filter, the cells are lysed, and their proteins immobilized on the filter. The so-constructed protein array offers a notable advantage over the widely used filter-immobilized cDNA expression libraries that are based on the bacteriophage λgt11 *(8,9)*. The advantage is immediate addressability, namely, the direct link between a given protein spot on the array and the corresponding clone in a well of a microtiter plate that can serve as a resource for unlimited future use. In addition, the protein arrays possibly contain more recombinant protein per spot area because many methyltransferase substrates remain undetected if an immobilized phage expression library is used instead of the protein array *(5)*.

Protein arrays from a cDNA expression library are available at the German Resource Centre *(10)*. The corresponding cDNA expression library was constructed from human fetal brain and was preselected as described under **Subheading 3.6.** for clones that express recombinant proteins.

2. Materials

2.1. Cloning of a cDNA Expression Library

2.1.1. RNA Preparation, cDNA Synthesis, and Escherichia coli Transformation

1. Polyadenylated (poly [A+]) RNA isolation kit.
2. cDNA Synthesis Kit (Invitrogen Life Technologies).
3. cDNA size-fractionation columns (Invitrogen Life Technologies).

2.2. Construction of Expression Clone Arrays

2.2.1. Colony Picking

1. Blotting paper: 3MM Whatman. Prepare 23 × 23 cm^2 sheets.
2. Dishes for large agar plates, 23 × 23 cm^2 (Bio Assay Dish, Nunc).
3. 40% (w/v) glucose: Dissolve 400 g D-glucose monohydrate in dH_2O to 1 L and sterilize by filtration through a 0.2 μ*M* pore-sized filter.
4. 2X YT broth: Add 16 g tryptone, 10 g yeast extract, 5 g NaCl per liter and autoclave. Cool to 50°C; add appropriate antibiotics and glucose to 2%.
5. 2X YT agar: Add 16 g tryptone, 10 g yeast extract, 5 g NaCl, 15 g agar per liter and autoclave. Cool to 50°C; add appropriate antibiotics and glucose to 2%.
6. Colony-picking robot and additional material for picking *(7)*. Alternatively, a smaller number of colonies can be picked manually with toothpicks or other devices.
7. 384-well microtiter plates with lids. These plates should have a well volume greater than or equal to 95 μL, such as Genetix polystyrene large-volume plates, product code X7001. Optionally, order microplates prelabeled with unique identifiers.
8. Cryolabels for the microtiter plates (e.g., Laser Cryo-Etiketten, Roth; http://www.carlroth.de).
9. 384-pinned replicators. Plastic and steel replicators are available from Genetix or Nunc.
10. Incubator at 37°C.

2.2.2. High-Density Spotting of Expression Clones onto Filter Membranes

1. Polyvinylidene fluoride (PVDF) filter membranes, 222 × 222 mm^2. Immobilon P (Millipore) or Hybond-PVDF (Amersham Biosciences) have been used successfully. The required filter size may have to be custom ordered.
2. Blotting paper, media and agar plates (*see* **Subheading 3.2.1.**).
3. Isopropyl-β-D-thiogalactopyranoside (IPTG) agar plates: Prepare 2X YT agar; add appropriate antibiotics and IPTG to 1 m*M*.
4. Incubators at 30°C and 37°C.
5. Lyophilized rabbit and mouse sera.
6. Black ink, such as TG1 Drawing Ink, Faber-Castell.
7. Forceps to handle the filters.
8. Spotting robot and additional material for spotting *(7)*.
9. Tris-buffered saline (TBS): 10 m*M* Tris-HCl, pH 7.5, 150 m*M* NaCl.
10. Ethanol.

2.2.3. Release of Cellular Proteins on the Membrane

1. Denaturing solution: 0.5 *M* NaOH, 1.5 *M* NaCl.
2. Neutralizing solution: 1 *M* Tris-HCl, pH 7.4, 1.5 *M* NaCl.
3. 20X standard sodium citrate (SSC): 3 *M* NaCl, 0.3 *M* sodium citrate, pH 7.0.
4. Blotting paper and dishes (*see* **Subheading 3.2.1.**).

2.2.4. Nondenaturing Release of Cellular Proteins on the Membrane

1. Lysis buffer: 50 m*M* Tris-HCl pH 8.0, 300 m*M* NaCl, 1 m*M* ethylenediaminetetraacetic acid (EDTA), 0.1% Triton X-100, 1 m*M* phenylmethylsulfonyl fluoride, 1 mg/mL lysozyme.
2. Blotting paper and dishes (*see* **Subheading 3.2.1.**).

2.3. Screening of the Array for Protein Antigens

1. Dry protein array filter.
2. TBS: 10 m*M* Tris-HCl, pH 7.5, 150 m*M* NaCl.
3. TBS+Tween+Triton (TBSTT): 20 m*M* Tris-HCl pH 7.5, 0.5 *M* NaCl, 0.1% (v/v) Tween-20, 0.5% (v/v) Triton X-100.
4. Nonfat dry milk powder.
5. Kimwipes paper towels (Kimberly-Clark).
6. Large plastic box that can accommodate the filters.
7. Primary antibody directed against the antigen of interest.
8. Secondary antibody directed against IgGs of the organism that the primary antibody was obtained from, conjugated with alkaline phosphatase (AP) (for example, Roche Antimouse Ig-AP for use with mouse monoclonal primary antibodies).
9. Attophos, available from Roche or Promega.
10. Attophos stock solution: 2.4 *M* diethanolamine, 5 m*M* attophos, 0.23 m*M* $MgCl_2$; set pH to 9.2 with HCl, sterilize by filtration through a 0.2 μ*M* pore-sized filter.
11. AP buffer: 1 m*M* $MgCl_2$, 100 m*M* Tris-HCl, pH 9.5
12. Fluorescence-scanning device or charge-coupled device (CCD) camera.
13. Ethanol.

2.4. Screening of the Array for Protein–Protein Interaction

2.4.1. Phosphate Incorporation into the Fusion Protein

1. 400–600 μg purified fusion protein with protein kinase A (PKA) site.
2. 1000 U cyclic adenosine monophosphate-dependent protein kinase (Sigma P-2645).
3. 40 m*M* dithiothreitol (DTT).
4. 10X kinase buffer: 200 m*M* Tris-HCl, 1 *M* NaCl, 120 m*M* $MgCl_2$, pH 7.5, 10 m*M* DTT.
5. Sephadex G50 (medium grade) gel filtration column (approx 2.5 mL bed volume) equilibrated in 20 μ*M* HEPES-KOH, 50 m*M* KCl, 0.1 m*M* EDTA, 2.5 m*M* $MgCl_2$, pH 7.4.
6. [γ-^{32}P] adenosine triphosphate (ATP) (25 μL 1 m*M* ATP, 20 dpm/nmol).
7. Liquid scintillation counter.

2.4.2. Blocking and Probing the Filter

1. Dry protein array filter (*see* **Subheading 3.2.2.**).
2. TBS: 10 m*M* Tris-HCl, pH 7.5, 150 m*M* NaCl.
3. TBST: TBS containing 0.05% (v/v) Triton X-100.
4. Blocking buffer (BB): 20 m*M* HEPES-KOH, 5 m*M* $MgCl_2$, 5 m*M* KCl, 0.1 m*M* EDTA, pH 7.4, 0.05% (v/v) Nonidet P-40, 4% (w/v) nonfat dry milk powder.
5. Hybridization buffer (HB): 20 m*M* HEPES-KOH, 50 m*M* KCl, 0.1 m*M* EDTA, 2.5 m*M* $MgCl_2$, pH 7.4, 0.05% (v/v) Nonidet P-40, 1% (w/v) milk.
6. Labeled fusion protein probe (*see* **Subheading 3.4.1.**).
7. Ethanol.
8. Storage phosphor screen plus scanner or autoradiography equipment.

3. Methods

3.1. Cloning of a cDNA Expression Library

A detailed description of cDNA library construction is beyond the scope of this chapter. Therefore, the authors provide only a short summary. Construction of a cDNA expression library requires extra consideration in comparison to standard libraries. cDNA synthesis should be primed with deoxythymidine oligonucleotides for directional cloning and for the production of recombinant proteins with their complete N-terminus. An average cDNA insert size of 1.4–1.8 kbp is recommended. This leads to an appropriate ratio of full-length and truncated clones and maximizes the chances that the protein or protein domain of interest is expressed in the library.

3.1.1. Choice of Expression Vector and E. coli Strain

3.1.1.1. Expression Vector and Screening for Expression Clones

A wide range of bacterial expression vectors is currently available. Choose a vector for expression of fusion proteins with a short N-terminal affinity tag to allow selection of expression clones after the library has been constructed ***(11)***. The hexahistidine tag is particularly well suited for this purpose because fusion proteins can easily be detected with antibodies (*see* **Fig. 1**). The authors used a derivative of the pQE-30 vector (Qiagen), namely pQE30NST (*see* **Fig. 2**) to express his-tagged proteins in *E. coli* and used antibodies against RGS(H_6) to detect them.

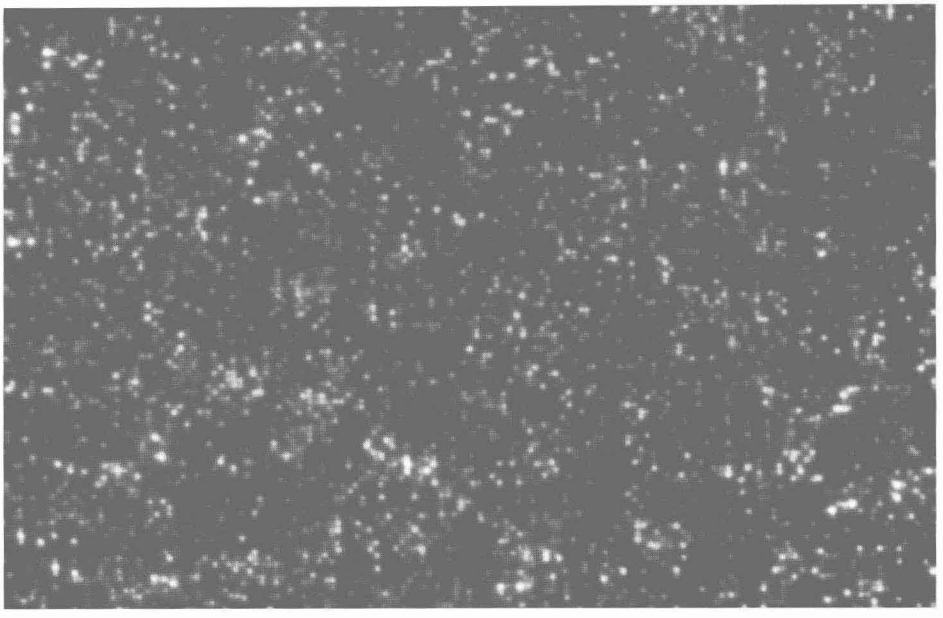

Fig. 1. Detection of recombinant proteins on an array with proteins from the human fetal brain expression library (hEx1). A section is shown of the array that was decorated with the RGS-His antibody according to **Subheading 3.3.**

3.1.1.2. *E. coli* Strain

The *E. coli* strain for the library has to be suitable for cloning, plasmid propagation, and protein expression. The authors recommend a robust K-21 strain with high transformation efficiency and the *endA* genotype for plasmid stability, for example, SCS1 (Stratagene).

3.1.1.3. *Lac* Repressor

If an IPTG-inducible vector with a promoter regulated by *lac* operators is used, consider that sufficient amounts of the repressor protein ***(12)*** have to be expressed in the host cells. A mutated form of the *lac* repressor gene, $lacI^Q$, enhances expression of the repressor protein and is included in many expression vectors. Alternatively, an *E. coli* strain carrying the $lacI^Q$ gene, for example, DH5αZ1 ***(13)***, can be used. Further, a helper plasmid that carries the $lacI^Q$ gene, and that is compatible with the expression vector, can be introduced into the preferred *E. coli* strain before the cells are transformed with ligated cDNA.

3.1.1.4. Rare Codons

Many eukaryotic genes contain codons that are rare in *E. coli*. This can strongly reduce the expression of the corresponding eukaryotic proteins in *E. coli*. To weaken