17

Antibody-Microarrays on Hybrid Polymeric Thin Film-Coated Slides for Multiple-Protein Immunoassays

Xichun Zhou and Jizhong Zhou

Summary

The development and characterization of protein microarrays fabricated on nanoengineered three-dimensional polyelectrolyte thin films (PET) deposited on glass slide by consecutive adsorption of polyelectrolytes in solutions through self-assembly process were described. Protein antibodies or antigens were immobilized in the PET-coated glass slides by electrostatic adsorption and entrapment of the porous structure of the three-dimensional polymer film and thus, establishing a platform for parallel analysis. A method for fabrication of cytokines antibody-based protein microarray for simultaneous detection of multiple cytokines on the PET-coated slides was described. Cytokines play an important role in a wide range of physiological process, such as innate immunity, apoptosis, angiogenesis, cell growth, and differentiation. Therefore, simultaneous measurement of multiple cytokine expression levels is vital to reveal the complex cytokine network and to understand the development of certain human diseases. The protein microarray was printed by robotically spotting nine human cytokine and growth factor capture antibodies onto planar glass substrates. The fluoroimmunoassay of printed cytokine antibody microarrays were performed by incubating with cytokine samples, then binding by biotin-conjugated detection antibodies, and detecting by fluorophore conjugated streptavidin. This sandwich immunoassay-based protein microarrays protocol was developed for detection of multiple expression levels simultaneously with commercial available biotin-labeled detection antibody, so that no labeling of sera samples in required. This method was also optimized specifically for the special requirements of the cytokine detection, with special attention paid to selecting the surface chemistry of array substrate, array printing buffer and blocking buffer, and the fluorescent detection settings that yielded the highest sensitivity and selectivity against the lowest background. The dynamic ranges of the parallel assay for cytokines were around two to three orders of magnitude with limit of detection <10 pg/mL. This cytokine detection protein microarray system can be extended to a larger menu of cytokines and growth factors for applications such as profiling of cytokine expression.

Key Words: Antibody–antigen interaction; antibody microarray; biochips; cytokines; polymeric thin film; protein microarrays.

From: *Methods in Molecular Biology, vol. 382: Microarrays: Second Edition: Volume 2*
Edited by: J. B. Rampal © Humana Press Inc., Totowa, NJ

1. Introduction

Microarray-based parallel detection of different biomolecules in complex biological samples has a wide range of potential applications in the diagnosis of allergies and autoimmune and infectious diseases, as well as in epitope mapping studies and the development of vaccines ***(1–5)***. In recent years, protein microarrays have evolved as powerful tools to address these high throughput requirements. However, there are several additional challenges with protein-based microarrays because in general, proteins are more sensitive to their surrounding environment than nucleic acids. Ideally, proteins should be immobilized on a slide such that their native format and their folded conformations are preserved.

Any strategy to construct protein microarrays requires two steps: (1) deposition of proteins in parallel format on a substrate surface and (2) immobilization of the arrayed capture probes on the substrate surface. Covalently coupling, physical adsorption, and specific affinity interaction are the proposed methods to immobilize proteins in array format. Although covalent linkage to an activated surface is generally the most stable method of immobilization protein on microarray, covalent coupling typically involves multiple-step surface chemistry treatments to obtain the activated substrates for subsequent protein immobilization and extensive processing protocols have to be followed by after protein microarray fabrication. One of the most popular approaches is the slide surface functionalized with aldehyde groups. These aldehyde groups readily form aldimine (schiff-base) bonds with primary amines in protein probes, which can be further stabilized by reduction. However, covalent coupling often results in some of the immobilized proteins to lose activity because of the direct chemical modification of the binding site and steric hindrance or strain from multiple attachment sites.

Protein microarray can also be fabricated onto a slide surface through a specific affinity interaction, where protein probes were fused with a high-affinity tag at their amino or carboxyl terminus for the attachment to the chip surface through this tag. However, the modification of protein probes with affinity tag and the modification of slide substrate with protein A or Streptavidin require a peculiar time-consuming process, and in general, an increase in the quantity of reagents.

In addition to the chemistry used to immobilize proteins, the binding capacity of protein probes on slides surface is also critical for the performance of a protein microarray because the protein samples are often very limited in supply and (unlike nucleic acid) cannot be amplified. In the use of aldehyde and PLL functionalized slides, the amount of immobilized protein/peptide is limited to a two-dimensional surface area, causing a low sensitivity and a low signal/noise level. Polymer-based three-dimensional (3D) films, such as activated agarose film, hydrogel polymer, sol-nitrocellulose film, plasma-polymerized film, and protein–gel chip were reported very recently to improve binding capacity and thus the sensitivity. In addition to the sophisticated processes of creating such

3D matrixes, which often include photolithography or photopolymerization process, the major disadvantage of these reported 3D protein microarrays is that the 3D coatings often have lower reproducibility and a higher background signal caused by autofluorescence of the polymer materials.

Thus, there is great demand for new slide surface which provides reliable attachment of protein probes for various functional analyses. Ideally, proteins should be immobilized on a slide so that their native format and their folded conformations are preserved. At present a simple procedure was reported to coat glass slides with polymeric thin films by self-assembly of polyelectrolyte multilayered thin films as a platform for fabrication cytokine antibody microarrays. Owing to the amphiphilic nature of polyelectrolyte, protein probes are immobilized in semi-wet environment by the combination of strong electrostatic adsorption, hydrophobic adsorption, and entrapment of the porous structure, which keep protein probes in an active form. Furthermore, the multilayered polyelectrolyte thin films provide 3D structures, in which high-binding capacity can be achieved and the direct contact of protein with hydrophobic glass surface was avoided. In addition to this, polyelectrolyte thin films (PET) film is chemically stable, and their adhesion to substrates is strong, which is critical in obtaining a reproducible immunoassay performance. We also demonstrated the fabrication of antigen and antibody microarrays on the PET-coated glass slides as well as the direct and indirect immunoassays on the protein microarrays for multiple analyte detection.

Cytokines are a group of proteins mediate communication among cells in the immune system which include cytokines, chemokines, growth factors, angiogenic factors, and proteases ***(6–8)***. Cytokines play an important role in a wide range of physiological process, such as innate immunity, apoptosis, angiogenesis, cell growth, and differentiation. Frequently, cytokine expression is coordinately regulated as multiple cytokines often share the same upstream signal pathway, and one cytokine can regulate the expression of other cytokines. Deregulation of cytokine expression is often associated with disease status, particularly cancer, cardiac disease, and arthritis. In cancer, cytokines have been implicated in diagnosis, treatment, and prognosis. Knowledge regarding the levels of multiple cytokines is critical to the understanding of immune processes. The interaction between cytokines and the cellular immune system is a dynamic process. The interactions of positive and negative stimuli and positive as well as negative regulatory loops are complex and often involve multiple cytokines. Therefore, simultaneous measurement of multiple cytokine expression levels is vital to reveal the complex cytokine network and to understand the development of certain human disease. Immunoassays (e.g., enzyme-linked immunosorbent assay [ELISA]) are typically used to detect the level of single cytokines in biological samples, thus the presence or absence of other

cytokines is unknown. Cytokine-based protein microarrays; however, provide a simple array format, and highly sensitive approach to simultaneously detect multiple cytokine expression levels from conditioned media, patients' sera, and other sources. Scientists can rapidly and accurately identify the expression profiles of multiple cytokines by using cytokine-based protein microarray technology in several hours in a cost-effective fashion.

This PET-based array approach has several advantages over the traditional ELISA for cytokine detection. First and most important one is that cytokine-based protein microarray can simultaneously and effectively detect many cytokines, and the noncovalent adsorption of capture proteins on PET film minimizes the denaturation of the biological function of the proteins. Second, the sensitivity of most cytokine is higher, which is at the pg/mL levels. For example, as low as 10 pg/mL of TNF-α can be detected in cytokine microarray format. Thirdly, the dynamic range of detection is much greater than ELISA. For example, the dynamic detection range of TNF-γ varies from 32 to 20,000 pg/mL, whereas, it varies only within 100–1000 pg/mL in a typical ELISA. Therefore, the detection range is about 100-fold greater in protein array in a typical ELISA. In addition, the variation is lower than ELISA. As detection by fluorometry, the intraslide variation in fluorescence, determined as the coefficient of variation (CV) among the 18 spots at each concentration, ranged from 5% at 100 ng/mL to 8% at 6.4 pg/mL. In contrast, variation in ELISA is much higher (about 20%). Finally, the protein microarray system can be much easier to extend to high-density protein microarray if more capture cytokine antibodies are available.

2. Materials

2.1. Preparing Thin Polymer Film-Coated Glass Slide

1. Microscope glass slides ($76 \times 26 \times 1$ mm^3).
2. Glass clean solution: 2.5 *M* NaOH/ethanol solution.
3. 0.5% Poly vinylsulfonic acid, sodium salt (PVS) solution.
4. 0.5% Polyallylamine hydrochloride (PAAH) (M_n 50,000–65,000) solution.

2.2. Capture Antibody and Detection Antibody

1. Recombinant human cytokine TNF-α, IFN-γ, IL-1β, IL-2, IL-6, IL-8, IL-10, MCP-1, TGF-β, and monoclonal antibodies against the above cytokines and their biotinylated derivatives (biotinylated antibodies against TNF-α, IFN-γ, IL-1β, IL-2, IL-6, IL-8, IL-10, MCP-1, TGF-β) were obtained from R&D Systems (Minneapolis, MN).

2.3. Printing Requirements

1. Printing stock buffer: 1X phosphate buffered saline (PBS) (pH 7.4) with 10% glycol.
2. PixSys 5500 robotic printer (Cartesian Technologies, Inc., Irvine, CA).

2.4. Buffer Solutions for Immunoassays

1. PBST buffer: 1X PBS, 0.5% Tween-20.
2. Blocking buffer: 1% bovine serum albumin (BSA) (w/v) in PBST buffer.
3. Immunoassay stock buffer: 2X PBS (pH 7.4).
4. Biotinylated Antibody Cocktail: mix biotinylated antibodies against TNF-α, IFN-γ, IL-1β, IL-2, IL-6, IL-8, IL-10, MCP-1, TGF-β in 1X PBST buffer to final concentration for each antibody at 10 μg/mL.
5. Staining solution: 100 μg/mL Cy3-labeled streptavidin in PBST buffer.
6. Washing buffer I: 1X PBST (pH 7.4).
7. Washing buffer II: 0.1X PBS (pH 7.4).

2.5. Scan Instrument and Data Analysis Software

1. Scanning laser confocal fluorescence microscope (ScanArray 5000 System, Packed Biochip Technologies LLC, Billerica, MA) with 530-nm wavelength channel.
2. Image analysis: ImaGene 6.0 (Biodiscovery, Inc., Los Angeles, CA). The mean signal intensity of each spot was used for data analysis.
3. Statistical analysis was performed with SigmaPlot 5.0 (Jandel Scientific, San Rafael, CA) or with Microsoft Excel® (Microsoft Corp., Redmont, WA).

3. Method

The protein microarray-based bioassay systems have some similarities to cDNA microarrays, which generally contain two steps: (1) deposition of purified capture reagents or samples in parallel format at spatially defined locations on a substrate surface and immobilization of the arrayed capture probes on the substrate surface. The state of functionality of immobilized proteins determines the usefulness of protein arrays for the appropriate applications. Because antibodies can be considered as the active binding partner, these must retain their specific binding properties upon immobilization. Therefore, protocols for the immobilization, storage, and assays need to be optimized. (2) Assay design and signal generation: besides the direct immunoassays, sandwich immunoassay is most used in protein microarrays. Sandwich immunoassay takes advantage of the proven utility of ELISA. In the sandwich assay, proteins captured on an antibody microarray are detected by a cocktail of labeled detection antibodies. Each antibody is matched to one of the spotted antibodies. Thus sandwich immunoassays are widely used for the detection of proteins found in very low concentrations, such as cytokines, growth factors, or hormones from biological specimens. **Figure 1** illustrated the schematic procedures of protein microarray-detection system.

3.1. Coating APTS-Functionalized Glass Slide

1. Glass slides were cleaned by sonication in 2.5 *M* NaOH/ethanol solution for 10 min and then thoroughly rinsed with distilled water.

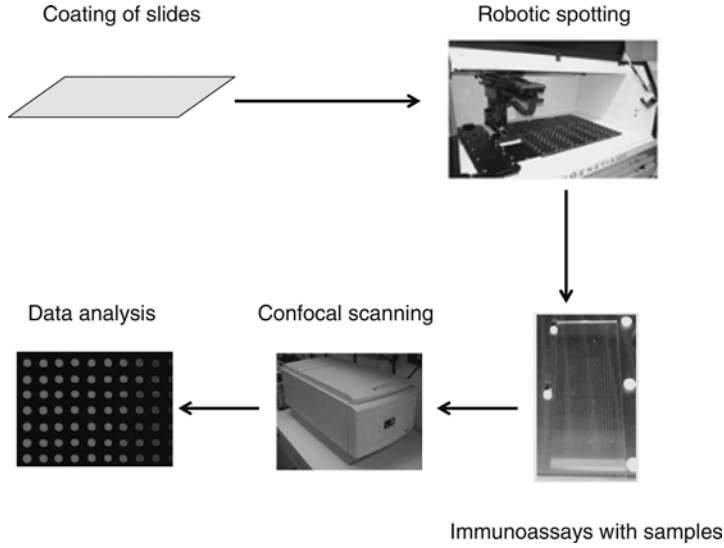

Fig. 1. Schematic procedures of protein microarray-based detection system.

2. Cleaned slides were immersed into solution of 0.5% PAAH, 1.0 *M* NaCl at pH about 6.0 for 15 min, followed by washing with distilled water, and air-drying.
3. The PAAH-coated slide was then exposed to solution of 0.5% PVS, 1 *M* NaCl at pH 8.0 for 15 min, followed by washing with distilled water, and air-drying.
4. The slide was then immersed into above PAAH solution for 15 min again. The surface was then washed again with distilled water.
5. This procedure was repeated until the desired number of polyelectrolyte pair layers $(PAAH/PVS)_6$/PAAH were deposited on the slide with the positively charged PAAH on the outer most layer (*see* **Note 1**).
6. Stored the coated glass slides in desiccator.

3.2. Protein Microarray Fabrication

1. Suspend cytokine antibodies in printing stock solution: 10 of cytokine antibodies as well as antigoat IgG (negative control) and biotinylated antigoat IgG (positive control) were buffered in 1X phosphate buffered saline (PBS) at concentration of 0.5 μg/μL and an equal volume of printing stock solution was added. The final concentration of cytokine antibody is 0.25 μg/μL (*see* **Note 2**). Mix the samples by pipetting up and down 10 times to make sure the protein samples are mixed thoroughly before printing. Transfer 10 μL of each protein printing solution into 384-well plate and stored in 4°C (*see* **Note 3**).
2. One nanoliter of the each cytokine antibody solution from a 384-well plate were printed onto the PET-functionalized glass slide using one pin with a distance of 250 μm between the centers of adjacent spots by using a PixSys 5500 robotic printer (Irvine, CA) in 60% relative humidity followed by incubation for 2 h. Each

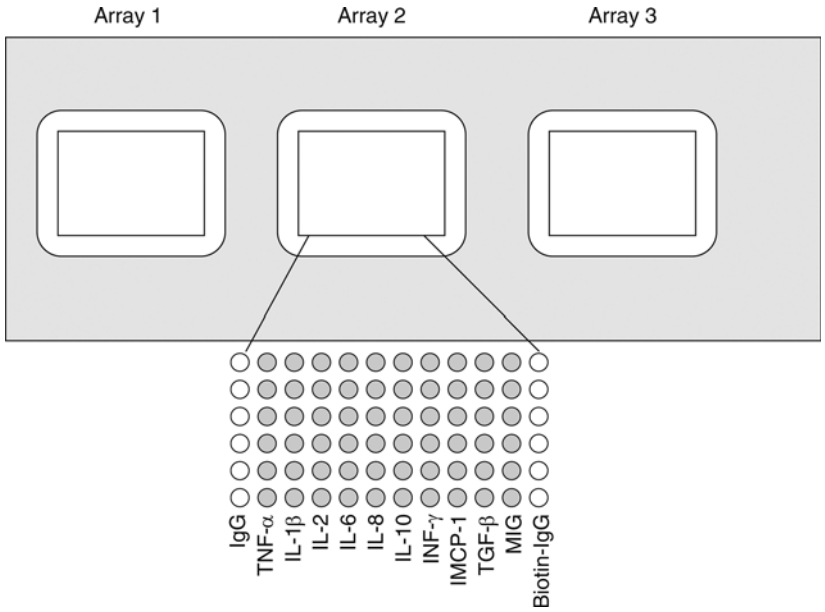

Fig. 2. Schematic illustration of the cytokine microarray arrangement on one PET slide.

cytokine antibody was printed with six replicate spots. **Figure 2** depicted the cytokine protein microarray arrangement on one slide.

3. Stored the printed slide in 4°C.

3.3. Blocking Nonspecific Adsorption

The slides were immersed into blocking buffer at room temperature for 30 min. The slides were stored in blocking buffer at 4°C unless they were used immediately. Before use for immunoassays, the slides were taken out from the blocking buffer solution and washed with washing buffer II for 1 min (*see* **Note 4**).

3.4. Generation of Standard Dose–Response Curves

1. Make 200 μL of serial dilutions of the purified cytokine antigens (from 100 ng/mL to 0.62 pg/mL at fivefold serial dilution) in the immunoassay stock buffer. Mix each concentration of cytokine antigen solution together to set up standard cytokine antigen solution.
2. Apply 20 μL of each mixed standard cytokine antigen solution on each of the three blocked cytokine antibody submicroarray surface and covered with frame seal. For each cytokine concentration, three replicate slides were tested. Total 21 slides were used to generate a seven-point standard curve (*see* **Note 5**). These slides were incubated at room temperature for 60 min.

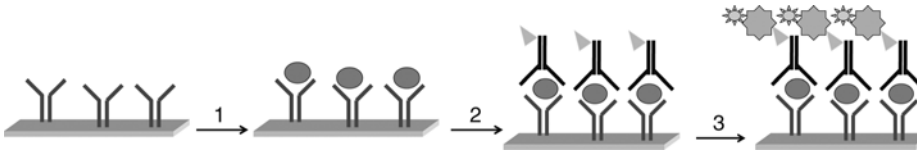

Fig. 3. Schematic representation of immunoassay procedures on cytokine protein microarrays: **(1)** samples (sera) were incubated with cytokine capture antibody microarray; **(2)** after removing unbound samples component, the chips are probed with biotin-labelled secondary antibody; and **(3)** after washing, the chips are probed with streptavidin labelled with fluorophore and specific signal are detected using fluorescent scanner.

3. After the slides were washed three times in PBST for 5 min each, a 10 μg/mL mixture of detection antibodies (biotinylated anticytokine antibodies) were applied to the slides and the slides were incubated for 1 h at room temperature. Slides were washed twice in PBST buffer for 8 min each, and then incubated with 100 μg/mL of Cy3-labeled streptavidin for 30 min. **Figure 3** shows the immunoassay procedures on cytokine protein microarrays.
4. Remove unbound Cy3-streptavidin by washing the slides three times in PBST buffer for 5 min each, followed by washing twice in 0.1X PBS for 2 min to remove detergent. The slides were dried by centrifugation at 500*g* for 3 min (*see* **Note 6**).
5. Scan the microarray to produce a fluorescent image. Microarrays were scanned at 10 μm resolution with the scanning laser confocal fluorescence microscope of a ScanArray 5000 System (*see* **Note 7**). The emitted fluorescent signal was detected by a photomultilier tube (PMT) at 570 nm for Cy3. For all microarray experiments, the laser power was 85% and the PMT gain was 75%. Save image as a high-resolution single image *.tif file (*see* **Note 8**). **Figure 4** is a typical scanning images of antibody microarrays exposed to 4 ng/mL of the 10 cytokines. The fluorescent signals were analyzed by quantifying the pixel density (intensity) of each spot using ImaGene 3.0. The local background signal was automatically subtracted from the hybridization signal of each separate spot and the mean signal intensity of each spot was used for data analysis. Statistical analyses were performed using SigmaPlot 5.0 (Jandal Scientific, San Ratael, CA) or by Microsoft Excel® (*see* **Note 9**).
6. The mean fluorescence intensity of the replicate spots for each cytokine concentrations were averaged and subsequently plotted to generate dose-response curves. **Figure 5A–C** show the typical standard dose response curves of IL-2, TNF-α, and MCP-1 cytokines. The data resulted in sigmoid curves having a linear range (the concentration range that gave the best fit to the linear equation $y = mx + b$) from 6.4 pg/mL to 20 ng/mL for IL-2, 32 pg/mL to 4 ng/mL for TNF-α and MCP-1, respectively. **Table 1** shows the limit of detection and the dynamic range of the cytokine protein microarray for individual cytokine (*see* **Note 10**).

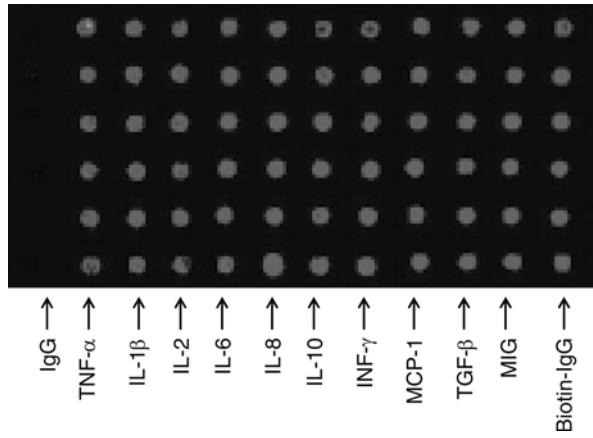

Fig. 4. Typical scanning images of antibody microarrays exposed to 4 ng/mL of the 10 cytokines.

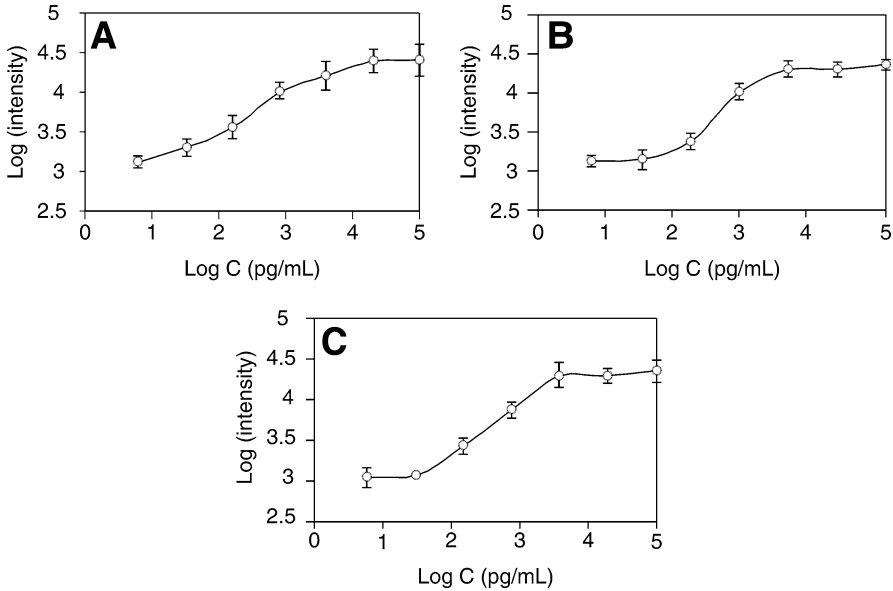

Fig. 5. Standard dose–response curve of cytokines IL-2 **(A)**, INF-γ **(B)**, and MCP-1 **(C)** oncytokine antibody microarrays.

Table 1
Limit of Detection and Standard Dose–Response Curve Range for Individual Cytokines

Cytokine	Limit of quantification 1 (pg/mL)	Suggested range for standard curve 2
TNF-α	<10	32–20,000
IL-1β	<10	6–4000
IL-2	<32	10–4000
IL-6	<10	32–4000
IL-8	<10	10–4000
IL-10	<50	50–10,000
IFN-γ	<50	33–4000
MCP-1	<100	160–10,000
TGF-β	<30	10–4000
MIG	<30	32–4000

3.5. Sandwich Immunoassays for Detection of Cytokines in Samples

1. Remove the cytokine microarray slides from Blocking Solution and briefly rinse slides twice with Wash Buffer II.
2. Samples (patients' serum or supernatant of cancer cell line culture medium) were diluted in 1X PBST buffer.
3. Apply 30 μL of the sample to be analyzed on the microarray surface and sealed with frame seal film (*see* **Note 11**). Incubate the sample with microarray for 60 min at room temperature.
4. Wash slide with washing buffer I twice for 5 min each at room temperature with gentle shaking.
5. Pipet 30 μL of 1X Biotin-conjugated anticytokine antibodies cocktail onto the slide. Incubate the cocktail solution with the slides at room temperature for 30 min.
6. Wash slide with washing buffer I twice for 5 min each at room temperature with gentle shaking.
7. Add 30 μL of 1X Streptavidin-Cy3 conjugate diluted in 1X PBST buffer. Incubate at room temperature for 30 min.
8. Wash slide with washing buffer I twice for 5 min each at room temperature with gentle shaking.
9. Wash slide with washing buffer II for 5 min each at room temperature with gentle shaking. Spinning dry the slide.
10. Image the cytokine microarray slide(s) with fluorescent imagers using the settings same as that used to generate standard curve. Each cytokine antibody is arrayed in six replicates to provide better reliability. Using imaging analysis software to determine the specific signal of each spot (signal-background). Averaging the signal of the replicate spots to determine the specific signal for each cytokine on the

array. Then determine the standard deviation of this averaged value. The average specific signal for each cytokine +/– the standard deviation is used to compare cytokine expression levels between samples. The average specific signal +/– the standard deviation for the cytokine expression in one sample can be compared to the same in other samples to determine if each sample has a different level of cytokine expression.

4. Notes

1. This procedure coats glass slides with polymeric thin films by self-assembly of polyelectrolyte multilayered thin films as a platform for fabrication protein microarrays. Owing to the amphiphilicity nature of polyelectrolyte, protein probes are immobilized in semi-wet environment by the combination of strong electrostatic adsorption, hydrophobic adsorption, and entrapment of the porous structure, which keep protein probes in an active form. Furthermore, the multilayered polyelectrolyte thin films provide 3D structures where high-binding capacity can be achieved and the direct contact of protein with hydrophobic glass surface was avoided. In addition to this, PET film is chemically stable, and their adhesion to substrates is strong, which is critical in obtaining a reproducible immunoassay performance. Compared with some of commercial slides such as epoxy-functionalized or aldehyde functionalized glass slides, the PET slides can maximally maintain the activity of protein probes, and provide a wider dynamic range for immunoassay.
2. Generally, background-subtracted fluorescent intensities for all the capture antibody spots (features) increased with increasing capture antibody concentration, however, higher concentration of capture antibody than 0.5 μg/μL will cause the cross reactivity in multiplex assay. The concentration of capture antibodies between 0.25 and 0.5 μg/μL was recommended to build the antibody arrays, which do not encounter nonspecific cross reactivity in a multiplex assay and should detect low-concentration samples, such as 32 pg/mL TNF-α.
3. Spotting buffer composition can influence the spot morphology, protein-binding capacity of a surface, the stability of proteins and the quality of the spots produced. Ten percent of glycerol was recommended to be used as additives to decrease the volatility of printing solution. However, higher glycol concentration in printing solution would produce smeared printing spots and reduce the binding of printed protein on PET slide.
4. Effective blocking of the microarray to protect nonspecific adsorption of target samples and cross-talk is important for microarray application. It is found that the use of classic blocking reagents of 1% BSA in PBS for 40 min can strongly block nonspecific adsorption. BSA concentration higher than 3% can increase the fluorescent background. To decrease the unspecific background by chemical modification of the surface coatings with 10 m*M* mercaptoethanol or 10 m*M* cysteamine is not recommended because it will reduce the activity of the immobilized capture protein. Preincubate the antibodies with the blocking reagents or add blocking reagents in washing solution might avoid antibodies contain reactivities to some

components in blocking reagents. Blocking the cytokine microarrays overnight if the blocking is not complete.

5. Each cytokine antibody is arrayed in six-repeated, so there should always be a group of six consecutive spots lighting up to a similar level. The typical design is to generate 48 replicate spots for each cytokine concentration to enable reliable quantitative data.
6. Several arrays on each slide can be reserved to run a standard curve to quantify level of cytokine antigen. A standard curve must be run for each antigen to be quantified. Purified antigens can be combined to generate multiple standard curves, simultaneously, within the same set of arrays.
7. The mean signal intensity of each spot was used for data analysis. The fluorescent protein microarray can be scanned or imaging using any of a number of high quality commercial detection instruments from Perkin Elmer (Wellesley, MA), Bio-Rad (Hercules, CA), Axon, API (Sunnyvale, CA), and many others. Instrument settings can be adjusted to optimize the imagine process.
8. Take care not to saturate the scanner-detection system by properly adjusting parameters such as the laser power, PMT power, and/or exposure settings to avoid saturation while maintaining sensitivity. The signals from the 800 pg/mL should not be saturated (>60,000 counts on many scanners), while the signals from the 32 pg/mL standard should be apparent in the scanning image.
9. Protein microarray data from the fluorescent image can be quantified, mined, and modeled using many different commercial software packages. Including those made by BioDiscovery (Marina del Ray, CA), and many others make excellent products.
10. Limit of detection is the cytokine concentration that generated fluorescent signal larger than the background plus three times of standard derivation. The dynamic range is the cytokine concentration range that gave the best fit to the linear equation $y = mx + b$.
11. Samples can be run neat or they can be diluted in 1X wash buffer II. Serum used for cell culture might contain proteins that interact nonspecifically with elements on the array. If the sample is in undiluted cell culture medium with serum, reserve one array to run the media with serum alone, thereby, controlling for possible cross-reactivity of the serum with the arrayed antibodies.

References

1. Zhu, H., Klemic, J. F., Chang, S., et al. (2000) Analysis of yeast protein kinases using protein chips. *Nat. Genet*. **26,** 283–289.
2. MacBeath, G. and Schreiber, S. L. (2000) Printing proteins as microarrays for high-throughput function determination. *Science* **289,** 1760–1763.
3. Liu, X. S., Brutlag, D. L., and Liu, J. S. (2002) An algorithm for finding protein-DNA binding sites with applications to chromatin-immunoprecipitation microarray experiments. *Nat. Biotechnol*. **20,** 835–839.
4. Madoz-Gurpide, J., Wang, H., Misek, D. E., Brichory, F., and Hanash, S. M. (2001) Protein based microarrays: a tool for probing the proteome of cancer cells and tissues. *Proteomics* **1,** 1279–1287.

5. MacBeath, G. (2002) Protein microarrays and proteomics. *Nat. Genetics* **32,** 526–532.
6. Lin, Y., Huang, R. C., Chen, L. P., et al. (2003) Profiling of cytokine expression by biotin-labeled-based protein-arrays. *Proteomics* **3,** 1750–1757.
7. Huang, R. P., Huang, R. C., Fan, Y., and Lin, Y. (2001) Simultaneous detection of multiple cytokines from conditioned media and patients' sera by an antibody-based protein array system. *Anal. Biochem.* **294,** 55–62.
8. Li, Y. W. and Reichert, W. M. (2003) Adapting cDNA microarray format to cytokine detection protein arrays. *Langmuir* **19,** 1557–1566.