Performance Characteristics of the Yeast ProtoArray™ Protein-Protein Interaction (PPI) Proteome Microarray

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Abstract

A reliable means of determining protein function is to map its interactions with other proteins. We describe here a new paradigm for studying protein interactions on a proteome scale, the Yeast ProtoArray™ PPI Proteome Microarray. This proteome microarray contains 4088 open reading frames (ORFs) from *Saccharomyces cerevisiae* expressed as N-terminal Glutathione-S-Transferase (GST) fusions, purified and spotted in duplicate on a nitrocellulose-coated 1 inch x 3 inch glass slide. Using the Yeast ProtoArray™ PPI Proteome Microarray, we show how proteins of interest can be screened for interactions with the majority of the yeast proteome in as little as four hours. Detection on the arrays is shown to be extremely sensitive—as little as 1 pg of protein on the array can be reproducibly detected with submicrogram quantities of probe protein. Since it is estimated that nearly half of yeast proteins have a human homolog, the Yeast ProtoArray™ PPI Proteome Microarray is a compelling model for investigating interactions in higher eukaryotic systems. In this paper, we describe both how this unique discovery platform is manufactured and rigorously quality controlled to ensure reproducible interaction screening results and an integrated system for the controlled and reproducible labeling of proteins with biotin. In further studies, we show that using Alexa Fluor® fluorescent labels to detect protein interactions on the Yeast ProtoArray™ PPI Proteome Microarray is superior to detection using a CyDye™ label. Finally, results of probing with human proteins are presented that clearly demonstrate the broad utility of the ProtoArray™ platform.

Introduction

It is now possible to study entire proteomes with the goals of elucidating protein expression, subcellular localization, biochemical activities, and protein pathways. There are a variety of approaches for simultaneously studying large numbers of proteins and protein variants, including two-dimensional gel electrophoresis, mass spectroscopy, and combinations of mass spectroscopy with liquid chromatography (1). Such methods have found important applications in the areas of basic biological research, drug target and disease marker identification, and in drug development. The drawbacks of these technologies are that they are time-consuming, require expensive and specialized equipment as well as considerable expertise to run the equipment, and require large amounts of sample. More recently, it has become possible to analyze the activities of thousands of proteins using protein microarrays (2,3). Protein microarrays contain a defined set of proteins spotted and analyzed at high density, and can be generally classified into two categories: protein profiling arrays consist of multiple antibodies printed on glass slides and are used to measure protein abundance and/or alterations (4); functional protein arrays can be made up of any type of protein, and therefore have a more diverse set of useful applications. Some of the advantages of these arrays

include low reagent consumption, rapid interpretation of results, and the ability to easily control experimental conditions. The key advantage, however, is the ability to rapidly and simultaneously screen large numbers of proteins for biochemical activities, protein-protein interactions, protein-lipid interactions, protein-nucleic acid interactions, and protein-small molecule interactions. Using these arrays, one can, in a single experiment, determine all of the substrates for a protein-modifying enzyme, build an entire protein interaction network, or determine all of the potential binding partners in a cell for a drug under development.

The ultimate form of a functional protein array consists of all of the proteins encoded by the genome of an organism; such an array is the "whole proteome" equivalent of the whole genome arrays now available. Snyder *et al.* recently described the preparation of a functional protein microarray that closely approaches this ideal (3). More than 80% of the 6280 annotated (5) genes from the yeast *Saccharomyces cerevisiae* genome were cloned, over expressed, purified, and arrayed in an addressable format on glass slides (3). This work represented the first time that the majority of proteins in a proteome had been individually isolated and transferred simultaneously to a solid surface. This "whole-proteome" microarray has proven to be a powerful tool for high-throughput and comprehensive measurements of protein-protein, protein-lipid, and protein-small molecule interactions (3). More recently, further development of this powerful protein microarray platform has been carried out to prepare it for commercialization, including additional sequence and protein characterization of the Snyder collection, increasing the quality and robustness of the protein and microarray production process, and developing a number of easy-to-use protocols for the use of the platform for biochemical assays.

This paper describes the result of two years of product development—the Yeast ProtoArray™ Protein–Protein Interaction (PPI) Proteome Microarray. Included are details of the unique process used to manufacture the Yeast ProtoArray™ microarray, a simple protocol to reproducibly prepare proteins for probing these arrays, and a sensitive method for detecting interactions on the array. Several examples of probing the arrays with yeast proteins are given, and the result of probing the Yeast ProtoArray™ PPI Proteome Microarray with a human protein is shown for the first time. These results clearly illustrate the uniqueness of the product in terms of the scope of its protein content, the breadth of its applications, and its ease-of-use.

Materials and Methods

Yeast Proteome Collection. The yeast proteome collection was derived from the yeast clone collection of 5800 yeast ORFs generated by the Snyder lab as described in Zhu *et al.* (3). The identity of each clone was verified at Invitrogen using 5'-end sequencing. In addition, expression of Glutathione-S-transferase (GST)–tagged protein by each clone was tested using Western blotting and detection with an anti-GST antibody. The 4088 clones that passed both quality control measures were purified using high-throughput affinity chromatography as previously described (xx).

Manufacture and QC of ProtoArray™ Microarrays. Following purification, the yeast proteins are distributed into 384-well plates along with a number of control proteins. Some of the controls relevant to the QC and performance of this product include a dilution series of purified GST, a dilution series of BSA used as negative control, an anti-biotin antibody used for measuring the efficacy of probe biotinylation (see below), a biotinylated protein used for a detection control, and a fluorescently labeled protein used as a fiduciary (marker). The arrayer used for production is a GeneMachines OmniGrid 100 (Genomic Solutions) equipped with 48 quill-type pins. The array substrate is in the form of a 1″ x 3″ glass microscope slide coated with a layer of nitrocellulose membrane (pad size 20 mm x 60 mm) (Schleicher & Schuell). The thickness of the membrane is 15-20 µm, and the nominal pore size is 0.2 µm. Each slide has a unique barcode that associates each array with information in our LIMS database such as lot number, protein content, etc. Samples are printed in 48 subarrays (4000x4000-µm each) and are equally spaced in both vertical and horizontal directions. An extra 500-µm gap exists between adjacent subarrays to facilitate subarray identification. To protect proteins in the source plates and on the arrays while they are being printed, the OmniGrid arrayer is located in a cold room that is temperature, (6˚C) humidity (<35% RH), and dust-controlled. Each yeast protein is spotted in duplicate, giving a total of 12,288 spots on the Yeast ProtoArray™ PPI Proteome Microarray, including control proteins. As will be described in more detail below, we also produce an array consisting of only control proteins; this array, called the Yeast ProtoArray™ PPI Control Microarray has a total of 3072 spots.

Following printing, the lot of arrays used for the experiments described in this paper were subjected to the following quality-control procedure: two slides from the beginning of the print run and two slides from the end were placed in a Nalgene tray, two slides per tray, slides lying flat with the printed side facing up. Slides were blocked with blocking buffer (50 ml PBST + 1% BSA) for one hour in the cold room with shaking (~50 rpm). Blocking buffer was removed and 20 ml of PBST, 200 µl 30% BSA, and 2 µl rabbit anti-GST were added to each tray and incubated for two hours in the cold room with shaking. Slides were washed two times, five minutes each, with 20 ml of PBST at +4˚C with shaking. Twenty milliliters of PBST, 200 µl 30% BSA, and 40 µl Alexa Fluor® 647 goat anti-rabbit antibody was added to each tray and incubated for two hours in the cold room with shaking. The slides were then rinsed three times with 20 ml PBST. Excess liquid was removed by gently tapping the edges of

the slides, handling only the edges of the slides. The slides were then placed into slide containers, placed into a centrifuge, and centrifuged at ~3220 x g for one minute. After the slides were completely dry, they were scanned using an Axon Scanner at the 635 nm wavelength setting, 100% Laser Power, and 500 PMT, 10 µm pixel size, lines to average setting at 1, and focus position set at 0 µm.

***In Vitro* Biotinylation.** Twenty microliters of yeast calmodulin kinase at a concentration of 2.5 mg/ml was aliquoted into three 0.5 reaction tubes. A fourth reaction tube containing 20 µl of 2.5 mg/ml BSA was also prepared. Two microliters of 1 M freshly prepared sodium bicarbonate solution was added to each tube to obtain a pH between 8-9. Ten microliters dH_2O was added to 100 µg (150 nmoles) of lyophilized biotin-XX sulfo-succinimidyl ester (Molecular Probes) to give a solution of 15 nmole/µl. This solution was 3-fold serially diluted to give solutions of 5 nmole/µl and 1.66 nmole/µl. 1.35 µl of the 5 nmole/ml solution of biotinylation reagent was added to the reaction tube containing BSA to give a biotinylation reagent:BSA molar ratio of 9:1. 1.8 µl of the 15 nmole/µl, 5 nmole/µl, and 1.66 nmole/µl solutions of biotinylation reagent were added to the tubes containing calmodulin kinase in order to give biotinylation reagent/protein molar ratios of 27:1, 9:1, and 3:1, respectively. After addition of the biotinylation reagent, samples were mixed, briefly centrifuged, and incubated at room temperature for 60 minutes. Following this incubation, each of the biotinylation reactions was loaded onto spin columns containing a gel filtration resin (exclusion limit: 6,000 Da). Spin columns were placed in a microcentrifuge, centrifuged at maximum speed for 1 minute at room temperature, and the eluate collected. Ten percent of the recovered material was used to determine protein concentration using a Bradford assay. A similar procedure was used to biotinylate other proteins used as probes in the experiments described below.

Assessment of Biotinylation. The degree of biotinylation of protein probes was assessed using a Western blot assay. The standard used for this assessment was BSA that had been biotinylated *in vitro* and the average number of biotins determined by mass spectroscopy. Briefly, 25 fmoles of protein biotinylated at molar ratios of 27:1, 9:1, and 3:1 along with 5 dilutions of standard ranging from 12.5 to 100 fmoles were boiled for 10 min at 100˚C, quick spun in a microcentrifuge, and then run on a NuPAGE® 4-12% Bis-Tris gel in MES buffer (Invitrogen). After transfer of the gel to nitrocellulose, blotting was performed with a WesternBreeze® Chemiluminescent Kit (Invitrogen). After blocking and washing, streptavidin-alkaline phosphatase was added at 1:4000 dilution in blocking solution and incubated 1 hr at room temperature with shaking. After washing, substrate was added, and the blot briefly exposed to film. After exposure and development, the film was imaged with an Alpha Ease FC imager and band intensity for each standard quantitated using the imager software to generate a standard curve.

Probing Arrays with Biotinylated Proteins. Microarray slides were placed in a Nalgene tray, two slides per tray, with slides lying flat and the printed side facing up. Arrays were blocked with 30 ml PBST/1% BSA for one hour in the cold room with shaking (~50 rpm). Arrays were then removed from the blocking buffer, tapped gently on a Kimwipe, and placed on a flat surface with the printed side of the array facing up. One hundred-twenty microliters of biotinylated protein (50 µg/ml) was pipetted on top of the slide and overlaid with a HybriSlip™ (Molecular Probes) cover-slip. The assembly was then inserted into a 50-ml conical tube with the printed side facing up. The tube was capped, making sure that the array was as level as possible, and incubated at +4-6°C for 90 minutes. After incubation, the slide was removed from the 50-ml conical tube and inserted into a slide-holding chamber (Evergreen Sciences). Thirty milliliters of Probe Buffer (PBS, 5 mM $MgCl_2$, 0.5 mM DTT, 0.05% Triton X-100, 5% glycerol, 1% BSA) were then added slowly down the chamber wall of the slide-holding container, avoiding pipetting directly onto the surface of the array, while keeping the chamber on ice. The HybriSlip™ coverslip was gently removed with forceps and discarded without touching the array with the forceps. The array was incubated in the Probe Buffer for ~1 minute on ice, then the wash buffer discarded by inverting the chamber. This process was repeated with another 30 ml of Probe Buffer. After discarding the wash buffer, 30 ml of streptavidin-Alexa Fluor® 647 diluted 1:5000 in Probe Buffer was immediately added to the chamber. This was incubated for 30 minutes on ice and then discarded by inverting the chamber. Without allowing the surface of the array to dry, 30 ml of Probe Buffer was added slowly down the chamber wall, avoiding pipetting directly onto the surface of the array, while keeping the chamber on ice. The array was incubated in the Probe Buffer for ~1 minute on ice, then the wash buffer discarded by inverting the chamber. This process was repeated two more times. After these washes, the array was removed from the chamber, the edge of the slide tapped on a Kimwipe to remove residual buffer, and the slide placed in a centrifuge slide holder. The assembly was then centrifuged (800 x g) in an Eppendorf centrifuge equipped with a plate rotor. Arrays were stored in the dark for ~30 minutes at room temperature to allow them to dry. Once slides were completely dry (no translucent areas), they were scanned on a GenePix® 4000B at 635 nm with a PMT gain of 600, a laser power of 100%, and a focus point of 0 µm.

Results and Discussion

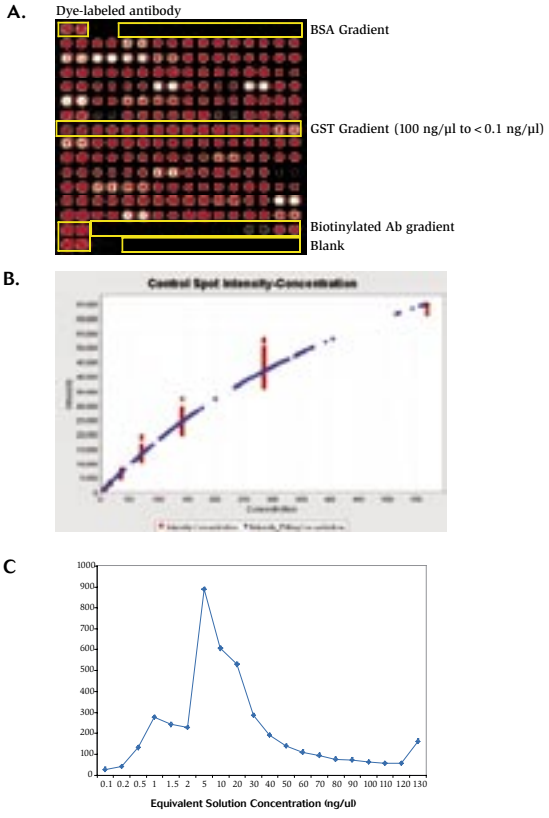
Preparation of the Yeast Proteome. Manufacturing high-quality proteome-scale protein arrays requires a high-throughput process for expression and purification that consistently generates good yields of pure and functional proteins. The proteins used to generate the Yeast ProtoArray™ Microarray are tagged with Glutathione–S– Transferase (GST). This tag features high affinity and selectivity for binding to glutathione-agarose to facilitate purification and elution under conditions that retain activity. Each step, including cell lysis, binding to affinity resins, washing, and elution, has been optimized to enable purification of sufficient amounts of as many proteins as possible. Protocol standardization and reproducibility is facilitated by the use of 96-well boxes. Processing of these 96-well boxes through the sequential steps of the purification process is carried out in a semi-automated fashion with operators moving boxes between pipetting robots, centrifuges, and/or filtration devices. All steps in the process are carried out at +4˚C; this feature in combination with the overall speed of the high-throughput purification process helps to ensure that proteins are purified in a functional form. After purification, a sample of every purified protein is printed onto arrays and the concentration of each protein determined as described in detail below. Each protein purification lot must have a median concentration of 1 ng/µl or greater before it is cleared for use on the protein microarrays.

Multiple copies of the Invitrogen™ Yeast Proteome Collection, consisting of 4088 different proteins, were expressed and purified in a high-throughput fashion and then pooled to provide sufficient material for multiple lots of ProtoArray™ manufacture. The purification yield was quantified by spotting each protein on a microarray, detecting the amount of GST-tagged protein deposited in every spot using an anti-GST antibody, and then comparing these signals to a dilution series of pure GST that was also printed on the array (Figure 1A). The intensities of the spots for the GST concentration gradient were used to calculate a standard curve (Figure 1B). The concentrations of yeast proteins were extrapolated from the standard curves using the average intensities for the duplicate spots for every yeast protein on the array. Therefore, all concentrations are normalized relative to 'molecules of GST'. The concentration distribution of all the proteins is shown in Figure 1C. The average protein concentration in this preparation was 24 ng/µl, with a median concentration of 7.3 ng/µl, well above the QC cutoff of 1 ng/µl previously determined as the criteria for a successful purification.

Manufacture and QC of the Yeast ProtoArray™ PPI Proteome Microarrays. The Yeast ProtoArray™ PPI Proteome Microarray is printed with the yeast proteins expressed and purified as described above. This array has a total of 12,288 spots, consisting of 4088 unique yeast proteins printed in duplicate, 2,056 process controls (both positive and negative), and assay controls, arranged in 48 separate subarrays. A contact-type printer equipped with 48 matched quill-type pins deposits each of these proteins along with a set of control elements in duplicate spots on a 1˝ x 3˝ nitrocellulose-coated glass slide (Schleicher & Schuell). A variety of different surface chemistries exist for protein microarray development. We, and others, have found non-covalent attachment of proteins on nitrocellulose-coated slides to be useful for immobilization of large numbers of different proteins (6-9).

A direct comparison of these slides with amine- or aldehyde-derivatized slides demonstrated that nitrocellulose-coated slides provided a nearly quantitative retention of the spotted proteins, and significantly higher detection sensitivity than the other surfaces (6,10). This increase in sensitivity was shown to be the result of the higher binding capacity of the nitrocellulose-coated surface (10). The printing of these arrays is carried out in a cold room under dust-free conditions to preserve the integrity of both samples and printed microarrays. A typical lot of microarrays generated from one printing run consists of 100 slides. Before releasing these protein microarrays for use, each lot of slides is subjected to a rigorous QC procedure. The first step in this procedure is a gross visual inspection of all the printed slides to check for scratches, fibers, smearing, etc. The second step consists of a more detailed characterization of each spot on the array. Since each of the proteins is tagged with the GST epitope, this QC procedure is accomplished by using a labeled antibody that is directed against this epitope. This procedure measures the variability in spot morphology, the number of missing spots, and the presence of control spots. An important objective of the QC process is to determine how much material is deposited on each spot. Every slide, therefore, is printed with a dilution series of known quantities of protein containing the epitope tag (e.g. purified GST) that is used to generate a standard curve. This enables the signal intensities for each spot to be converted into the amount of protein deposited.

Figure 1. Determination of protein yields for the yeast proteome.



A. Subarray of Yeast ProtoArray™ PPI Proteome Microarray showing GST concentration gradient used to generate standard curve. B. Standard curve (blue circles) generated from the GST gradients in every subarray (red circles). C. Distribution of concentrations of the yeast proteome collection.

Results and Discussion, continued

Four Yeast ProtoArray™ PPI Proteome Microarrays were subjected to the QC procedure described in Materials and Methods. An image obtained by scanning one of the arrays incubated with an anti-GST antibody is shown in Figure 2. Qualitatively, it is seen that the spotting pattern is uniform and there are very few "missing" spots, except for areas where non-GST-tagged controls (e.g., BSA) are printed. Quantitative analysis provided the data shown in Table 1, which confirms this qualitative analysis and demonstrates the reproducibility of the procedure used to manufacture the array product. Notable among the values shown in Table 1 are the low intra-array CVs for the GST controls (an indication of the pin-to-pin variation) of 16% and the low inter-array CV for all of the proteins of 13%.

Figure 2. Anti-GST QC of the Yeast ProtoArray™ PPI Proteome Microarray.

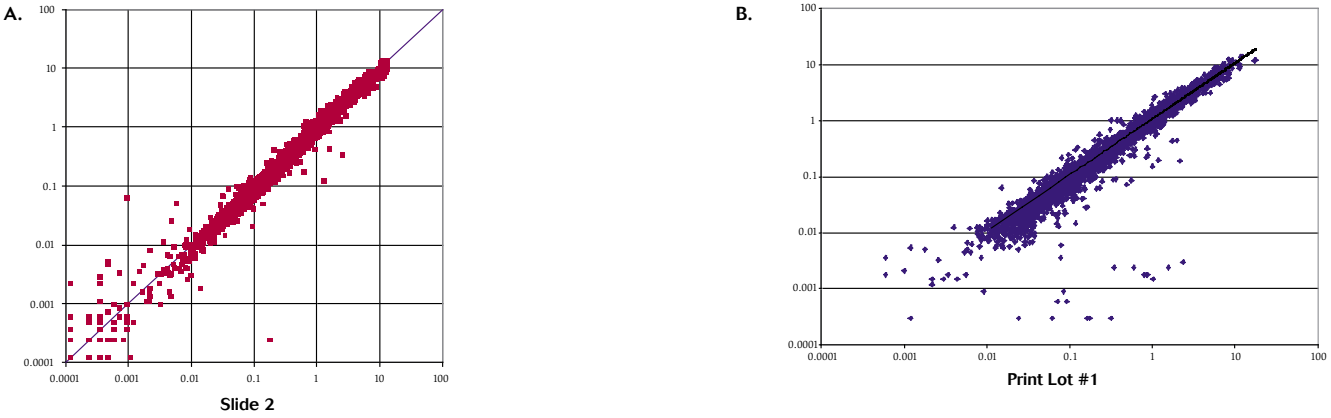
A representative array from one lot of slides was incubated with an anti-GST antibody, detected with a fluoresently labeled anti-rabbit antibody, and scanned.

The reproducibility of the process used to manufacture ProtoArray™ Microarray can also be visualized by graphically comparing the GST signals for all proteins on two different arrays. Figure 3A shows an example of this comparison between two Yeast ProtoArray™ PPI Microarrays from the same lot, while Figure 3B shows a comparison between two Yeast ProtoArray™ PPI Microarrays from different lots. In both cases, the correlation (R2) values were very high—0.97 for the intra-lot comparison and 0.95 for the inter-lot comparison. This high degree of reproducibility is obviously important for comparing results from different experiments when using different protein probes or validating results obtained with the same protein.

Table 1. Quantitative analysis of Yeast ProtoArray™ PPI Proteome Microarrays.

Category	Group	Measurement	Criteria	Actual
Inter-array	Among all 4 arrays	Median CV of Intensity	≤ 20%	13%
		Percentage of CV <30%	≥ 70%	83%
	Adjacent arrays	Median CV of Intensity	≤ 15%	8%
		Percentage of CV <25%	≥ 70%	89%
Intra-array	All 4 individual arrays	Average of GST Control Intensity CVs	≤ 25%	16%
		Median Intensity of GST Controls	≥ 1200	1660
		Median Intensity of Proteome	≥ 1200	7345
		Percentage of Proteome Intensity >500	≥ 75%	90%
		Median Diameter (µm)	150±10	150
		Percentage of Diameter within Median ±10	≥ 75%	85%

Figure 3. Intra-lot and inter-lot reproducibility of yeast proteins on the Yeast ProtoArray™ PPI Proteome Microarray.



GST signals for all yeast proteins are shown for two arrays from the (A) same lot, or (B) different lots.

***In Vitro* Biotinylation.** Detection of protein-protein interactions on the Yeast ProtoArray™ PPI Proteome Microarray requires the use of some type of label to visualize the interaction of the probe protein with proteins on the array. A widely used approach for labeling proteins is to covalently attach biotin molecules to the protein using simple conjugation chemistry. Protein probes labeled in this fashion can be detected using streptavidin pre-labeled with a fluorophore. Since most proteins are labeled with several biotins per molecule and each streptavidin molecule is labeled with approximately 5 fluorophore molecules, this method of detection provides a significant level of signal amplification, increasing sensitivity. The extremely high affinity of the biotin-streptavidin interaction also makes this approach fast and robust. Invitrogen FluoReporter® Mini-Biotin-XX Protein Labeling Kit provides a method for efficiently biotinylating small amounts of antibodies or other proteins. The water-soluble biotin-XX sulfosuccinimidyl ester contained in this kit reacts with a protein's amines to yield a biotin moiety covalently attached via two aminohexanoic chains ("XX"). Also included in the kit are ready-to-use spin columns, which provide a convenient method for purifying the biotinylated protein from excess biotinylation reagents. We have formulated a scaled-down version of the standard biotinylation kit to allow for the labeling of as little as 20 µg of protein with biotin.

Table 2. Results of in vitro biotinylation of bovine calmodulin using a mini-biotinylation kit.

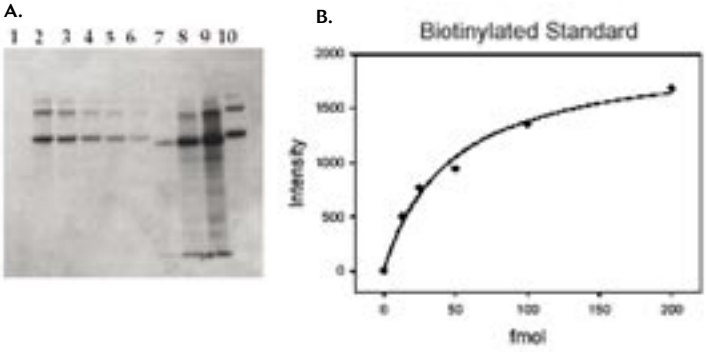
A. 20 µg Starting Material			
Sample	µg/ml	Amount Recovered (µg)	% Recovery
1	176	13.2	66
2	184	12.0	60
3	190	12.7	63
4	147	11.6	58
5	173	11.9	60
6	121	9.8	50
7	178	10.3	52
8	181	11.9	59
9	135	9.3	46
	Mean = 165	Mean = 11.4	Mean = 57.1
	S.D. = 24	S.D. = 1.3	S.D. = 6.5
B. 100 µg Starting Material			
Sample	µg/ml	Amount Recovered (µg)	% Recovery
1	840	76.0	76
2	730	73.0	73
3	820	74.0	74
4	830	81.0	81
5	760	76.0	76
	Mean = 796	Mean = 76.0	Mean = 76
	S.D. = 48	S.D. = 3.1	S.D. = 3.1

Protein concentrations were determined using a Quant-iT™ kit (Molecular Probes). Biotinylation levels were determined using a fluorescent assay.

Nine samples of 20 µg bovine calmodulin were biotinylated using the ProtoArray™ Mini-Biotinylation Kit. In parallel, 5 samples of 100 µg bovine calmodulin were also biotinylated. The results of these experiments are summarized in Table 2. The data show that both sample sets are biotinylated to the same extent despite the lower concentration of the 20-µg samples. In addition, the consistency of the degree of biotinylation was comparable between the two sample sets. Percent recovery of the 20-µg samples was lower than the 100-µg samples, but the average amount recovered (11.4 µg) was sufficient for carrying out ProtoArray™ probings.

We have developed a simple procedure for assessing the level of biotinylation using Western blot analysis. Briefly, a dilution series of a control protein conjugated to a known number of biotins is run on an SDS-PAGE gel. In parallel, the protein of interest biotinylated at three different molar ratios of biotinylation reagent is run on the gel. After Western blotting, detection of biotinylated protein is carried out using alkaline phosphatase-conjugated streptavidin. The degree of protein biotinylation can be determined qualitatively by comparison with the standards, or more quantitatively by densitometry. For a protein with an average lysine content of 8% like BSA, biotinylating at a molar ratio of 9:1 should incorporate 3-5 biotin molecules/protein. As shown in Figure 4, the intensity of the band from the 25 fmoles of BSA biotinylated at this ratio is indeed similar to the band intensity of 100 fmoles biotinylated BSA. Figure 4 also shows the results of biotinylating calmodulin kinase at 3:1, 9:1, and 27:1 molar ratio. Inspection of Figure 4 reveals that the protein biotinylated at the 9:1 molar ratio is closest in intensity to the 100 fmoles standard, and is therefore the preparation that should be used in probing experiments.

Figure 4. Assessment of biotinylation of yeast calmodulin kinase using Western blot analysis.



A. Western blot. Lane 1: SeeBlue® Plus2 Pre-Stained Standard (Invitrogen); Lane 2: Biotinylated BSA Standard (200 fmoles); Lane 3: Biotinylated BSA Standard (100 fmoles); Lane 4: Biotinylated BSA Standard (50 fmoles); Lane 5: Biotinylated BSA Standard (25 fmoles); Lane 6: Biotinylated BSA Standard (12.5 fmoles); Lane 7 CaMK (25 fmoles) biotinylated at 3:1 molar ratio; Lane 8: CaMK (25 fmoles) biotinylated at 9:1 molar ratio; Lane 9: CaMK (25 fmoles) biotinylated at 27:1 molar ratio; Lane 10: BSA (25 fmoles) biotinylated at 9:1 molar ratio. B. Curve generated by densitometry quantitation of Lanes 2-6.

Use of Yeast ProtoArray™ PPI Control Microarray. A protein used to probe the Yeast ProtoArray™ PPI Proteome Microarray should be conjugated with approximately 1-5 biotins so that sufficient detection sensitivity can be achieved. Furthermore, achieving optimal results using *in vitro* biotinylated proteins requires that unconjugated biotin is removed from the probe preparation, since free biotin can lead to high background signals when detection is carried out using streptavidin. We have developed a simplified ProtoArray™ Control Microarray that allows users to test the quality of their protein probes before using the Yeast ProtoArray™ Proteome Microarray. These Yeast ProtoArray™ PPI Control Microarray contain all of the control proteins printed on the proteome arrays, but not the yeast proteins. To demonstrate the use of the ProtoArray™ Control, six of the ten 20-µg samples biotinylated using the mini-biotinylation kit were used as probes. As shown in Figure 5, each of the protein probes exhibited the expected interaction with CaMK. Background signal was low, showing removal of sufficient free biotin from the probe preparation using the spin columns. An anti-biotin antibody is also present on the ProtoArray™ Control; if a probe protein has greater than one conjugated biotin, it will be captured by this antibody and detected using streptavidin unless the binding is inhibited by the presence of high concentrations of free biotin. The biotinylated calmodulin probe was indeed captured by this antibody and detected using labeled streptavidin (Figure 5), indicating removal of sufficient free biotin by the spin column to prevent competition for the antibody binding sites. These experiments also demonstrate that the mini-biotinylation kit can reproducibly biotinylate proteins for use as probes on ProtoArray™ Microarrays, even with limited sample amounts.

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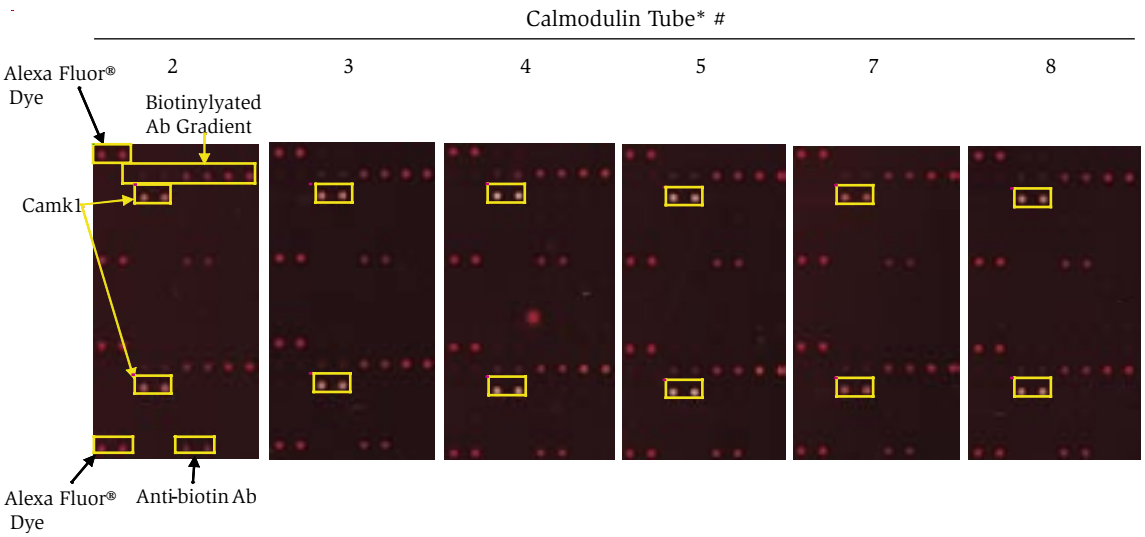
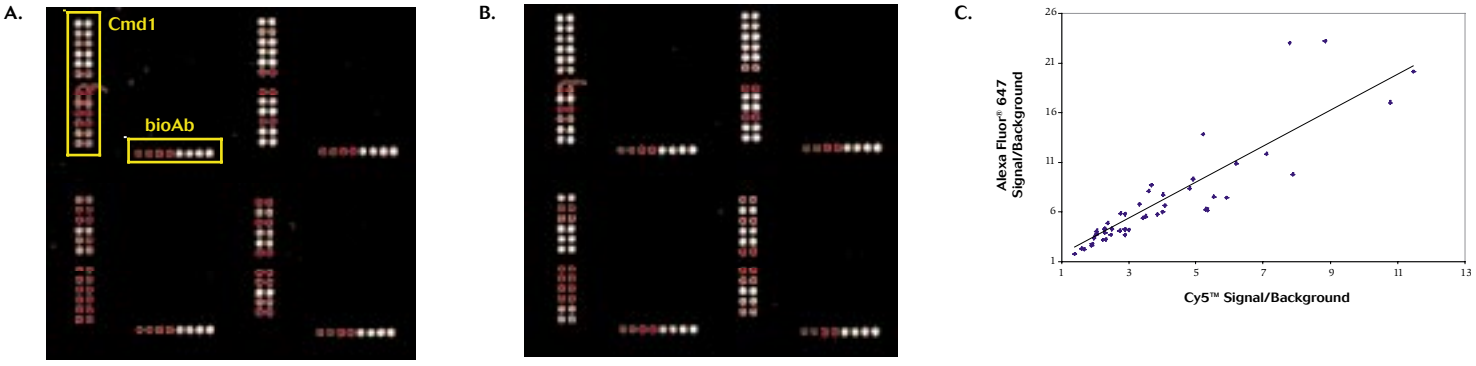
Figure 5. Probing Yeast ProtoArray™ PPI Control Microarrays with calmodulin biotinylated using the mini-biotinylation kit.



*Calmodulin tube numbers correspond to the sample numbers in Table 2.

Comparison of Cy5™ and Alexa Fluor® 647 Detection on the Yeast ProtoArray™ PPI Proteome Microarray. Detection of protein-protein interactions on ProtoArray™ Microarrays can be carried out using a variety of approaches including fluorescence, chemiluminescence, colorimetry, and radioactivity. Of these, fluorescence detection is used most often due to its high sensitivity, low background, stable signal, and availability of fluorescence-based microarray scanners. A number of different fluorescent dyes are available, including the Cy™ and Alexa Fluor® dyes. To determine which of these fluorescent dyes is most suitable for use with Yeast ProtoArray™ Microarrays, Yeast ProtoArray™ PPI Proteome Microarrays were probed with biotinylated yeast calmodulin kinase (CaMK) and detection of bound CaMK carried out with either Alexa Fluor® 647-streptavidin or Cy5™-streptavidin. As negative controls, assays were carried out using detection reagent alone. Arrays were scanned on a GenePix® 4000B fluorescence scanner and the signal/background determined for spots representing binding of CaMK to calmodulin. The results clearly demonstrate the ability to detect protein-protein interactions using Alexa Fluor® 647-streptavidin (Figure 6). Slides probed with biotinylated CaMK and detected using Alexa Fluor® 647-streptavidin revealed the expected interaction with calmodulin (CMD1; YBR109C) at an average signal/background that was at least 1.5-fold higher than detection with Cy5™-streptavidin. Negative controls (i.e. no CaMK) revealed only fiduciary spots (not shown). Preliminary experiments indicated that a 1:5000 (0.2 µg/ml) dilution of Alexa Fluor® 647-streptavidin gave lower background than the 1:500 dilution (3.0 µg/ml) used for Cy5™-streptavidin in our standard probing protocol. It is interesting to note that even though the Alexa Fluor® 647-streptavidin solution was 15-fold more dilute than the Cy5™-streptavidin, the signal/background observed for the CaMK/CaM interaction using Alexa Fluor® 647 was significantly greater.

Figure 6. Comparison of Cy5™ and Alexa Fluor® 647 detection on the Yeast ProtoArray™ PPI Proteome Microarray.

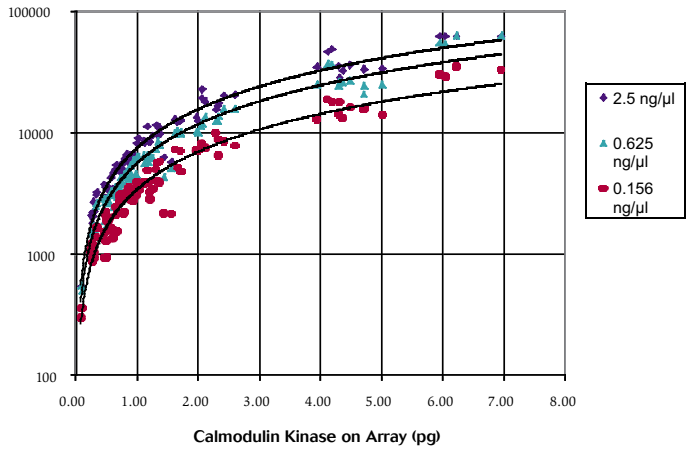


A. Subarray showing interactions of yeast CaMK with yeast calmodulin (Cmd1) with Cy5™ detection. B. Subarray showing interactions of yeast CaMK with yeast calmodulin (Cmd1) with Alexa Fluor® 647 detection. C. Quantitation of Alexa Fluor® 647 and Cy5™ detection for all Cmd1/CaMK interactions.

Sensitivity of Detecting Protein Interactions on the Yeast ProtoArray™ PPI Proteome Microarray. In most cases, users of the Yeast ProtoArray™ PPI Proteome Microarray will have access to sufficient amounts of probe protein so that ultrasensitive detection of protein-protein interactions should be unnecessary. It is useful, however, to have some idea about the limit of detection. In the case of the Yeast ProtoArray™ PPI Proteome Microarray, determining this limit is complicated by the fact that not all yeast proteins are printed at the same concentration. Thus, the limit of detection of an interaction of a probe protein with one protein on the array may be different from an interaction with a protein printed at a different concentration on the array. To investigate this further, the Yeast ProtoArray™ PPI Proteome Microarray, which has 48 pairs of calmodulin kinase spots printed at a range of concentrations, was probed with three different concentrations of biotinylated calmodulin. As shown in Figure 7, signals with intensities greater than 10,000 were observed at the higher concentrations of printed calmodulin kinase even with the lowest concentration (0.156 ng/µl) of the calmodulin probe. Signals significantly over background were also observed at spots printed with less than 1 pg (or an equivalent solution concentration of less than 1 ng/µl) of protein. This amount of printed protein is approximately 50-fold lower than the average concentration of yeast protein printed on the array (*vida supra*). These results suggest that interactions on the Yeast ProtoArray™ PPI Proteome Microarray should be observable even if the concentration of the probe and/or the printed protein is relatively low.

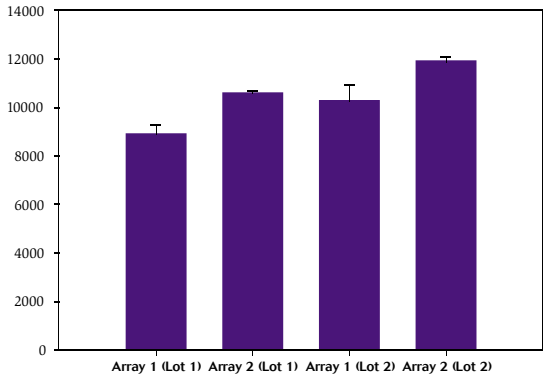
Reproducibility of Detecting Protein Interactions with the Yeast ProtoArray™ PPI Proteome Microarray. It is clearly desirable for protein microarray assays to give reproducible results. This is especially important in cases where the sample source is limited, multiple replicates are required for a higher level of statistical reliability, or the comparison of past and current results is necessary. An example of the type of reproducibility obtainable with the Yeast ProtoArray™ PPI Proteome Microarray is shown in Figure 8. Two pairs of arrays from two different print lots were probed with biotinylated calmodulin and the interaction of the probe with YML057W, an annotated interactor with calmodulin and a homolog of human calcineurin, quantitated. It can be seen from the data presented in Figure 8 that the reproducibility of the results was quite high, both between arrays from the same printing lot as well as between arrays from different print lots. In fact, the CV of the fluorescent signal for all of the arrays was 12%. This degree of reproducibility is comparable to that typically seen with DNA microarrays, and is more than sufficient for the types of experiments for which ProtoArray™ Microarrays will be used.

Figure 7. Sensitivity of detection on the Yeast ProtoArray™ PPI Proteome Microarray.



Arrays were probed with three different dilutions of biotinylated calmodulin and the signals from all of the calmodulin kinase spots on the array quantitated.

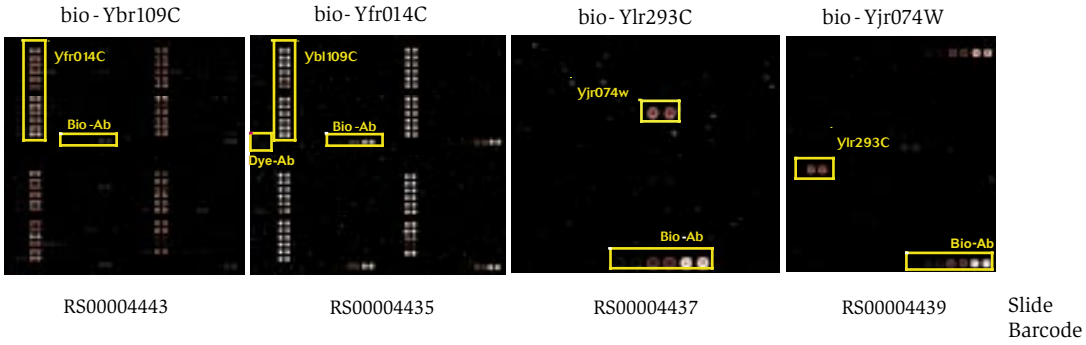
Figure 8. Intra-lot and inter-lot reproducibility of the Yeast ProtoArray™ PPI Proteome Microarray.



Four slides from two lots of arrays were probed with biotinylated calmodulin. Quantitation of the signals obtained from the interaction of calmodulin with a known interactor is shown.

Probing Yeast ProtoArray™ PPI Proteome Microarray with Yeast Proteins. In addition to bovine calmodulin, four yeast GST-fusion control proteins were biotinylated using the mini-biotinylation kit and purified using a spin column to remove free biotin. As shown in Figure 9, all four proteins showed the expected interactions when used to probe the Yeast ProtoArray™ PPI Proteome Microarray and detected with Alexa Fluor® 647-streptavidin. It is worthwhile to point out that the interactions shown in Figure 9 are reciprocal; in other words, biotinylated Ybr109C (calmodulin) interacts with Yfr014C (calmodulin kinase) on the array, and biotinylated Yfr014C interacts with Ybr109C on the array. The same relationship is observed with the GTP binding protein GSP1 (Ylr293C) and the nuclear transport protein MOG1 (Yjr074W). This latter interaction has been well annotated in the literature using a variety of different approaches (see http://www.yeastgenome.org for further details). These types of reciprocal interactions are important for demonstrating the validity of the observed interactions and the functionality of the proteins on the array.

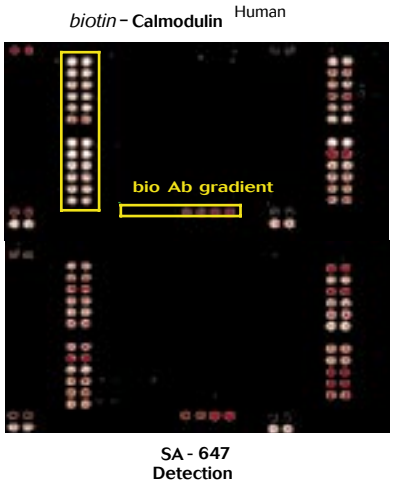
Figure 9. Probing Yeast ProtoArray™ PPI Proteome Microarrays with *in vitro* biotinylated yeast proteins.



Subarrays showing expected interactions with biotinylated control yeast proteins. Proteins were concentrated to 2.5 µg/ml, biotinylated using the mini-biotinylation kit, and purified using the spin columns as described in Materials and Methods.

Probing Yeast ProtoArray™ PPI Proteome Microarrays with a Human Protein. In addition to the yeast proteins, a human version of calmodulin was also biotinylated using the mini-biotinylation kit, used to probe the array, and found to give the same specific interaction on the array as the yeast protein (Figure 10).

Figure 10. Probing a Yeast ProtoArray™ PPI Proteome Microarray with an *in vitro* biotinylated human protein.



Subarray showing expected interaction of biotinylated human calmodulin with yeast calmodulin kinase.

Conclusion

High-density, functional protein microarrays hold enormous potential to revolutionize biological and drug discovery research by enabling the study of proteins on a scale not before possible. Until recently, the development of functional protein microarrays has been hindered primarily by the limitation of protein content. Here we have presented the Yeast ProtoArray™ PPI Proteome Microarray, which contains nearly the full protein complement of the yeast *S. cerevisiae* and demonstrated its utility for the study of protein-protein interactions. The launch of the ProtoArray™ technology ushers in a novel paradigm for the rapid study of protein interactions and biochemical activities on a proteome scale.

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