

Protein Microarrays to Detect Protein–Protein Interactions Using Red and Green Fluorescent Proteins¹

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Proteomics, the study of protein function on a global scale, will play an important role in furthering our understanding of gene functions, complex biological pathways, and discovery of novel drug targets. A number of techniques have been developed for proteomic studies to identify and analyze proteins, compare protein expression levels, and study protein–protein interactions. Recent developments have applied a DNA array-type approach to immobilize proteins on a surface for high-throughput analysis. Here we report the development and construction of protein chips using derivatized glass and nitrocellulose-coated slides and the employment of recombinant proteins fused with green and red fluorescent proteins for detection. Fluorescent signals were found to be proportional to the amount of arrayed proteins and could be readily detected with a conventional fluorescence slide scanner. This technique allows the investigation of protein–protein interactions without the need for additional labeling steps of probe proteins. © 2002 Elsevier Science (USA)

Key Words: microarrays; protein interactions; fluorescent proteins; green fluorescent protein; red fluorescent protein.

With the first draft of the human genome sequences in hand, as well as a number of other organisms' genomes completely sequenced, an increasing number of proteins of unknown structure and function are avail-

able in need of characterization. Proteomics, the study of the entire expressed proteins of an organism or cell, is important for understanding various diseases as well as identifying new therapeutic targets (1, 2). Recent work has focused on developing protein arrays or chips that will allow the high-throughput analysis of protein expression levels, function, and interactions among other possibilities (3–6).

Several strategies have been employed to construct protein chips that retain the structural integrity and functional activity of the arrayed proteins. Polyacrylamide gel pads have been used to absorb protein samples, but require manufactured masks and electrophoresis to accelerate diffusion of molecules (1). Groups have also used polyvinylidene difluoride (5, 7) and nitrocellulose (4, 8) filters to array antibodies and antigens. Recent work by MacBeath and Schreiber used glass slides derivatized with aldehyde groups or activated bovine serum albumin to attach proteins to the surface (6).

Another important consideration in the development of protein chips is the method of detection. In most of the above work, protein or antibody detection was accomplished with probes that were labeled with fluorescent dyes or traditional enzyme-linked immunosorbent assay (ELISA) techniques. In the present report, we have described an alternative technique for preparing protein arrays and detecting protein–protein interactions using red and green fluorescent proteins. Since their discovery, GFP³ and other fluorescent proteins have become an important tool in many areas of bio-

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³ Abbreviations used: GFP, green fluorescent protein; RFP, red fluorescent protein; PCR, polymerase chain reaction; IPTG, isopropylthiogalactoside; PBS, phosphate-buffered saline; BSA, bovine serum albumin; PBST, PBS containing 0.1% Tween 20.

chemistry and molecular biology. GFP has been used as a marker for gene and protein expression (9), cell signaling (10), protein trafficking (11), and many other applications (12). We have investigated the use of GFP and RFP as luminescent labels for use in protein arrays. Proteins to be used as probes were expressed with a green or red fluorescent protein (G/RFP) tag. Protein microarrays were constructed by spotting antibodies and antigens onto aldehyde or nitrocellulose-coated (FAST) glass slides using a DNA arrayer. After application of probes to the arrays, interactions between proteins could be easily visualized with commercially available slide scanners used for DNA microarrays. This method allows the construction and analysis of protein chips without the need for fluorescent dye-labeling and purification steps and can be used for numerous applications including high-throughput immunoassays, novel protein interaction profiling, and antigen-antibody studies.

EXPERIMENTAL PROCEDURES

Expression and purification of proteins fused with GFP and RFP. GFP and RFP coding regions were amplified with PCR and subcloned in a modified bacterial expression vector pBet which contains dual IPTG-regulated promoters and a N-terminal polyhistidine tag for metal affinity chromatography (Gu *et al.*, in preparation). *Escherichia coli* B834 cells expressing recombinant GFP, RFP, and their fusion proteins were grown at 30°C for 4–5 h in LB medium until an O.D. value of 0.8 was reached with a freshly inoculated seed culture before 1 mM of IPTG was added. Overnight-cultured cells were harvested by centrifugation and frozen at –80°C until further use. Protein purification procedure involves application of an *E. coli* extract, prepared in 20 mM sodium phosphate buffer, pH 7.6, containing the recombinant proteins on a column of Ni-nitriloacetate-agarose equilibrated with the extraction buffer. The column is washed batchwise with buffer containing increasing concentrations of NaCl from 0 to 2 M and lastly with buffer containing 5 mM imidazole, pH 7.4. The proteins, eluted with an imidazole gradient from 5 to 150 mM, were applied to a column of DEAE (Bio-Rad) previously equilibrated with 20 mM sodium phosphate buffer, pH 7.6. After the column was washed with equilibration buffer, the proteins were eluted with a linear salt gradient from 0 to 1 M and concentrated/dialyzed in a Centricon 30 concentrator (Amicon). The purified enzyme was either used immediately or stored at –80°C in 20 mM sodium phosphate buffer, pH 7.6, containing 40% of glycerol.

Application of proteins to glass slides. Super aldehyde substrates and aldehyde glass microscope slides used to make arrays were purchased from TeleChem International (Cupertino, CA). Proteins were diluted

into a mixture of 60% PBS, 40% glycerol (pH 7.5) at varying concentrations (1 mg/ml to 7.8 µg/ml). The proteins were then spotted onto aldehyde glass slides or nitrocellulose (FAST) slides using a GMS 417 arrayer (Affymetrix, Santa Clara, CA) or a MicroGrid II DNA arrayer (BioRobotics, Woburn, MA). Glass slides were incubated overnight in humid chambers. Unbound protein was removed by rinsing the slide twice with PBS. Aldehyde and imine groups were reduced with a NaBH₄ solution (20 mg NaBH₄ in 4 ml PBS plus 1 ml ethanol) for 10 min and washed twice with PBS containing 0.1% Tween 20 (PBST). Slides were then incubated for 1 h with a solution of PBS containing 1% BSA and were ready for probing.

FAST slide protocol. Nitrocellulose-coated FAST slides were obtained from Schleicher & Schuell (Keene, NH). Proteins were prepared and arrayed as described above. After printing, FAST slides were allowed to dry overnight, then blocked with PBST plus 1% BSA for an hour.

Probing of slides for protein-protein interactions. Either a hydrophobic pen (PAP PEN from The Binding Site, San Diego, CA) was used to draw a frame around the protein array or a hybridization chamber (Schleicher & Schuell) was used to retain solution on the slide. Proteins to be used as probes were diluted into PBS containing 1% BSA and 0.1% Tween 20 at various concentrations. Four hundred microliters of protein was added to each slide and incubated for 1 h, the protein solution was removed by suction, washed once with PBS, three times with PBST, and once with PBS. Excess PBS was removed and slides were visualized.

Fluorescence detection. Slides were scanned using a GMS 418 scanner (Affymetrix) or a ScanArray 5000 (Packard BioScience). Using the GMS 418, RFP was detected using the default settings for Cy 5. For the ScanArray, GFP-tagged proteins were visualized using 488-nm excitation and 510-nm emission settings (default setting for Alexa₄₈₈). RFP-tagged proteins were visualized at 532- and 550-nm emission settings. Slide images were analyzed using QuantArray (Packard BioScience, Boston, MA) or ScanAnalyze (Eisen, Stanford, CA).

RESULTS AND DISCUSSION

To study the practicality of using fluorescent proteins with protein arrays we printed and immobilized purified RFP on aldehyde- or nitrocellulose-coated (FAST) glass slides using a GMS 417 or a BioRobotics MicroGrid II DNA arrayer. Multiple serial dilutions of the protein, ranging in concentration from 1 mg/ml to 7.8 µg/ml, were arrayed to determine the reproducibility of the procedure and the limit of detection for the fluorophore. Since the excitation and emission maxima

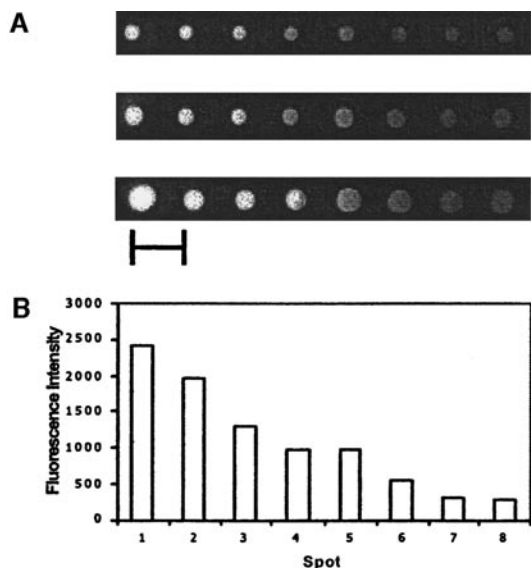


FIG. 1. Detection of RFP printed on aldehyde slides. (A) Image of slide after arraying proteins, fixing, and scanning as described under Experimental Procedures. Approximately 2-nl volumes of solutions containing 1, 0.5, 0.25, 0.13, 65, 31.25, 15.6, and 7.8 ng/μl RFP were printed in triplicate, corresponding to spots 1 through 8, respectively. (B) Relative fluorescence intensity of signal from above slide image. Values are averages of three sets of data. The distance between spots was 1000 μm and is represented by the bar.

of RFP (558 and 583 nm, respectively) fall within the same range as those of Cy-5, conventional two-laser DNA array scanners such as the GMS 418 can easily detect RFP or RFP fusion proteins. The image and fluorescence intensity of the slides after printing and washing with buffer are shown in Fig. 1. The signal for RFP was easily detected and was found to be proportional to the amount of arrayed protein. Since each protein contains one fluorophore the fluorescence signal can also be used to quantify the amount of protein arrayed or interacting. In this series, the lowest detected signal (spot 8) theoretically contained 2 nl of a 7.8 μg/ml solution of RFP or 15.6 pg of protein. However, we hypothesized that the actual amount of detected protein may be less due to losses associated with inefficient protein binding and subsequent washing steps.

The use of RFP and GFP allowed us to address this hypothesis and investigate the amount of protein that was retained on the arrays once printed, fixed, blocked, probed, and washed. This information is an important factor that can affect the usefulness, reliability, and sensitivity of protein arrays in various applications. The fluorescence intensity of arrays containing serial dilutions of RFP was compared immediately postarraying with that after the arrays were fixed, blocked, probed, and washed. These readings were taken using the same laser power and gain settings to make the

values comparable. As hypothesized, after probing and washing, protein microarrays printed on aldehyde slides still showed a concentration-dependent fluorescence, but had decreased in intensity by approximately 20-fold (Fig. 2a). FAST slides, however, showed only a slight decrease in fluorescence intensity after blocking and washing, indicating that they retained a much larger amount of the arrayed protein (Fig. 2b). This difference in protein binding capacity is most likely due to the three-dimensional polymeric surface of the FAST slide compared with the flat, two-dimensional nature of the aldehyde glass slide surface. The ability of FAST slides to retain greater amounts of protein samples may make them a better substrate for use in applications that require high sensitivity such as immunoassays and the detection of weak protein interactions.

Also of note in Fig. 2 is the apparent initial higher fluorescence intensity of the aldehyde slides compared with the FAST slides. This is a result of the nature of FAST slides. Since the nitrocellulose coating causes some light scattering and thus a higher background, FAST slides need to be scanned using lower scanner settings (power = 10, gain = 10) compared with the aldehyde slides (power = 40–60, gain = 20–40). This limitation does not seem to negatively affect the performance of these slide, since after printing and washing, FAST slides still give a higher signal than aldehyde slides while using lower laser power and gain settings (Fig. 2).

After showing that fluorescent proteins could be easily detected on protein microarrays, we tested their use in visualizing antigen–antibody interactions. An array was designed that contained serial dilutions of an affinity-purified polyclonal antibody to RFP. This array was first printed on aldehyde or FAST slides, then probed with RFP, washed, and scanned as described under Materials and Methods. RFP was captured and detected using both aldehyde- and nitrocellulose-based arrays; however, a higher signal was obtained using the nitrocellulose-coated glass slides (Fig. 3). Additional experiments were also conducted where the concentration of RFP probe applied to the array was varied to determine the limits of detection for this system. A reliable and reproducible signal was detected over the entire probe concentration range tested (1 mg/ml to 1 μg/ml). These concentrations represent a reasonable range that may be encountered in biological systems and smaller concentrations of analyte may be detected since we did not reach a limit over the range we examined. However, the limit of detection will vary for each different system investigated depending on the binding constants of antibodies and protein interactions, among other factors.

To further characterize the protein array system we had developed, serial dilutions of an affinity-purified

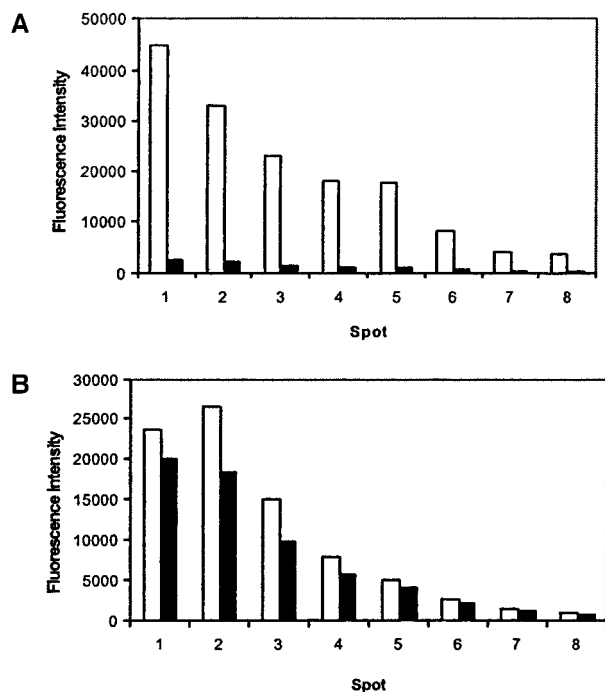


FIG. 2. Quantification of immobilized protein samples. (A) Graph of the fluorescence intensity of a protein array containing a serial dilution of RFP printed on an aldehyde slide after arraying (white bars) and after blocking and washing (black bars). GMS 418 scanner settings: laser power = 40, gain = 20. (B) Graph of the fluorescence intensity of the same protein array in (A), printed on a FAST nitrocellulose slide. GMS 418 scanner settings: laser power = 10, gain = 10.

antibody to GFP were arrayed. The protein chip was then probed with a protein in which GFP and RFP had been fused together. If the arrayed antibody functioned properly and recognized the GFP epitope, we would be able to detect a signal for GFP and RFP in the same spot. As anticipated, the protein was captured by the antibody and could be easily visualized (Fig. 4). GFP and RFP were detected at separate, individual wave-

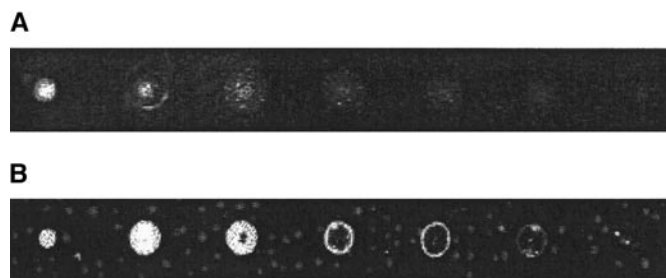


FIG. 3. Detection of antibody-antigen interaction. A protein microarray containing serial dilutions of an RFP-antibody was printed on (A) aldehyde glass slides or (B) FAST slides, then probed with 400 μ l of a solution containing 10 μ g/ml RFP and visualized. (A) GMS 418 scanner settings: laser power = 40, gain = 20. (B) GMS 418 scanner settings: laser power = 10, gain = 10.

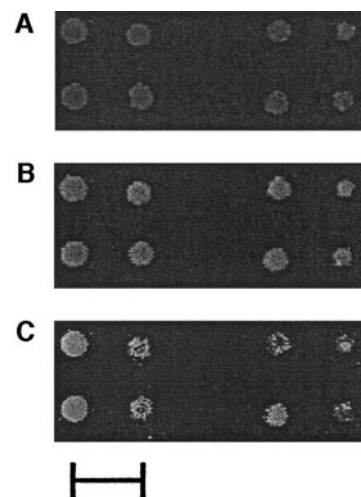


FIG. 4. Detection of protein-protein interactions. (A) A microarray printed using ~ 1 nl of GFP-antibody was probed with a GFP-RFP fusion protein. The fluorescence (red) signal of RFP was imaged using excitation at 532 with appropriate emission filters. (B) GFP (green signal) was detected using FITC excitation and emission filter settings. (C) Combined image showing colocalization of GFP and RFP fluorescence. The distance between spots was 350 μ m.

lengths (Figs. 4a, 4b) or detected simultaneously to form a composite image (Fig. 4c). These distinct spectral properties of RFP and GFP will allow their use for dual labeling and comparative hybridization experiments.

In conclusion, we have demonstrated that recombinant proteins labeled with RFP or GFP can be conveniently used in protein microarrays as tags to investigate antigen-antibody binding and other protein-protein interactions. This system allows the quantification and detection of arrayed or interacting proteins using commercially available slide scanners. The use of fluorescent proteins instead of conventional dye labeling also eliminates the extra cost of dyes and the labor of purifying labeled proteins. Both aldehyde-derivatized glass slides and nitrocellulose-coated slides were shown to be effective substrates for the construction of functional protein arrays. However, FAST slides retained a greater amount of protein, gave higher signals after probing, and may prove to be more sensitive for some applications.

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