

Protein Microarray-Based Screening of Antibody Specificity

Rhonda Bangham, Gregory A. Michaud, Barry Schweitzer, and Paul F. Predki

Summary

The increased use of antibodies as therapeutics, as well as the growing demand for large numbers of antibodies for high-throughput protein analyses, has been accompanied by a need for more specific antibodies. An array containing every protein for the relevant organism represents the ideal format for an assay to test antibody specificity since it allows the simultaneous screening of thousands of proteins in relatively normalized quantities. Indeed, the use of a yeast proteome array to profile the specificity of several antibodies directed against yeast proteins has recently been described. In this chapter, we present a detailed description of the methods used to probe protein arrays with antibodies as well as the technical issues to consider when carrying out such experiments.

Key Words: Microarrays; antibody specificity; crossreactivity.

1. Introduction

DNA microarrays have become powerful tools for characterizing the relationships between large groups of genes. Although this type of microarray has become an established tool for expression profiling technology in biological and medical research, many aspects of cellular dynamics (such as tissue-specific protein expression, posttranslational modifications, protein activity, and protein-protein interactions), cannot be studied at the DNA level. Consequently, attention has turned more recently to proteomics—the study of all the proteins expressed by a given genome.

It is now possible to study entire proteomes with the goals of elucidating protein expression, subcellular localization, biochemical activities, and protein pathways. A variety of approaches exist to carry out such studies, including 2D gel electrophoresis, mass spectroscopy, high-throughput immunofluorescence studies with cell mounts, and yeast two-hybrid methods (reviewed in **ref. 1**).

From: *Methods in Molecular Medicine*, Vol. 144, *Microarrays in Clinical Diagnostics*
Edited by: T. Joos and P. Fortina © Humana Press Inc., Totowa, NJ

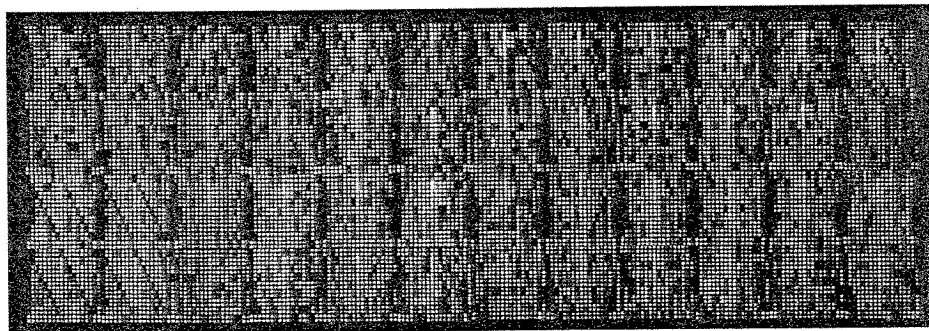
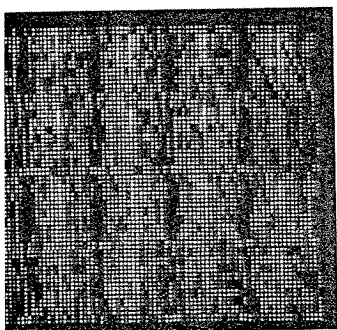


Fig. 1. Fluorescent image of the yeast ProtoArray™ (see Color Plate 16 following p.178). Microscope slides were spotted with over 4000 different proteins that were cloned, expressed and purified from yeast. Each protein on the array was detected using a Cy5-labeled antibody directed against an attached epitope tag. Slides were scanned using an Axon 4000B microarray laser scanner. A protocol for antibody probing of protein microarrays is given in **Subheading 3**. (Copyright 2003 Protometrix Inc.)

More recently, it has also become possible to analyze the activities of hundreds to thousands of different proteins using protein microarrays (2,3). Cloning, expression, and purification strategies for high-throughput protein generation that do not discriminate among the diverse characteristics of individual proteins have been developed (2–4). In addition, many new surface chemistries are available for protein immobilization that permit high detection sensitivity while preserving the functionality of the printed proteins. These include, but are not limited to, modified glass slides (2,5), nitrocellulose-coated slides (6), oriented immobilization via affinity tags (3,7) and 3D polyacrylamide gel pads (8). These advances have fostered the development of high-content protein arrays such as a recently reported functional array consisting of nearly the entire proteome of the yeast *Saccharomyces cerevisiae* (3) (see also **Fig. 1**).

Protein microarrays have the potential to make significant contributions to both basic and applied research (9,10). The breadth of this potential was first demonstrated in the work of MacBeath and Schreiber, who used relatively simple protein arrays to demonstrate a variety of applications such as protein–protein interactions, protein–small molecule interactions, and protein kinase substrate phosphorylation (2). The first reported use of functional proteome-scale microarrays for biological discovery was reported by Snyder and coworkers (3). In this study, arrays of thousands of yeast proteins were manufactured and used for protein–protein interaction and lipid binding screens. Since this publication, the number of discovery-based papers using protein microarrays has been increasing steadily (9).



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One application in which protein microarrays have enormous potential is the profiling of antibodies in clinical specimens for the diagnosis of disease, monitoring of disease progression, assessing response to treatment, and developing therapies to diseases such as cancer and autoimmune disorders. In fact, several studies have already shown sensitive and specific detection of antibodies for certain autoimmune diseases (11,12) Using an indirect fluorescence assay, specialized protein arrays consisting of a subset of proteins and peptides related to connective tissue diseases have demonstrated strong autoantibody reactivity with a four-to eightfold increase in sensitivity over conventional enzyme-linked immunosorbent assays (ELISAs).

A related area in which protein microarrays may have great utility is in profiling antibody specificity. New applications such as antibody arrays (15,16) and monoclonal antibody therapeutics (17) have increased the demand for more target-specific antibodies that exhibit minimal crossreactivity with disparate biomolecules. The ability to isolate and screen antibodies against a large number of different proteins is critical for providing sufficiently specific antibodies. A variety of approaches currently exist for antibody profiling including immunoblotting, immunoprecipitation, ELISAs, and radioimmunoassays (RIAs). These techniques are labor intensive and costly and consume large volumes of sample. Applying protein microarray technology to profiling antibodies has many advantages over these more conventional methods, including the ability to screen thousands of proteins simultaneously for crossreactivity in a quick and simple procedure. The identity of all represented proteins is known, including sequence information, minimizing the need for extensive analyses aimed at identifying the crossreactive species and protein concentration. In addition, the sample size needed to perform these experiments is very small, which is critical when the sample to be screened (i.e., patient sera) is precious.

The first demonstration of the use of positionally addressable protein microarrays for antibody specificity profiling was recently reported by Michaud et al. (18). In this study, polyclonal and monoclonal antibodies generated against yeast proteins were probed against an array of more than 4000 purified yeast proteins. Not surprisingly, monoclonal antibodies showed greater specificity than polyclonal antibodies. Even monoclonal antibodies, however, exhibited some degree of crossreactivity. Further analyses indicated that the nature of the crossreactivity could not be predicted strictly on the basis of sequence analysis, indicating that this type of experimental approach to profiling antibody specificity will be an important tool for developing or using antibodies for research or medical purposes. This chapter describe a method that utilizes protein microarrays as a screening tool for the characterization of antibody binding, specificity, and crossreactivity.

2. Materials

1. Blocking buffer: 1X phosphate-buffered saline (PBS), 0.1% Tween-20, 1% protease-free bovine serum albumin (BSA). Prepare fresh as required.
2. 30% Protease-free BSA solution (Sigma, St. Louis, MO).
3. PBS probe buffer: 1X PBS, 5 mM $MgCl_2$, 5% glycerol, 0.05% Triton X-100, 1% protease-free BSA. Can be stored at 4°C indefinitely. (We recommend adding BSA just prior to use).
4. Primary antibody.
5. Fluorescent-labeled secondary antibody (reactive with primary antibody).
6. Nalgene trays or equivalent chambers for antibody incubations with slides.
7. Platform shaker.
8. Slide rack.
9. Microarray fluorescent scanner.

3. Methods

The method described in this chapter is a protein-antibody sandwich assay where by proteins have been spotted onto the surface, and two antibodies, one to recognize a specific target and a second to detect the primary antibody, are used as probes.

1. An unlabeled primary antibody is incubated with the protein microarray, and the array is then washed and incubated with a secondary antibody labeled with a fluorescent dye.
2. All steps can be completed in 1 d, including data processing. All steps should be performed at 4–6°C to maximize detection sensitivity and to preserve the integrity of the proteins on the array. All reagents should also be at this temperature when you are ready to begin the experiment.
3. It is very important to consider environmental conditions such as humidity and air quality before working with microarrays. Small changes in humidity may result in condensation on the surface of the array, leading to irregular spot morphology and subsequent difficulties in data analysis. Particulate matter in the air can also adversely affect data quality. Dust particles not visible to the eye can autofluoresce, leaving artifacts on the final scanned image that can mask spots and affect data extraction. Therefore, the experimenter should make every attempt to perform protein array experiments under clean conditions.

3.1. Probing Protein Arrays With Antibodies

3.1.1. Preparation of Protein Arrays

Preparation of the yeast proteome microarrays used in our examples is only described here briefly.

1. More than 4000 different yeast proteins were purified using affinity chromatography and printed on FAST (nitrocellulose pad size, 20×60 mm) slides (Schleicher & Schuell) with a Genemachines Omnigrid arrayer. All features were spotted in duplicate.

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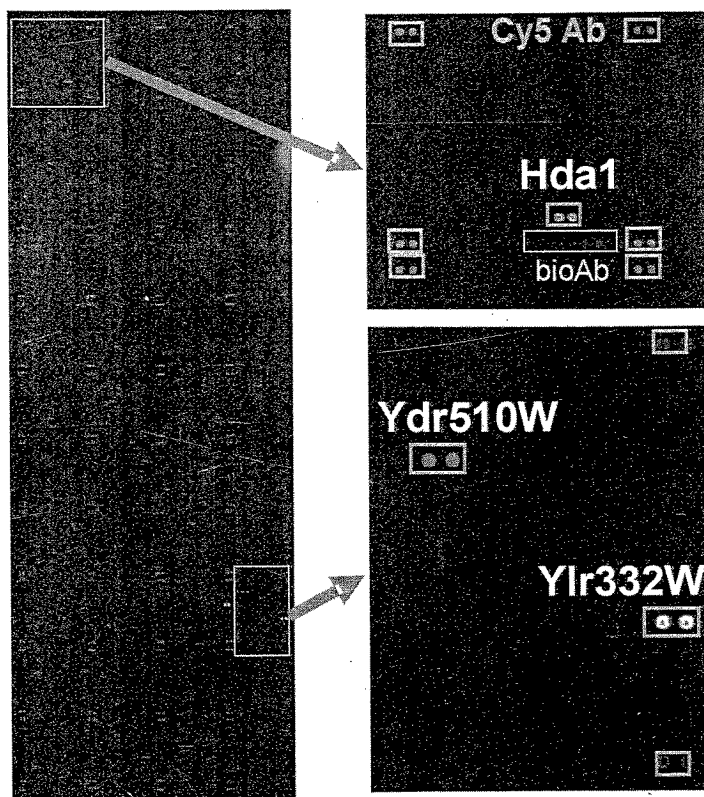


Fig. 2. Fluorescent images of antibody probing of the yeast proteome microarray (see Color Plate 17 following p.178). The expanded subarray on the top shows the anti-Hda1 antibody reacting with its cognate protein. The blue boxes are drawn around spots of Cy5-labeled antibody used as fiduciary markers. The yellow box is drawn around the spots representing biotinylated antibody used as a positive control. The expanded subarray on the bottom shows the anti-Hda1 antibody crossreacting with two other proteins.

2. Fluorescently-labeled antibody was printed in every subarray to facilitate gridding during data acquisition (**Fig. 2**; see Color Plate 17 following p.178). Each array contains 48 subarrays with 18×18 geometry with 250- μm center-to-center spacing. A gradient of BSA has been included as a negative control for assessing non-specific protein interactions.
3. To calculate the amount of protein represented in each spot, a gradient of pure glutathione-S-transferase (GST) was printed in every subarray to generate a standard curve. Probing slides with an antibody that recognizes GST allows for the calculation of the equivalent solution protein concentrations for every protein printed on the array by extrapolating the concentrations from this standard curve.
4. Slides are stored at -20°C in a sealed bag until ready to use.

3.1.2. Blocking the Protein Microarrays

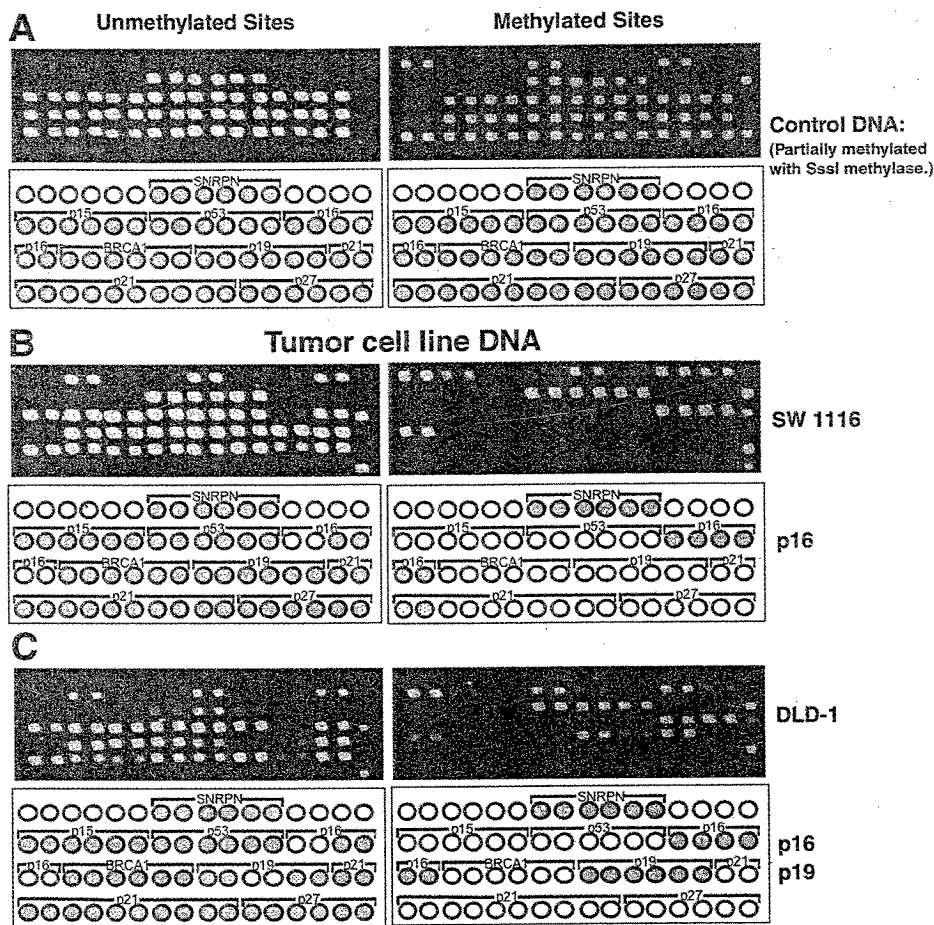
The first step in probing a protein array with antibodies is to block sites on the microarray surface with a nonspecific blocking agent such as BSA. This will prevent nonspecific binding of proteins to the microarray substrate, effectively increasing the specificity of protein-protein interactions and expanding the dynamic range of the data.

1. Prepare blocking buffer fresh (*see Note 1*). Buffer must be cold (4–6°C) prior to use.
2. Transfer enough blocking buffer to a tray or slide rack to ensure complete coverage of the slides. Slides can be blocked lying flat or upright depending on choice of tray.
3. Place the slides in the blocking buffer (*see Note 2*).
4. Incubate slides for 1 h with gentle agitation on a platform shaker.
5. Dispose of the blocking buffer, and immediately incubate with the primary antibody (*see Note 3*).

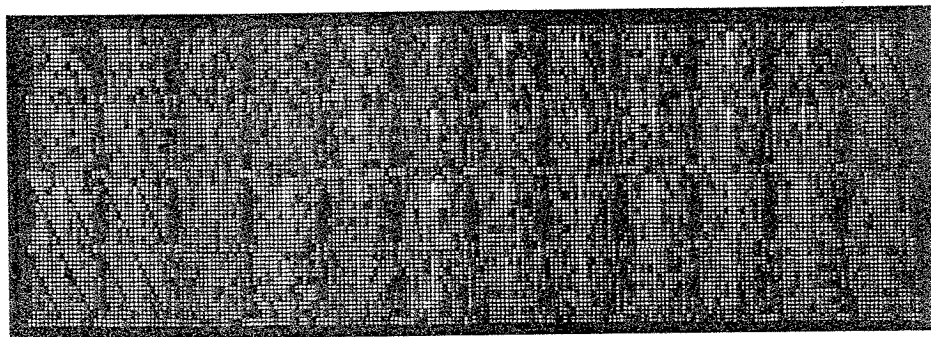
3.1.3. Primary and Secondary Antibody Binding to Array

Conditions should be optimized for probing the arrays with any antibody. Titering the antibody will determine the optimal concentration needed to achieve maximal sensitivity with minimal background. The most favorable dilution should be determined empirically from the manufacturer's recommendation when using a purchased antibody. Three 10-fold dilutions are recommended. If the signal for the cognate protein is weak, or no protein signals are detectable on the array, consider using a higher concentration of antibody. Consequently, if background intensity is masking recognition of spots on the array or there are large numbers of nonspecific interactions, consider using a more dilute antibody concentration.

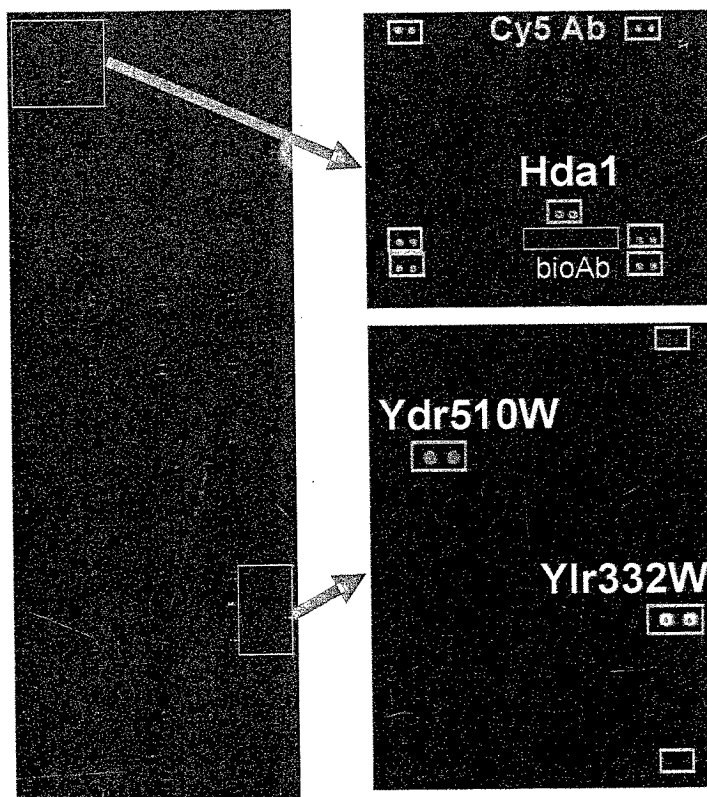
1. Add 20 mL probe buffer with 1% BSA and primary antibody to slides in the trays. For the negative control slide, add buffer with 1% BSA only (*see Note 4*).
2. Incubate while shaking for 2 h.
3. Discard buffer.
4. Wash the slides with 20 mL of probe buffer. Mix gently for 5 min on a platform shaker. Discard the buffer and repeat two more times.
5. Centrifuge the secondary antibody (16,000g, 5 min) to sediment any dye-labeled protein aggregates.
6. Add 20 mL of probe buffer with 1% BSA and a secondary antibody (directed against the primary antibody) to the tray. Also add secondary antibody only to a slide that will serve as the negative control slide. A titer of the secondary antibody should be performed to determine the concentration required for minimizing background and optimizing sensitivity.
7. Incubate while shaking on a platform shaker for 2 h.
8. Discard buffer.



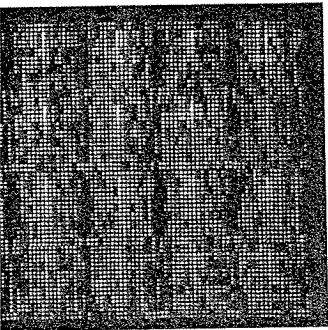
Color Plate 15. Universal array images of methylation profiles of selected promoter regions in normal and colorectal tumor cell line genomic DNAs (Chapter 2, Fig. 8, *see* pp. 48, 49).



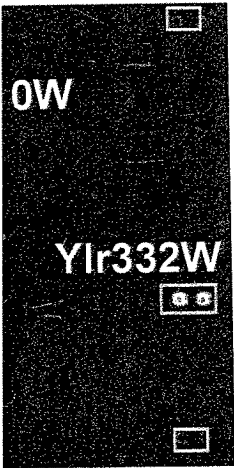
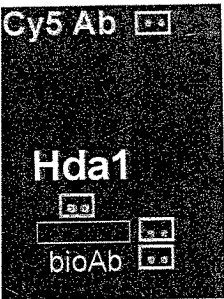
Color Plate 16. Fluorescent image of the yeast ProtoArray™ (Chapter 9, Fig. 1, *see p. 174*).



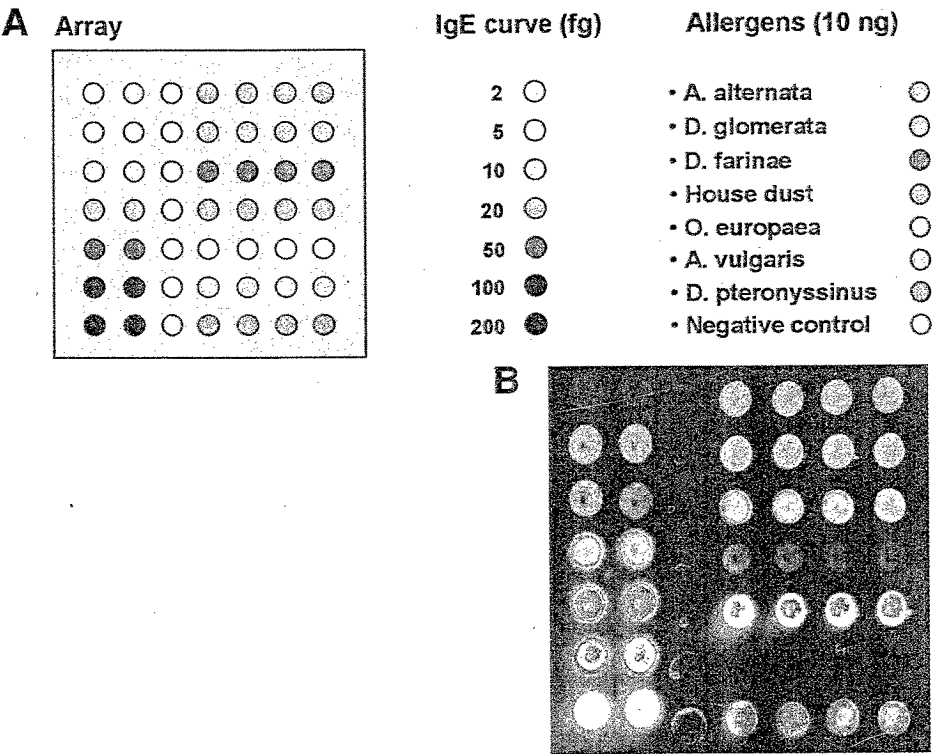
Color Plate 17. Fluorescent images of antibody probing of the yeast proteome microarray (Chapter 9, Fig. 2, *see p. 177*).



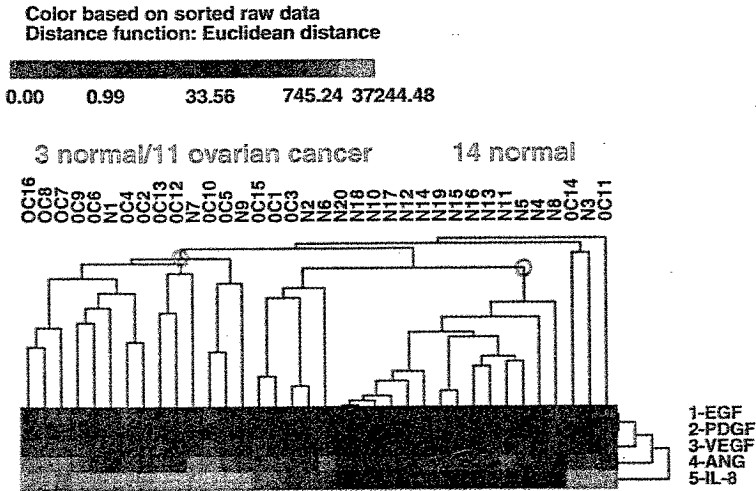
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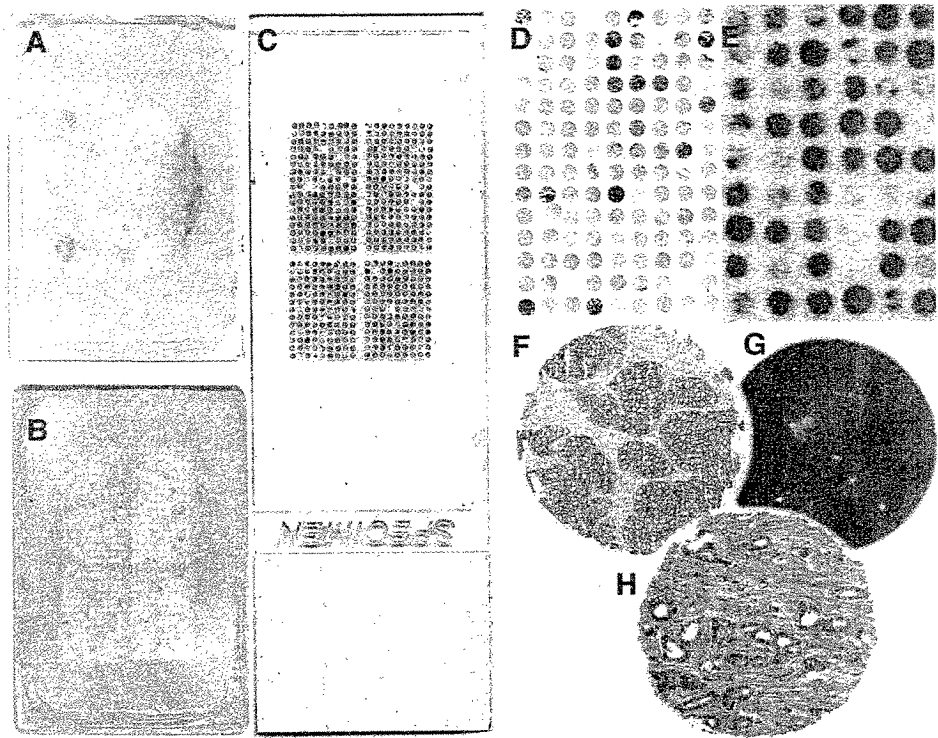
probing of the yeast proteome



Color Plate 18. Schematic array format and fluorescent scan for allergen investigation (Chapter 11, Fig. 1, see pp. 201, 202).



Color Plate 19. Clustering analysis of angiogenic factors measured by enhanced protein profiling arrays between normal subjects and patients with gynecological diseases (Chapter 12, Fig. 3, see p. 219).



Color Plate 20. Tissue microarray manufacturing and applications (Chapter 15, Fig. 2, *see* p. 264, 265).

9. Wash the slides with 20 mL of probe buffer for 10 min with gentle shaking on a platform shaker. Discard the buffer and repeat two more times.
10. After the final wash, remove slides from the tray by grasping the slide edges (do not touch center of array with any object), and remove excess liquid by tapping lightly onto a dry cloth. Place slides into a slide box.
11. Centrifuge at ($<1800g$) for 1 min in a table-top centrifuge equipped with a rotor for handling slide boxes for 1 min. Be sure to provide a balance.
12. Place slides in a dark place until ready to be imaged. (We recommend scanning slides within 12 h after completion of experiment.)

3.2. Array Imaging

The following protocol utilizes the Axon 4000B scanner for acquiring images. Steps can be modified for other microarray laser scanners. Cy5 labels are scanned with a red excitation laser at 635 nm and Cy3 with a green laser at 532 nm (*see Note 5*). Images are analyzed using Genepix Pro 4.0 software (Axon Instruments).

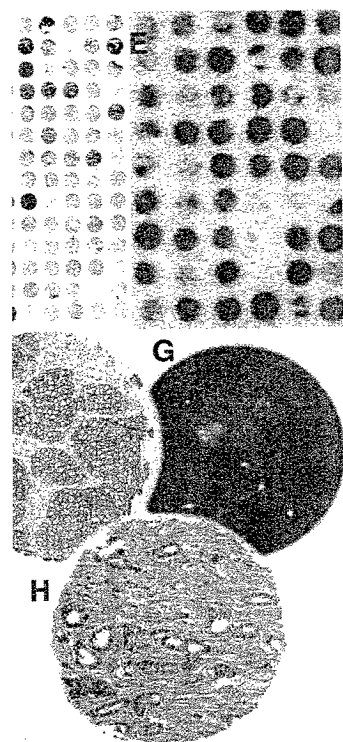
Slides must be completely dry before scanning.

3.2.1. Acquiring the Image

1. Turn on the Axon 4000B scanner and then open Genepix software from the desktop.
2. Slide the door of the scanner open and lift the slide holder up. Place the slide face down with the barcode nearest the front of the machine. Close the slide holder and door. Slide is ready to be scanned.
3. Select the wavelength appropriate to the fluorescence excitation/emission spectrum of the dye.
4. Set the photomultiplier tube (PMT) gain. Recommended starting PMT is 600.
5. Set laser power to 100%, pixel size to 10 μm (or highest resolution), and focus position to 0 μm (*see Note 6*).
6. Perform a preview scan (*see Note 7*). While the scan is being performed, the PMT can be adjusted. If the cognate protein is present on the array and is saturated (spots will be white), the PMT should be adjusted so that these spots are just below saturation (65,535). Be sure the scan area encompasses all the spots on the array.
7. Scan the image (*see Notes 8 and 9*).

3.2.2. Extracting the Data With Genepix Pro Software

1. Once the image has been acquired and saved, load an array list file (.gal file). This file can be made with the Genepix Pro software. Basically, it will transform a text file with the identities of the spots into a file (.gal) that can be overlaid on the image as a grid that assigns each spot on the chip its identity based on the printing parameters. Refer to the Help menu for details on how to make this array list file.
2. Overlay the grid to roughly cover the spots on the array (*see Note 10*).



and applications (Chapter 15,

3. Go to "Align Blocks" and select "Align features in all blocks." Accuracy of the alignment should be ensured and manually reconfigured if necessary.
4. Click on "Analyze Data," and Genepix Pro will generate a report for all feature and background pixel intensities for the array.
5. Save results as an Excel (.xls) file.

3.3. Analyzing the Data

The following method uses Microsoft Excel and Access software to process the data. If a number of slides are going to be analyzed, it is best to write a macro to process the data more quickly.

1. Calculate median F-B for each spot. Use the values appropriate for the scanning wavelength used (i.e., 532 for Cy3 or 635 for Cy5). This is the signal-background ratio for each spot.
2. Average the F-B values for duplicate spots of the same protein.
3. Calculate the standard deviation and coefficient of variance (CV) for the duplicate spots.
4. Filter out the control spots from the protein spots of interest.
5. Calculate the average signal-background for the slide (minus control spots) by averaging the signal-background ratios for all the yeast proteins.
6. Calculate the standard deviation for the yeast proteins on the slide by measuring the standard deviation of the signal-background ratios for all the yeast proteins.
7. Determine the statistical significance of the features by setting an F-B cutoff value. In general, we have assigned hits as significant if the F-B value is greater than or equal to the average signal-background ratio of the slide plus three standard deviations ($3 \times \text{SD} [\text{step 7}] + \text{Average} [\text{step 6}]$). However, the user should consider the signal distribution for each slide when determining statistical significance.

Once features have been assigned some statistical significance (*see Note 11*), it is important to consider the CV values that were calculated for every feature when using an automated method of data analysis. CVs greater than 30% generally indicate artifactual problems on the array. It is best to refer back to the image to see whether local artifacts such as dust particles or anomalous local variance have influenced the data. Import the Excel files into Access.

1. Design queries to align (based on array location) the features for each protein. The output of these queries should contain the name of each protein, array location, F-B background value, and standard deviation of F-B for every feature from each slide.
2. Export results from queries files into Excel.
3. Proteins having signals (F-B) that are greater than two fold over the signal generated on the negative slide are also considered significant.

4. Notes

1. Many alternative reagents are commonly used to reduce nonspecific binding to the slide. Nonfat dry milk, normal serum to the host of the secondary antibody, and gelatin

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are a few examples. Different microarray surface chemistries may perform differ-
ently with different blocking solutions. Protease-free BSA is highly recommended.

2. Always wear powder-free gloves when handling the slides and do not touch the
printed surface of the array. Also, do not wear clothing that is capable of leaving
fibers on the array. Hair coverings are recommended. Pay special attention to the
cleanliness of the experiment.
3. Do not allow slides to dry at any point in the experiment, particularly between
buffer changes. If slides do dry during the course of the experiment, it is likely that
background will be very high.
4. Maintaining BSA in the buffers throughout the process will help keep background
low and also increase the specificity of protein interactions.
5. A variety of other dyes can be scanned by these lasers. Refer to the scanner man-
ufacturer's recommendations for dye compatibility. Many scanners also come
equipped with added lasers for excitation of other fluorescent dyes commonly used
for microarray imaging.
6. These settings have been optimized for glass and nitrocellulose-coated slides. If
other microarray substrates are used; these settings will probably differ and should
be optimized.
7. If no signals are observed on the array, particularly dye-labeled positive control
spots, the scanner may not be set to the correct wavelength.
8. If the PMT is lowered to 400 and the image is still too bright, we recommend leav-
ing the PMT at 400 and beginning to reduce the laser power.
9. Dyes can be light sensitive and thus susceptible to photobleaching. It is not rec-
ommended to scan any part of the array numerous times prior to data extraction.
10. The grid may not align accurately throughout the slide because of minor variations
that occur during the printing process. The blocks should be individually repositioned
before allowing the software to find features.
11. The method of analysis illustrated above is only for consideration. The user
should determine the best method for data analysis and assignment of statistical
significance.

Acknowledgments

We are grateful to Michael Salcius, Jaclyn Bonin, and Fang Zhou for their
expertise in the process of manufacturing the yeast protein arrays that made all
of these experiments possible.

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