Low-Density Protein Microarray on Nitrocellulose Membrane for the Detection of Human Cytokines

Chi-Yang Yu, Mao-Lung Liu, I-Ching Kuan and Shiow-Ling Lee

Department of Bioengineering, Tatung University, Taipei, Taiwan, Republic of China

A low-cost, low-density protein microarray on nitrocellulose membrane was developed for the detection of human cytokines. The microarray was derived from chemiluminescent sandwich-type immunoassay. Anti-human IL-2 and anti-human IL-4 polyclonal antibodies were arrayed and immobilized on the membrane. After the membrane was blocked and then incubation with human cytokines IL-2 and IL-4, biotinylated anti-human IL-2 and anti-human IL-4 monoclonal antibodies were added to form complexes with the cytokines and the immobilized polyclonal antibodies. Streptavidin-horseradish peroxidase conjugate was applied to detect the level of biotin. Finally, substrates for peroxidase were added to initiate chemiluminescence. The printed spots were uniform and consistent in size. Under optimized conditions, the limits of detection for human IL-2 and IL-4 were 0.5 and 0.125 ng/ml, respectively. The linear range of the calibration curves spanned over two orders of magnitude. The application of 10 ng/ml streptavidin-peroxidase polymer was found to facilitate the analysis process.

Key words: Chemiluminescence, cytokine, microarray, nitrocellulose

Introduction

Protein microarray has become an important research tool in proteomics. While the combination of two-dimensional gel electrophoresis and mass spectrometry is very useful for discovery-oriented proteomics, protein microarray is well suited for the study of a collection of related proteins [1]. The most important advantage of protein microarray is the ability to perform multiplexed analysis. Other advantages include small sample volumes, low reagent consumption, low limit of detection (LOD), and high sensitivity [2].

Although protein microarray is a powerful research tool, one major disadvantage is its high cost. The cost of a commercial system ranges from 25,000 to 220,000 USD [3]. Colorimetric detection has been applied to protein microarray as a low-cost alternative [4]. However, the LOD remains high compared to fluorescence or chemiluminescence.

We developed a low-cost, low-density protein microarray system suitable for protein-detecting applica-

Materials and Methods

Substrate Preparation and Printing
Protein microarray for cytokine detection

Polyclonal antibody were used. Controls. For the detection of cytokines, 0.1 and 1 mg/ml anti-IL-4 monoclonal antibody were printed as positive controls, 0.5 mg/ml horseradish peroxidase and 0.1 mg/ml PBS buffer was printed as negative control; 0.1 mg/ml cytokines were purchased from Pierce (Rockford, USA). The capillary action of the porous membrane may enlarge the printed spots. To examine this effect, we printed 0.3 % methylene blue on the membrane. Maximal printing density was obtained without overlapping spots. The spots were almost circular under microscope (4x10 magnification). Using ocular micrometer, the average diameter of the spots was 634.6 ± 12.3 μm (CV=1.9 %, n=60). The arrayer has eight printing pins. We used average diameter from each grid (spots printed by the same pin, n=8) to determine the variation in diameter caused by individual printing pins. The CV between grids was only 0.5 %, suggesting the pins were almost identical in terms of printing size.

The radial diffusion of the chemiluminescent reaction intermediates often results in a larger spot image than the actual printed size. This effect may cause larger variation in spot diameter and decrease the practical arraying density. We printed 25 μg/ml horseradish peroxidase onto the membrane and initiated the chemiluminescence reaction by incubating the membrane with SuperSignal West Femto substrate for 5 min. The diameter of the chemiluminescent spot determined in pixel numbers using Scion Image software (www.scioncorp.com) was uniform (CV=3 %, n=60). Maximal printing density (768×24×32) could not be achieved due to spot overlapping. The highest printing density was decreased to 192 (12×16; x-axis pitch=1500 μm and y-axis pitch=1125 μm) in a 1.8×3.6 cm area. The sensitivity of the antibodies was examined by incubating the microarrays with 12.5 ng/ml IL-2 or IL-4 in PBS containing 1 % (w/v) BSA. No cross-reaction be-

Results and Discussion

MicroCaster hand-held microarray system has a maximal printing density of 768 spots (24×32; x-axis pitch=750 μm and y-axis pitch=1125 μm) in a 1.8×3.6 cm area. The capillary action of the porous membrane may enlarge the printed spots. To examine this effect, we printed 0.3 % methylene blue on the membrane. Maximal printing density was obtained without overlapping spots. The spots were almost circular under microscope (4x10 magnification). Using ocular micrometer, the average diameter of the spots was 634.6 ± 12.3 μm (CV=1.9 %, n=60). The arrayer has eight printing pins. We used average diameter from each grid (spots printed by the same pin, n=8) to determine the variation in diameter caused by individual printing pins. The CV between grids was only 0.5 %, suggesting the pins were almost identical in terms of printing size.

The radial diffusion of the chemiluminescent reaction intermediates often results in a larger spot image than the actual printed size. This effect may cause larger variation in spot diameter and decrease the practical arraying density. We printed 25 μg/ml horseradish peroxidase onto the membrane and initiated the chemiluminescence reaction by incubating the membrane with SuperSignal West Femto substrate for 5 min. The diameter of the chemiluminescent spot determined in pixel numbers using Scion Image software (www.scioncorp.com) was uniform (CV=3 %, n=60). Maximal printing density (768×24×32) could not be achieved due to spot overlapping. The highest printing density was decreased to 192 (12×16; x-axis pitch=1500 μm and y-axis pitch=2250 μm). A 10×10 pixel area within the spot was selected to determine the uniformity of chemiluminescence. The CV of the intensity ranged from 1.5 to 5 % (n=60), suggesting the printed spots were uniform.

The complete system can be built for less than 10,000 USD. The cost of the printing substrate is under 1 USD, which is at least ten times lower than most commercial products [3].

Optimization of Cytokine Detection

The specificity of the antibodies was examined by incubating the microarrays with 12.5 ng/ml IL-2 or IL-4 in PBS containing 1 % (w/v) BSA. No cross-reaction be-
between the antibody pairs was observed when compared to the blank.

To optimize the detection, 0.1 or 1 mg/ml polyclonal antibody, 20, 62.5, or 200 ng/ml biotinylated monoclonal antibody, 2 or 10 ng/ml SA-HRP were tested. Using 0.1 mg/ml polyclonal antibody resulted in poor response to the cytokines (Fig. 1, grid 3 and 4). For IL-2, the chemiluminescence increased slightly, compared to the blank when the highest concentration of cytokine standard was applied. For IL-4, the spots could not be observed until 0.5 ng/ml IL-4 was applied. The result suggests that 0.1 mg/ml polyclonal antibody could be insufficient for detection. This observation was more pronounced for IL-2, which may be due to the lower binding efficiency of the antibody pairs. The faint spots from 0.1 mg/ml polyclonal antibody also made the analysis difficult since the ScanAlyze software requires the user visually identify the locations and contours of the spots. We therefore conclude that 1 mg/ml polyclonal antibody was optimal. Similar result was also reported by others [7].

Although the membrane was blocked in PBS containing 1 % (w/v) BSA for 1 h, the background increased significantly as the concentration of the biotinylated antibody or SA-HRP increased (Fig. 2). This might result from the dynamic exchange between the adsorbed BSA molecules and the biotinylated antibodies (or SA-HRP) since protein adsorption is generally considered reversible. Extended blocking period and/or different blocking

![Chemiluminescence images of the protein microarrays at different cytokine concentrations. Numbered white boxes indicate the locations of each grid. Grid 1: 0.1 mg horseradish peroxidase/ml; grid 2: PBS; grid 3: 0.1 mg anti-IL-2 polyclonal antibody/ml; grid 4: 0.1 mg anti-IL-4 polyclonal antibody/ml; grid 5: 1 mg anti-IL-2 polyclonal antibody/ml; grid 6: 1 mg anti-IL-4 polyclonal antibody/ml; grid 7 and 8: 0.5 μg biotinylated anti-IL-4 monoclonal antibody/ml. Concentrations for each grid were the printing concentrations. Biotinylated antibody and SA-HRP applied to the membranes were 62.5 and 10 ng/ml, respectively.](image)
Protein microarray for cytokine detection

reagent may reduce the undesirable increase of the background. For 200 ng/ml biotinylated antibody and 10 ng/ml SA-HRP, the background almost reached the saturation level of the camera (around 65,000 arbitrary unit for a 16-bit image). The high background precludes the application of such combination.

To select the optimal reaction condition, the slope (calibration sensitivity) and $R^2$ value of the calibration curve were used as criterions. Among the six combinations of biotinylated antibody and SA-HRP we tested, 62.5 ng/ml biotinylated antibody and 10 ng/ml SA-HRP were optimal for the detection of both IL-2 and IL-4 (Fig. 3). The linear range spanned over two orders of magnitude. The LOD under optimal condition was determined

![Fig. 2. Effect of biotinylated antibody and SA-HRP on the background (□: 2 ng SA-HRP/ml; ▒: 10 ng SA-HRP/ml). The background was averaged from the negative control grid (PBS, n=6). AU=arbitrary unit.](image)

![Fig. 3. Calibration curves for IL-2 (▲) and IL-4 (□) under optimal reaction condition (62.5 ng biotinylated antibody/ml and 10 ng SA-HRP/ml). Cytokine standard (mixture) 0.125, 0.5, 1.25, 5, and 12.5 ng/ml were prepared in PBS containing 1 % (w/v) BSA. The fitted lines have slopes of 1826 ($R^2=0.92$) and 4058 ($R^2=0.92$) for IL-2 and IL-4, respectively. The printing concentration of the polyclonal antibody was 1 mg/ml.](image)
by further diluting the standards to 0.005, 0.0125, 0.05, 0.125, and 0.5 ng/ml cytokine. The LOD (~ 95% confidence level) is defined as the concentration that produces a chemiluminescence signal greater than blank+3SD [8]. The LOD for IL-2 and IL-4 were 0.5 and 0.125 ng/ml, respectively.

Compared to a fluorescent protein microarray system, which also utilized nitrocellulose as the printing surface, the LOD for IL-4 was similar [7]. However, the LOD for IL-2 in our system was an order of magnitude higher. The difference may be caused by the lower binding affinity between the antigen and antibody pairs, the amount of antibody immobilized, or the nature of the detectors. The linear range of the fluorescence system spanned over two to three orders of magnitude, which was similar to ours.

The LOD for both cytokines were similar to early immunoassays [9,10]. However, the LOD of recent commercial ELISA can reach several picograms per milliliter when testing standards (see www.piercenet.com or www.idsltd.com). It has been shown that membrane protein microarray coupled with radiographic film may lack the sufficient sensitivity to detect cytokine levels in complex biological fluids when compared to ELISA [11].

One possible explanation for the high LOD observed in microarray is the amount of capture antibody used. For 1 mg/ml printing concentration, approximately 3 ng capture antibody was printed (calculated from the delivery volume of 3 nl of the arraying pin). The amount of the capture antibody used per microtiter plate well in commercial ELISA is approximately two logs higher, in the sub-microgram range. However, when fluorescent label and laser microarray scanner were applied to microarrays, the reproducibility and LOD for sixteen different human cytokines were similar to ELISA results [12]. For limited number of analytes, protein microarray has no clear advantage over traditional ELISA in terms of performance and ease of use. Nevertheless, protein microarray would be an appropriate method when a complex concentration profile is needed due to its ability to perform multiplexed analysis.

In addition to protein microarray, several new technologies for cytokine detection have also emerged in the past two decades. The enzyme-linked immunospot (ELISPOT) uses the same concept from traditional ELISA [13]. Instead of measuring the cytokine concentration in the sample, the ELISPOT assay allows the quantitative measurement of frequency of cytokine secreting cells at the single cell level. Another immunologically based method, the multiplex bead array assays (MBAA), has the advantages of multiplexing and small sample volume requirement. By immobilizing capture antibody on fluorescence-coded beads, the analyte being measured on individual bead can be identified and the corresponding fluorescence signal from the antibody-antigen sandwich can be recorded when the bead passes through a flow cytometer. Hill and Martin were able to analyze ten different cytokines by MBAA using only 75 μl patient serum [14]. A recent review has shown that similar quantitative results can be obtained from both ELISA and MBAA if identical antibody pairs, similar diluents, and blockers are used [15]. Although MBAA appears to outperform protein microarray, it has several disadvantages including cumbersome immobilization procedure, lower multiplexity (up to one hundred analytes for current commercial assays), and the requirement of an expensive flow cytometer. In addition to antibodies, aptamers have been used as ligands to bind cytokines in various formats [16]. Bock et al. developed a fluorescent photoaptamer microarray for seventeen target proteins including several cytokines [17]. Since photoaptamers bind to the target analytes covalently, the microarray can withstand vigorous washing to improve signal to noise ratio and limit of quantification. Photoaptamers can also be applied to the chemiluminescent protein microarray described in this manuscript to improve its performance. The immunoproteomics approach is perhaps the most recent development in cytokine detection [18]. Immunoproteomics uses a single reagent (proACTR) for immobilization of antibody and capture of antigen. The immobilized antibody-antigen complex is then applied to the protein chip and analyzed directly by SELDI-TOF mass spectrometry. Comparing to protein microarray, the major advantage is its speed. The assay can be completed in less than 1 h. However, the current degree of multiplexing is low and expensive instrumentation is necessary.

**Effect of Streptavidin-Peroxidase Polymer**

The polymer is made by covalently linking multiple streptavidin and horseradish peroxidase molecules to a hydrophilic polymer backbone according to the supplier. The number of enzyme labels per biotin binding site can be significantly increased and thus enhance the chemiluminescence signal. Rolling circle amplification and silver enhancement have been applied to amplify the signal from protein microarrays [4,19].

We tested the potential signal enhancing effect of 10, 20, and 33 ng/ml polymer. Both 20 and 33 ng/ml polymer showed no response to IL-4 since the chemiluminescence already reached the saturation level of the digital camera (Fig. 4). When 10 ng/ml polymer was
used, chemiluminescence intensity increased about two fold for all standards, compared to the optimal 10 ng/ml SA-HRP. However, the LOD remained the same since there was a coherent increase in the blank signal (from 16432 ±1328 to 30595±2071, n=6). Similar result was also observed for IL-2. Although the polymer did not lower the LOD, the images were easier to analyze due to brighter and clearer spots.

Acknowledgements

Financial support by National Science Council (grant NSC 92-2213-E-036-003) and Tatung University (grant B9208-S05-073) is gratefully acknowledged.

References

低密度硝化纖維膜蛋白質微陣列應用於人類細胞激素檢測

游吉陽 劉茂隆 官宜靜 李錦鈴
大同大學生物工程系

本研究發展出低成本、低密度之硝化纖維膜蛋白質微陣列並應用於人類細胞激素檢測。此微陣列係由冷光三明治式免疫分析法衍生而來。首先將抗人類IL-2及抗人類IL-4多株抗體打點並固定於薄膜表面。薄膜經blocking後與人類IL-2及人類IL-4反應，再加入經生物素標定之抗人類IL-2及抗人類IL-4單株抗體與細胞激素及固定於表面之多株抗體形成複合物。以streptavidin-horseradish peroxidase conjugate偵測生物素，最後加入peroxidase之基質以產生冷光。微陣列中之點大小一致且產生均勻冷光。在最適條件下，對人類IL-2及人類IL-4之偵測極限分別可達0.5及0.125 ng/ml。標準檢量線之線性範圍跨越兩個級數。反應中使用10 ng/ml之streptavidin-peroxidase polymer可使數據分析更為容易。

關鍵詞：冷光、細胞激素、微陣列、硝化纖維膜