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Protein Arrays From cDNA Expression Libraries

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Summary

This chapter describes the production of a cDNA expression library from human fetal brain, the construction of a high-density protein array from such a library, and two applications to screen the array for binding proteins. After producing the library and decollating the expression clones, one can pick thousands of expression clones with a laboratory robot and can deposit them into microtiter plates in an ordered manner. Such ordered clone libraries are the starting material for the construction of a high-density protein array. This array is constructed by spotting the expression clones onto a protein-binding membrane. Following cell growth and induction of protein expression on the membrane, the cell spots are lysed and their recombinant protein immobilized on the membrane. The so-constructed array carries thousands of proteins without the need to clone, express, and spot individual proteins. Such arrays allow one to screen for numerous protein functions in a high-throughput manner.

Key Words:

Protein array; cDNA expression library; high-density spotting; clone array; protein antigen; protein function; protein–protein interaction; posttranslational modification; high-throughput screening.

1. Introduction

Arrays of complementary DNA (cDNA) expression libraries carry thousands of proteins without the need to clone, express, and spot individual proteins ***(1)***. These arrays are practical formats to screen en masse for a given protein function, that is, to identify protein antigens ***(1,2)***, including autoantigens ***(3)***, binding proteins ***(4)***, and substrates for arginine methyltransferases ***(5)***. Although not yet demonstrated, the arrays may also permit studies on posttranslational modifications other than protein methylation, that is, to find substrates for certain protein kinases.

The protein arrays described here are made using cDNA libraries that are constructed in expression vectors. With the help of a laboratory robot, one can pick thousands of library clones and can deposit them into microtiter plates in an ordered

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manner. Such ordered clone libraries are the starting material for the construction of high-density DNA or protein arrays that require additional robotics *(1,6,7)*. The arrays are constructed by spotting thousands of bacterial clones onto a protein-binding filter membrane. On cell growth and induction of protein expression on the filter, the cells are lysed, and their proteins immobilized on the filter. The so-constructed protein array offers a notable advantage over the widely used filter-immobilized cDNA expression libraries that are based on the bacteriophage λgt11 *(8,9)*. The advantage is immediate addressability, namely, the direct link between a given protein spot on the array and the corresponding clone in a well of a microtiter plate that can serve as a resource for unlimited future use. In addition, the protein arrays possibly contain more recombinant protein per spot area because many methyltransferase substrates remain undetected if an immobilized phage expression library is used instead of the protein array *(5)*.

Protein arrays from a cDNA expression library are available at the German Resource Centre *(10)*. The corresponding cDNA expression library was constructed from human fetal brain and was preselected as described under **Subheading 3.6.** for clones that express recombinant proteins.

2. Materials

2.1. Cloning of a cDNA Expression Library

2.1.1. RNA Preparation, cDNA Synthesis, and Escherichia coli Transformation

1. Polyadenylated (poly [A+]) RNA isolation kit.
2. cDNA Synthesis Kit (Invitrogen Life Technologies).
3. cDNA size-fractionation columns (Invitrogen Life Technologies).

2.2. Construction of Expression Clone Arrays

2.2.1. Colony Picking

1. Blotting paper: 3MM Whatman. Prepare 23 × 23 cm^2 sheets.
2. Dishes for large agar plates, 23 × 23 cm^2 (Bio Assay Dish, Nunc).
3. 40% (w/v) glucose: Dissolve 400 g D-glucose monohydrate in dH_2O to 1 L and sterilize by filtration through a 0.2 μ*M* pore-sized filter.
4. 2X YT broth: Add 16 g tryptone, 10 g yeast extract, 5 g NaCl per liter and autoclave. Cool to 50°C; add appropriate antibiotics and glucose to 2%.
5. 2X YT agar: Add 16 g tryptone, 10 g yeast extract, 5 g NaCl, 15 g agar per liter and autoclave. Cool to 50°C; add appropriate antibiotics and glucose to 2%.
6. Colony-picking robot and additional material for picking *(7)*. Alternatively, a smaller number of colonies can be picked manually with toothpicks or other devices.
7. 384-well microtiter plates with lids. These plates should have a well volume greater than or equal to 95 μL, such as Genetix polystyrene large-volume plates, product code X7001. Optionally, order microplates prelabeled with unique identifiers.
8. Cryolabels for the microtiter plates (e.g., Laser Cryo-Etiketten, Roth; http://www.carlroth.de).
9. 384-pinned replicators. Plastic and steel replicators are available from Genetix or Nunc.
10. Incubator at 37°C.

2.2.2. High-Density Spotting of Expression Clones onto Filter Membranes

1. Polyvinylidene fluoride (PVDF) filter membranes, 222 × 222 mm^2. Immobilon P (Millipore) or Hybond-PVDF (Amersham Biosciences) have been used successfully. The required filter size may have to be custom ordered.
2. Blotting paper, media and agar plates (*see* **Subheading 3.2.1.**).
3. Isopropyl-β-D-thiogalactopyranoside (IPTG) agar plates: Prepare 2X YT agar; add appropriate antibiotics and IPTG to 1 m*M*.
4. Incubators at 30°C and 37°C.
5. Lyophilized rabbit and mouse sera.
6. Black ink, such as TG1 Drawing Ink, Faber-Castell.
7. Forceps to handle the filters.
8. Spotting robot and additional material for spotting *(7)*.
9. Tris-buffered saline (TBS): 10 m*M* Tris-HCl, pH 7.5, 150 m*M* NaCl.
10. Ethanol.

2.2.3. Release of Cellular Proteins on the Membrane

1. Denaturing solution: 0.5 *M* NaOH, 1.5 *M* NaCl.
2. Neutralizing solution: 1 *M* Tris-HCl, pH 7.4, 1.5 *M* NaCl.
3. 20X standard sodium citrate (SSC): 3 *M* NaCl, 0.3 *M* sodium citrate, pH 7.0.
4. Blotting paper and dishes (*see* **Subheading 3.2.1.**).

2.2.4. Nondenaturing Release of Cellular Proteins on the Membrane

1. Lysis buffer: 50 m*M* Tris-HCl pH 8.0, 300 m*M* NaCl, 1 m*M* ethylenediaminetetraacetic acid (EDTA), 0.1% Triton X-100, 1 m*M* phenylmethylsulfonyl fluoride, 1 mg/mL lysozyme.
2. Blotting paper and dishes (*see* **Subheading 3.2.1.**).

2.3. Screening of the Array for Protein Antigens

1. Dry protein array filter.
2. TBS: 10 m*M* Tris-HCl, pH 7.5, 150 m*M* NaCl.
3. TBS+Tween+Triton (TBSTT): 20 m*M* Tris-HCl pH 7.5, 0.5 *M* NaCl, 0.1% (v/v) Tween-20, 0.5% (v/v) Triton X-100.
4. Nonfat dry milk powder.
5. Kimwipes paper towels (Kimberly-Clark).
6. Large plastic box that can accommodate the filters.
7. Primary antibody directed against the antigen of interest.
8. Secondary antibody directed against IgGs of the organism that the primary antibody was obtained from, conjugated with alkaline phosphatase (AP) (for example, Roche Antimouse Ig-AP for use with mouse monoclonal primary antibodies).
9. Attophos, available from Roche or Promega.
10. Attophos stock solution: 2.4 *M* diethanolamine, 5 m*M* attophos, 0.23 m*M* $MgCl_2$; set pH to 9.2 with HCl, sterilize by filtration through a 0.2 μ*M* pore-sized filter.
11. AP buffer: 1 m*M* $MgCl_2$, 100 m*M* Tris-HCl, pH 9.5
12. Fluorescence-scanning device or charge-coupled device (CCD) camera.
13. Ethanol.

2.4. Screening of the Array for Protein–Protein Interaction

2.4.1. Phosphate Incorporation into the Fusion Protein

1. 400–600 μg purified fusion protein with protein kinase A (PKA) site.
2. 1000 U cyclic adenosine monophosphate-dependent protein kinase (Sigma P-2645).
3. 40 m*M* dithiothreitol (DTT).
4. 10X kinase buffer: 200 m*M* Tris-HCl, 1 *M* NaCl, 120 m*M* $MgCl_2$, pH 7.5, 10 m*M* DTT.
5. Sephadex G50 (medium grade) gel filtration column (approx 2.5 mL bed volume) equilibrated in 20 μ*M* HEPES-KOH, 50 m*M* KCl, 0.1 m*M* EDTA, 2.5 m*M* $MgCl_2$, pH 7.4.
6. [γ-^{32}P] adenosine triphosphate (ATP) (25 μL 1 m*M* ATP, 20 dpm/nmol).
7. Liquid scintillation counter.

2.4.2. Blocking and Probing the Filter

1. Dry protein array filter (*see* **Subheading 3.2.2.**).
2. TBS: 10 m*M* Tris-HCl, pH 7.5, 150 m*M* NaCl.
3. TBST: TBS containing 0.05% (v/v) Triton X-100.
4. Blocking buffer (BB): 20 m*M* HEPES-KOH, 5 m*M* $MgCl_2$, 5 m*M* KCl, 0.1 m*M* EDTA, pH 7.4, 0.05% (v/v) Nonidet P-40, 4% (w/v) nonfat dry milk powder.
5. Hybridization buffer (HB): 20 m*M* HEPES-KOH, 50 m*M* KCl, 0.1 m*M* EDTA, 2.5 m*M* $MgCl_2$, pH 7.4, 0.05% (v/v) Nonidet P-40, 1% (w/v) milk.
6. Labeled fusion protein probe (*see* **Subheading 3.4.1.**).
7. Ethanol.
8. Storage phosphor screen plus scanner or autoradiography equipment.

3. Methods

3.1. Cloning of a cDNA Expression Library

A detailed description of cDNA library construction is beyond the scope of this chapter. Therefore, the authors provide only a short summary. Construction of a cDNA expression library requires extra consideration in comparison to standard libraries. cDNA synthesis should be primed with deoxythymidine oligonucleotides for directional cloning and for the production of recombinant proteins with their complete N-terminus. An average cDNA insert size of 1.4–1.8 kbp is recommended. This leads to an appropriate ratio of full-length and truncated clones and maximizes the chances that the protein or protein domain of interest is expressed in the library.

3.1.1. Choice of Expression Vector and E. coli Strain

3.1.1.1. Expression Vector and Screening for Expression Clones

A wide range of bacterial expression vectors is currently available. Choose a vector for expression of fusion proteins with a short N-terminal affinity tag to allow selection of expression clones after the library has been constructed ***(11)***. The hexahistidine tag is particularly well suited for this purpose because fusion proteins can easily be detected with antibodies (*see* **Fig. 1**). The authors used a derivative of the pQE-30 vector (Qiagen), namely pQE30NST (*see* **Fig. 2**) to express his-tagged proteins in *E. coli* and used antibodies against RGS(H_6) to detect them.

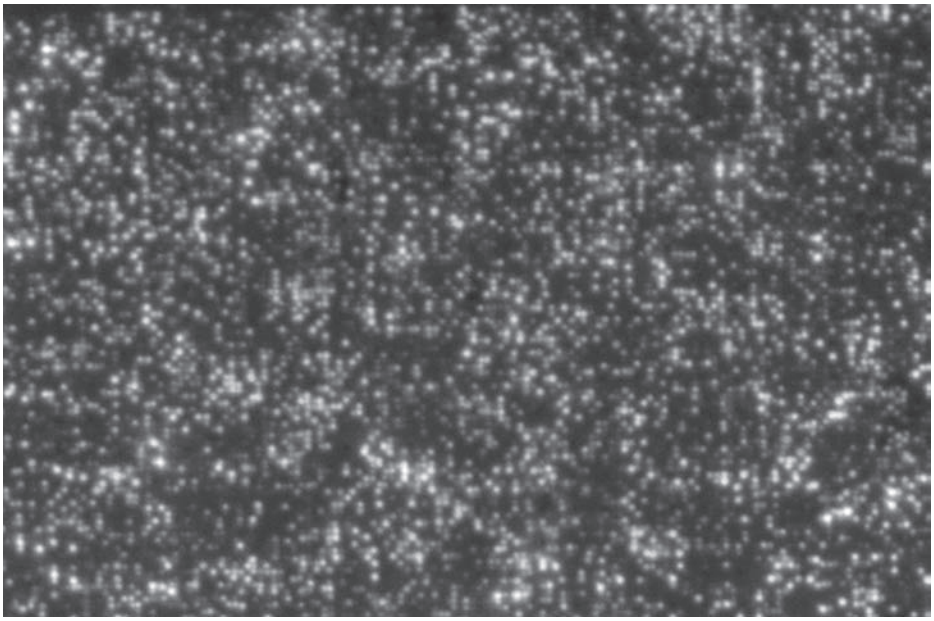


Fig. 1. Detection of recombinant proteins on an array with proteins from the human fetal brain expression library (hEx1). A section is shown of the array that was decorated with the RGS-His antibody according to **Subheading 3.3.**

3.1.1.2. *E. coli* Strain

The *E. coli* strain for the library has to be suitable for cloning, plasmid propagation, and protein expression. The authors recommend a robust K-21 strain with high transformation efficiency and the *endA* genotype for plasmid stability, for example, SCS1 (Stratagene).

3.1.1.3. *Lac* Repressor

If an IPTG-inducible vector with a promoter regulated by *lac* operators is used, consider that sufficient amounts of the repressor protein ***(12)*** have to be expressed in the host cells. A mutated form of the *lac* repressor gene, $lacI^Q$, enhances expression of the repressor protein and is included in many expression vectors. Alternatively, an *E. coli* strain carrying the $lacI^Q$ gene, for example, DH5αZ1 ***(13)***, can be used. Further, a helper plasmid that carries the $lacI^Q$ gene, and that is compatible with the expression vector, can be introduced into the preferred *E. coli* strain before the cells are transformed with ligated cDNA.

3.1.1.4. Rare Codons

Many eukaryotic genes contain codons that are rare in *E. coli*. This can strongly reduce the expression of the corresponding eukaryotic proteins in *E. coli*. To weaken

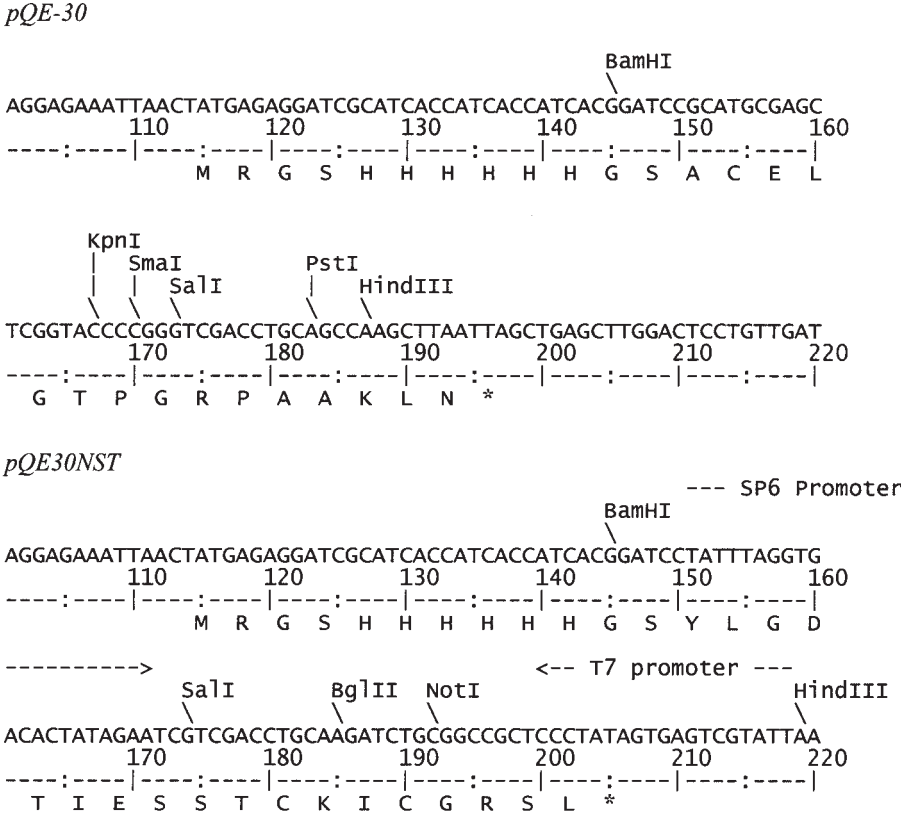


Fig. 2. Multicloning sites of vectors for expression of his-tag fusion proteins. Vectors pQE-30 (Qiagen) and pQE30NST (Genbank accession AF074376) are shown.

the problem, transfer RNA genes that are rare in *E. coli* should be introduced. The "Rosetta" and "CodonPlus" *E. coli* strains with plasmids that carry such genes are available from Stratagene or Novagen, respectively. The plasmids can be isolated and introduced into the preferred *E. coli* strain.

3.1.2. RNA Preparation, cDNA Synthesis, and E. coli Transformation

1. Extract total RNA from a tissue sample according to Chomczynski and Sacchi ***(14)*** and isolate poly(A)$^+$ mRNA with immobilized deoxythymidine oligonucleotides. Various kits are available for this purpose.
2. Start first-strand cDNA synthesis with at least 0.5 μg poly (A+) RNA and use a oligo-(dT) primer with a *Not*I site: for example, p-GAC TAG TTC TAG ATC GCG AGC GGC CGC CC $(T)_{15}$.
3. Construct double-stranded, blunt-ended cDNA according to the kit's instructions. Ligate-cloning adapters compatible to the 5' restriction site of the expression vector to the cDNA.

Here is an example for a *Sal*I adapter:
5'-TCG ACC CAC GCG TCC G-3'
3'-GG GTG CGC AGG C-p-5'.

4. Ligate the adapter to the cDNA, digest with *Not*I to produce cDNA with *Sal*I and *Not*I overhangs that can be ligated into the expression vector. Before the ligation, fractionate the cDNA on a sizing column, and, preferably, ligate the largest cDNA fragments.
5. Transform *E. coli* with the ligated cDNA by electroporation (*see* **Subheading 3.1.1.** for *E. coli* strains and additional considerations).

3.2. Construction of Expression Clone Arrays

The picking and arraying of expression libraries follows protocols that are well established for general DNA libraries ***(7)***. The picking of thousands of colonies into microtiter plates and spotting of the clones as arrays requires robotic equipment. Picking and spotting robots are available from Kbiosystems, Genetix, and other manufacturers. The German Resource Centre offers clone-picking and arraying services ***(10)***.

3.2.1. Colony Picking

The clones of the expression library are stored individually in the wells of microtiter plates. Take care to label the plates properly before use, for example, with barcodes. Print identifiers onto cryolabels and attach them to the plates; alternatively, prelabeled microplates can be purchased.

1. Fill 384-well microtiter plates with 65 µL 2X YT broth supplemented with antibiotics and glucose.
2. Plate transformed *E. coli* cells at a density of 3000 clones/plate onto square 23 × 23 cm^2 2X YT agar plates supplemented with antibiotics and glucose, and incubate at 37°C overnight.
3. Pick colonies into individual wells of the microtiter plates.
4. Wrap plates in plastic foil and incubate for approx 16 h at 37°C for bacterial growth.
5. Copy the plates by inoculating fresh plates with sterile 384-pin replicators (*see* **Note 1**). Store plates at –80°C (*see* **Note 2**).

3.2.2. High-Density Spotting of Expression Clones onto Filter Membranes

1. Optional: Prepare serial dilutions of rabbit or mouse serum in TBS with a maximum of about 70 mg protein per milliliter, and spot each dilution alongside the clones. The serum spot will show up as guide dots and as a control (*see* **Fig. 3**), if the filter is decorated with secondary antibodies according to **Subheading 3.3.** Alternatively, use black ink to spot guide dots. Later on, such dots may be extremely helpful for image analysis.
2. Thaw the plates (stored at –80°C) and prepare the PVDF filter membrane for spotting. Note that such filters are rather hydrophobic and have to be wetted properly before use. Wet the filter in ethanol for at least 5 min, then wash twice in dH_2O and, finally, in 2X YT broth. Place the filter on blotting paper soaked with 2X YT broth and remove air bubbles and excess liquid by rolling with a long glass pipet. The filter is now ready for spotting. Let the robot spot each clone in duplicate as described previously ***(7)***. *See* **Fig. 4** for recommended spotting patterns.
3. Place filter on a square 2X YT agar plate supplemented with antibiotics and glucose. Let colonies grow on the filter overnight at 30°C to a size of approx 1 mm diameter. Transfer

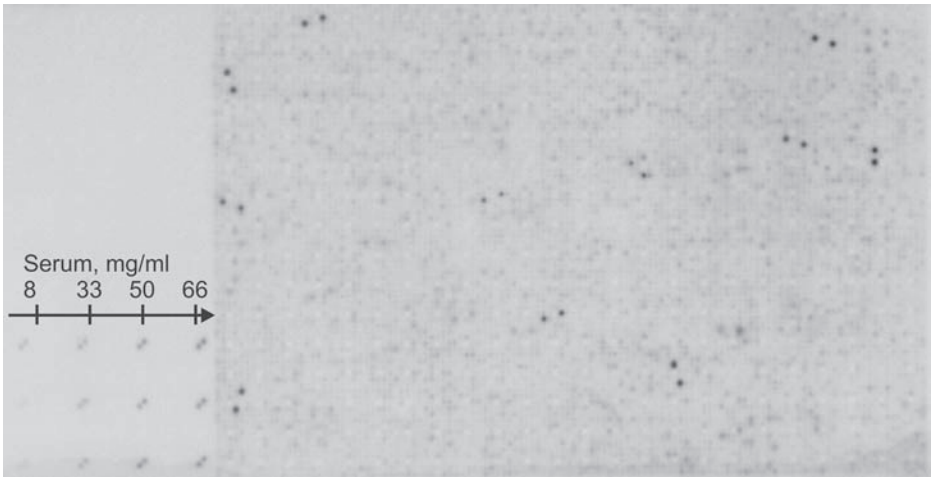
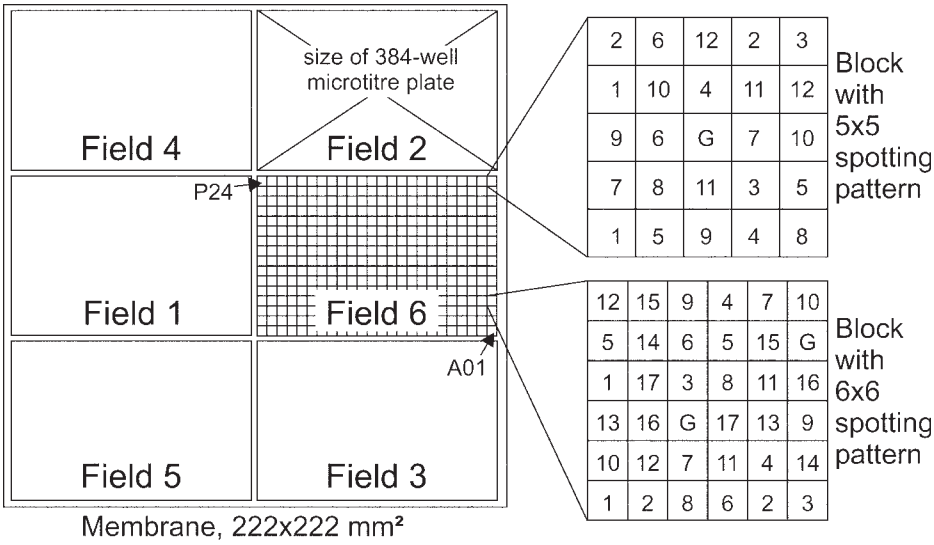


Fig. 3. Detection of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) on an array with proteins from the hEx1 library. The array was screened with a rabbit antibody against human GAPDH as described in **Subheading 3.3.** Eleven GAPDH cDNA clones were detected. The right part shows an array of the size of a standard 384-well microtiter plate. The 222 × 222 mm^2 filter format accommodates six such arrays. The array contains duplicates of proteins from 6528 clones that were spotted by the German Resource Centre in the "6 × 6 pattern" (**Fig. 4**). The left part shows the signals from a serial dilution of rabbit serum that was spotted to obtain guide dots (**Subheading 3.2.2.**) and to check if the intensity of strong GAPDH signals was limited by the secondary antibody.



the filters onto IPTG agar plates (prewarmed) to induce protein expression for 3 h at 37°C.

3.2.3. Release of Cellular Proteins on the Membrane

The standard protocol uses alkaline conditions to release cellular proteins on the filter. If denaturation of cellular proteins during the lysis step must be prevented, the protocol in **Subheading 3.2.4.** may be used.

Place a sheet of blotting paper in the lid of an agar plate dish and add denaturing solution. Pour off excess liquid and transfer the filter to the blotting paper with forceps. Incubate 10 min (*see* **Note 3**). Place the filter twice for 5 min on blotting paper soaked with neutralizing solution and finally on 2X SSC for 15 min. Place the filter on a dry blotting paper and allow to air-dry. Dry filters can be stored for several months at room temperature between sheets of blotting paper.

3.2.4. Nondenaturing Release of Cellular Proteins on the Membrane

Place the filter at 4°C on blotting paper soaked with lysis buffer; incubate for 1 h. Wash the filter in 1 L of TBS in a plastic box on a rocker. Do not let the filters dry out. The filters will deteriorate quickly; therefore, store them at 4°C, and use them no later than the next day.

3.3. Screening of the Array for Protein Antigens

This protocol uses AP-conjugated antibodies and the phosphatase substrate attophos for the detection of primary antibodies (*see* **Fig. 3**). Alternative detection systems may be used as well. Use forceps to handle the filters. Washing steps are performed by shaking the filters in a plastic box on a rocker submerged in a large volume, approx 0.5 L, of the respective buffer.

1. Soak dry protein filters in ethanol. Submerge filters in TBST-T in a plastic box, and wipe off bacterial debris with Kimwipes. Wash twice for 10 min in TBST-T, followed by two brief washes in TBS and a 10-min wash in TBS.

Fig. 4. (*Left*) Robotic spotting. A spotting pattern was developed to permit assignment of on a given signal on the array to the microplate and well number of the corresponding clone. Each clone is spotted twice onto the membrane in this pattern, namely, as a doublet at a certain location (*see* below). The robot uses a 384-pin gadget. The filter accommodates six fields of the size of the microtiter plate. The robot starts to spot bacteria from the first 384-well microplate into field 1 on two positions that are denoted with the number *1* within the 5 × 5 blocks (or, alternatively, within the 6 × 6 blocks). The next microplate is spotted into field 2 on exactly the same positions, and so on until plate 6. The seventh plate is spotted into field 1 on the position 2 within the blocks, and so forth. Each array contains 48 × 48 blocks. The 5 × 5 pattern consumes 12 × 6 microtiter plates (384-well) and spots 27,648 clones in duplicate. Position **G** denotes guide dots spotted with black ink. **A0** and **P24** denote microplate well positions. The left side on the top of the filter can be labeled with a unique number and the date of production.

2. Incubate the filters for 1 h in BB (3% nonfat dry milk powder in TBS). Dilute the primary antibody in BB. *See* **Note 5** for the required volume. A suitable concentration of the antibody has to be determined beforehand. A good starting point is a dilution that works well for enzyme-linked immunosorbent assay or Western blot experiments. A dilution of 1:5000 (v/v) might be suitable for an antiserum. Incubate 2 h or overnight with the diluted antibody (*see* **Note 6**).
3. Wash filters twice for 10 min in TBST-T, followed by two brief washes in TBS and a 10-min wash in TBS. Incubate with a suitable secondary antibody and conjugate with AP for 1 h. Wash three times for 10 min in TBST-T, once briefly in TBS and once in AP buffer.
4. Incubate in 0.25 m*M* attophos (*see* **Note 4**) in AP buffer for 5 min (*see* **Note 5**).
5. The fluorescent attophos dephosphorylation product can be detected on the filters by illumination with long wave ultraviolet light. Take a picture with a CCD camera or a suitable scanning device (*see* **Note 4**).
6. Continue with protocol in **Subheading 3.5.**

3.4. Screening of the Array for Protein–Protein Interaction

A recombinant protein covalently labeled with ^{32}P at a particular site is used here to probe the array for binding proteins. Such labeling avoids the problems associated with multisite labeling (iodination or biotinylation) or secondary detections (antibodies). The protein probe is a glutathione-*S*-transferase (GST) fusion in that the phosphorylation site of PKA is inserted between the GST and the protein part of interest. Vectors for the expression of affinity-tagged fusion proteins that contain a PKA site are commercially available (Novagen, Amersham Biosciences). The fusion protein has to be phosphorylated by PKA ***(9)*** and can then be used to decorate the filter (*see* **Notes 7–9** and **Fig. 5**).

3.4.1. Phosphate Incorporation into the Fusion Protein

1. Reconstitute 200 U PKA in 20 µL 40 m*M* DTT; leave at room temperature for 10–15 min.
2. Dilute approx 500 µg of the purified fusion protein in 160 µL 1X kinase buffer and add to the reconstituted PKA.
3. Start phosphorylation by adding 20 µL of the [γ-^{32}P]ATP.
4. After 1 h at 25°C apply the reaction mix (200 µL) to the gel filtration column, elute with equilibration buffer, and collect 10 fractions each of 200 µL. Monitor the Cerenkov counts in each fraction. Two peaks of radioactivity usually elute from the column and are well separated from each other. Only the first peak contains the phosphorylated fusion protein.

3.4.2. Blocking and Probing the Filter

1. Wet the dried protein filter with ethanol as described in **Subheading 3.3.**, and wash two times for 5 min each in TBST.
2. Block filter in BB in the cold room for 3–4 h on a rocker, and then equilibrate in HB for 15 min.
3. Dilute the radioactively labeled fusion protein in 20 mL HB, and add the blocked filter from **step 2** (*see* **Note 5**).
4. Incubate in the cold room as in **step 2** for at least 12 h to help detect slow-binding proteins.
5. Wash filter three times, each for 15 min and with 50 mL HB.

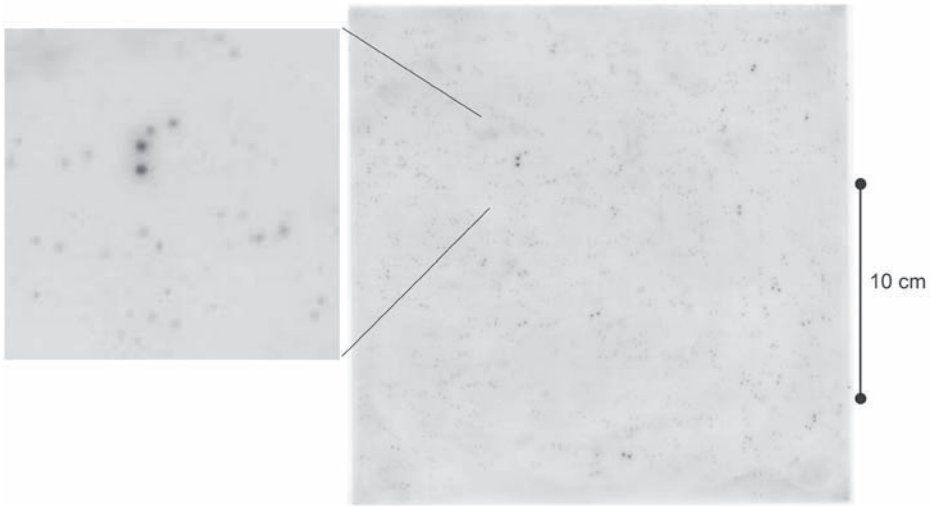


Fig. 5. Detection of endophilin-1 binders. This array contains proteins from 27,648 clones of a subset (**Subheading 3.6.**) of the hEx1 library that were spotted in doublets in a 5 × 5 pattern (**Fig. 4**). The array was decorated with a ^{32}P-labeled GST fusion protein of human endophilin-1 as described in **Subheading 3.4.** The magnified section shows the decorations in more detail.

6. Air-dry, cover with Saran wrap (*see* **Note 10**) and expose to a storage phosphor screen followed by scanning or autoradiography film.
7. Continue with protocol in **Subheading 3.5.**

3.5. Image Analysis and Clone Identification

The Xdigitise software is recommended for analysis of the array image ***(15)***. This software runs on UNIX or Linux computers and is available for free ***(16)***. Xdigitise can be used to score positive clones on the filter and to retrieve their microtiter plate position. As alternatives to Xdigitise, ImageJ or GIMP can be used. Both run on a Windows platform and are also available for free on the Internet. However, only *x* and *y* coordinates can be obtained with these programs. The position of the corresponding clone in the microtiter plates must be retrieved by other means. If the array was purchased from the German Resource Centre, enter the *x* and *y* coordinates of the detected doublet signal at their Web site to retrieve the corresponding clone. If the array was produced elsewhere, use Xdigitise to find the plate and well positions that correspond to a given signal. Identify clones by DNA sequencing and a Basic Local Alignment Search Tool search against the database of interest. In addition, retest important clones to confirm that the results are caused by the expected recombinant protein—via Laemmli gel fractionation, by binding studies with the recombinant protein immobilized on Western blottings, or by a solution-binding test.

3.6. Rearraying of Expression Clones

cDNA expression libraries usually contain many clones that do not produce a recombinant protein. Such clones are unwanted for the production of protein arrays and should, therefore, be detected and removed. In the library described here, all clones that express a hexahistidine-tagged fusion protein can be detected with the RGS-His antibody (Qiagen) according to the protocol in **Subheading 3.3.**, whereas the unproductive clones cannot. As shown in **Fig. 1**, about 20% of the library clones are detected. A list of the so-detected expression clones can be compiled with Xdigitise and can then be rearrayed to produce a subcollection of the library clones and, eventually, to produce an improved protein array. Colony-picking robots and many laboratory pipetting robots are capable of clone rearraying, also called *cherry picking*.

4. Notes

1. The handling and storage of microtiter plates containing bacterial cultures requires great care to avoid well-to-well contamination and to ensure cell viability *(7)*.
2. Microtiter plates should ideally be frozen quickly by laying them on dry ice in a single layer. However, freezing blocks of plates in a –80°C freezer is also acceptable. Microtiter plates stored in the freezer should be packaged well. The lids must not come off. Bacteria will only survive a limited number of freezing and thawing cycles. Therefore, a sufficient number of copies have to be stored frozen at any time.
3. If air bubbles get trapped underneath the filter, lift off and replace the filter from time to time.
4. The Fuji LAS-1000 video documentation system with a 470-nm top light works well with the attophos system.
5. Use a plastic container with a perfectly flat bottom, such as the lid of a large agar plate dish. A minimum volume of 15 mL is required to overlay the filters with reagent solution in such a container. Use a cover to prevent evaporation. Even smaller volumes of approx 2 mL can be used by either spraying the solution onto the semidry filter with an air brush device, or by the following technique: Place the semidry filter between two sheets of plastic. Lift the upper sheet, pipet the reagent solution onto one edge of the filter, and slowly lower the sheet onto the filter starting from the same edge.
6. In the present case (*see* **Fig. 3**), the specificity of antigen detection was increased by reducing the concentration of the primary antibody and incubation with this antibody overnight.
7. To confirm that the identified clones were detected as a result of binding to the protein of interest, but not to the GST part of the fusion protein, one should carry out control experiments with GST fused to an unrelated protein or with GST alone.
8. To reduce background and nonspecific signals, the stringency of the screen can be changed by varying incubation times during individual steps, the concentrations of salt or detergents, and the number of washing steps. Note that this filter-binding assay only detects protein–protein interactions. No information will be obtained about binding strength. So a strong signal does not necessarily mean strong binding, and likewise, a weak signal does not correspond to a weak interaction.
9. It is not uncommon to detect many protein–protein interactions on such an array. As shown in **Fig. 5**, at least 250 endophilin-1 binders can be scored. This is not surprising, because the arrayed proteins are redundant and because endophilin-1 is known to bind to

itself and to many other proteins. However, many of the so-detected protein–protein interactions may not be physiologically relevant. Therefore, any protein–protein interaction of interest must be confirmed by an independent technique such as a solution-binding assay.

10. To avoid wrinkling the Saran wrap, lay the filter on a thin, square piece of plastic, 24 × 24 cm^2, and pull the Saran wrap over the filter as flat as possible and without trapping air.

Acknowledgments

Several people at the Max Planck Institute of Molecular Genetics took part in the development of arrayed expression libraries, notably Gerald Walter, Wilfried Nietfeld, Dolores Cahill, and Hans Lehrach. The authors thank Timothy Lee Kam Yiu (National University of Singapore) for his contribution to the nondenaturing filter processing protocol.

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2

Protein Expression Arrays for Proteomics

Michele Gilbert, Todd C. Edwards, and Joanna S. Albala

Summary

As biology approaches the 50th year of deciphering the DNA code, the next frontier toward understanding cell function has protein biochemistry in the form of structural and functional proteomics. To accomplish the needs of proteomics, novel strategies must be devised to examine the gene products or proteins, emerged as *en masse*. The authors have developed a high-throughput system for the expression and purification of eukaryotic proteins to provide the resources for structural studies and protein functional analysis. The long-term objective is to overexpress and purify thousands of proteins encoded by the human genome. This library of proteins—the human proteome—can be arrayed in addressable format in quantities and purities suitable for high-throughput studies. Critical technology involved in efficiently moving from genome to proteome includes parallel sample handling, robust expression, and rapid purification procedures. Automation of these processes is essential for the production of thousands of recombinant proteins and the reduction of human error.

Key Words:

Protein array; baculovirus; insect cell; protein expression; purification; automation; robotics.

1. Introduction

1.1. Overview: Array-Based Proteomics

The key advantage to array-based methods for protein study is the parallel analysis of samples in a high-throughput fashion. Similar to the DNA microarray, this approach requires miniaturization technologies, high sample throughput, and automation. Array-based methods for protein analysis afford a high-throughput format by which to screen protein–protein, protein–DNA, and protein–small molecule interactions and provides important functional information for newly identified genes that are derived from genome projects. Protein arrays hold the potential to identify these interactions as well as provide a means for differential expression and protein profiling between different cell types.

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1.2. Generation of Protein Arrays

Proteins, peptides, and antibodies have been analyzed using a microarray format, and protein arrays have been produced using various media and a diversity of immobilization chemistries on surfaces such as nitrocellulose, polyvinylidene fluoride, silicone, glass, and plastic (for review, *see* **refs. *1–5***). Use of a standard glass microscope slide to bind proteins or antibodies provides a cheap, easily manipulated format that is amenable to many chemical modifications, as surface chemistry is critical when preparing protein arrays. Proteins, peptides, or antibodies can be applied to the array surface by ink-jet or contact printing in a similar manner to those used in spotting a DNA array ***(6)***. Generally, most analyses use fluorescent or radiolabeled targets for capture by proteins bound to the array, enzymatic or colorimetric analysis for functional assay, and mass spectrometry or surface plasmon resonance for detection.

1.3. Protein Production for Generation of Protein Arrays

The earliest bottleneck to the generation of protein arrays is obtaining large numbers of soluble, purified, functional proteins for direct application onto the array or for the generation of antibodies. Recombinant expression in *Escherichia coli* has become the standard because of robust production, low cost, and ease of use. Several laboratories to date have successfully produced and purified large numbers of proteins using high-throughput strategies in *E. coli* either by recombinant or in vitro means ***(7–10)***. To overcome many of the limitations arising from prokaryotic expression, such as formation of inclusion bodies, misfolding of proteins, and lack of posttranslational modifications, several eukaryotic systems have been developed using either yeast, insect, or mammalian cells for host expression. Dual-use methods for recombinant expression of prokaryotic and eukaryotic systems have also been devised as well as cell-free systems to expand recombinant protein production capabilities ***(11)***.

Automation is key to providing the throughput needed for proteomic studies involving hundreds to thousands of proteins. Many protein production methodologies lend themselves to robotic manipulation because of the repetitive nature of the procedures, such as plasmid isolation, polymerase chain reaction (PCR), DNA quantitation, cell culture, and affinity purification. The authors have developed an automatable system for high-throughput protein production in baculovirus ***(12,13)***. Using complementary DNA (cDNA) clones from the LLNL-I.M.A.G.E. collection ***(14)***, they can produce recombinant protein in a miniaturized, high-throughput format to derive large numbers of recombinant proteins for downstream functional applications, such as protein microarrays, antibody production, or pathway reconstitution (**ref. *15***; *see* **Note 1**).

2. Materials

2.1. PCR Production of cDNA Clone Inserts

1. *E. coli* from LLNL-IMAGE cDNA Collection.
2. 96-well round-bottom plates.
3. Luria Bertani (LB) broth/ampicillin/glycerol medium.
4. Cloned *Pfu* polymerase (Stratagene).
5. *Asc*I and *Fse*I enzymes (New England Biolabs).

6. 10X PCR buffer.
7. Deoxynucleotide-triphosphates (dNTPs).
8. QIAquick 96-well PCR purification kit (Qiagen).

2.2. Transfer Vector Design and Ligation of cDNA Inserts

1. pBacPAK9 (Clontech).
2. Shrimp alkaline phosphatase (SAP) (Fermentas).
3. One Shot TOP10 chemically competent *E. coli* (Invitrogen).
4. LB/ampicillin/agar 100-mm plates.
5. Wizard miniprep kit (Promega).
6. LB/ampicillin.

2.3. Transfection and Viral Amplification

1. *Sf9* insect cells.
2. Superfect transfectant (Qiagen).
3. IPL-41 insect cell media.
4. Linearized baculoviral DNA (Baculogold, Pharmingen).
5. SF900II insect cell media (Invitrogen).
6. Fetal bovine serum (FBS).

2.4. Deep-Well Viral Amplification and Protein Expression

1. 96-deep-well plate (Marsh Bioproducts).
2. 2.38-mm stainless steel beads (V& P Scientific).
3. 1% Pluronic F68.
4. Gas-permeable seal (Marsh Bioproducts).
5. Carousel Levitation Magnetic Stirrer (V& P Scienctific).
6. Sorvall RT-6000 centrifuge.

2.5. Protein Purification and Analysis

1. Lysis buffer: 20 m*M* Tris-HCl pH 8.0, 1 m*M* ethylene glycol *bis* (2-aminoethyl ether)-*N*,*N*,*N*'*N*'-tetraacetic acid (EGTA), 1 m*M* $MgCl_2$, 0.5% v/v *N*-octoglucoside.
2. Microplate mixer MT-360 (TOMY).
3. Sodium chloride.
4. Immunoaffinity beads.
5. Wash buffer: 20 m*M* Tris-HCl pH 8.0, 1 m*M* EGTA, 1 m*M* $MgCl_2$, 100 m*M* NaCl.
6. Elution buffer: wash buffer plus 5 μg/mL peptide.
7. 96-well filter plate (Whatman, 0.45 μ*M* cellulose acetate filter).
8. Vacuum manifold (Whatman).
9. ECL Plus kit (Amersham).
10. 10% Tris-HCl denaturing gels (Novex).
11. Coomassie blue dye.

3. Methods

The methods developed for miniaturized protein production in baculovirus are described in the following sections. The steps are (a) PCR production of cDNA clone inserts, (b) transfer vector design and ligation of cDNA inserts, (c) transfection and viral amplification, (d) deep-well viral amplification and protein expression, and (e) protein purification and analysis (*see* **Note 2**).

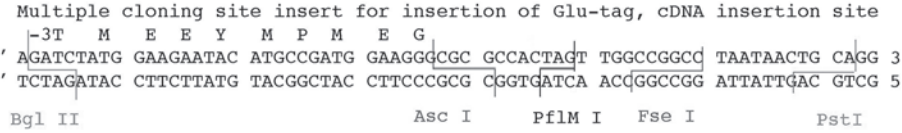

Fig. 1. The multiple-cloning site of pMGGlu, which contains the *Asc*I and *Fse*I cloning sites and the MEEYMPMEG (Glu) epitope tag.

3.1. PCR Production of cDNA Clone Inserts

The upstream molecular biology of the baculovirus-based system relies on many of the same techniques that have been applied for production of recombinant proteins in *E. coli.* These methods can also be used to subclone the genes of interest into an appropriate transfer vector for recombination with the baculovirus genome. The authors' scheme for amplification of cDNA clones begins by the generation of 5' gene-specific primers that are paired with a 3' vector-specific primer. The 5' gene-specific PCR primer is designed to contain the rare cutter *Asc*I site, and the 3' vector-specific primer contains a rare cutter *Fse*I site (*see* **Fig. 1**).

1. Aliquot 5 µL *E. coli* containing the cloned genes of interest into 96-well round-bottom plates containing 95 µL LB/ampicillin/glycerol medium and grow overnight at 37°C.
2. Perform PCR directly on a 1:100 dilution of the bacterial cultures using *Pfu* polymerase. The PCR conditions are 96°C for 3 min, 35 cycles of 96°C for 30s, 50°C for 30 s, then 6 min at 72°C, and they have been tested on genes ranging in size from 386 bp to 2409 bp. This cycle is followed by a final extension at 72°C for 10 min.
3. The PCR reaction includes the following: 10X PCR buffer diluted to a final concentration of 1X, dNTPs (25 m*M* each), 0.5 µ*M* final concentration of 5' primer and 3' primer, a 1:100 final dilution of *E. coli* in ddH_2O, and 5 U cloned *Pfu* polymerase in a final reaction volume of 50 µL.
4. Purify the PCR products using a Qiagen 96-well format (QIAquick 96 PCR purification kit) and elute into 50 µL ddH_2O.
5. Enzymatically digest the resulting PCR products with *Asc*I and *Fse*I, and purify the digested samples with the QIAquick 96 PCR purification kit.

3.2. Transfer Vector Design and Ligation of cDNA Inserts

For the creation of recombinant baculoviruses, a modified transfer vector was designed based on the pBacPAK9 transfer vector from Clontech (*see* **Fig. 2**). A "Glu" immunoaffinity tag ***(16)*** followed by exonuclease sites for the rare cutters *Asc*I, *PflM*I, and *Fse*I were added between the *Bgl*II and *Pst*I site of the multiple-cloning site of the pBacPAK9 transfer vector to generate the modified transfer vector called pMGGlu.

1. Linearize the pMGGlu vector with *Asc*I and *Fse*I.
2. Dephosphorylate the vector with SAP in preparation for inserting the clones of interest.
3. Ligate each of the clones into the cut and dephosphorylated pMGGlu vector in 96-well format.

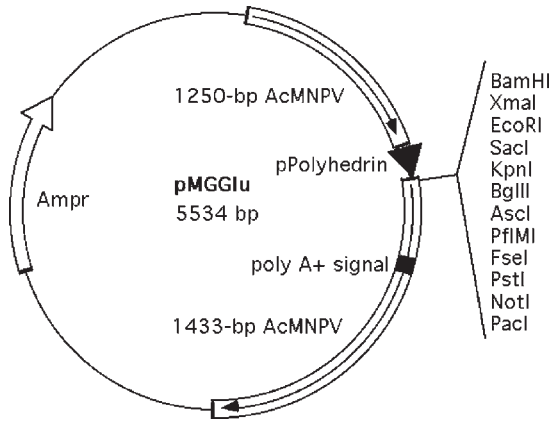

Fig. 2. Schematic diagram of pMGGlu, which is derived from pBAKPAK9 from Clonetech (which contains the Glu Immunoaffinity site followed by the rare cutter sites AscI and FseI for cloning).

4. Inactivate the reaction by heating at 65°C for 10 min.
5. Transform the ligation reactions into TOP10 cells from a One Shot kit, and then plate each transformation onto LB/ampicillin/agar (*see* **Note 3**).
6. Isolate two *E. coli* colonies for each cDNA clone and grow overnight in 3 mL LB/ampicillin.
7. Isolate plasmid DNA using the Wizard miniprep kit (*see* **Note 4**).
8. Screen the plasmid DNA by enzymatic digestion with *Asc*I and *Fse*I followed by agarose gel electrophoresis to determine if the correct size insert for the PCR gene product of interest is contained within the pMGGlu transfer vector.

3.3. Transfection and Viral Amplification

Once the genes of interest are inserted into the baculoviral transfer vector, pMGGlu, the vectors containing the cloned cDNAs are transfected into *Sf9* insect cells along with linearized baculoviral DNA. The cDNA is transferred from the transfer vector to the baculoviral genome by homologous recombination using the cellular machinery of the host insect cell.

1. Place *Sf9* insect cells into a 96-well flat-bottomed tissue-culture plate at 0.5×10^5 cells/well, and allow the cells to adhere for at least 30 min in a humidified 27°C-incubator.
2. Prepare a 1:50 dilution of SuperFect transfectant in IPL-41 media, and allow the solution to interact for a minimum of 10 min for micelle formation to facilitate transfection.
3. After 10 min, combine 5–10 ng of recombinant transfer vector and 5–10 ng of linearized baculoviral DNA per well, and incubate with the SuperFect solution at a final dilution of 1:100 in IPL-41 media (34 μL transfection cocktail per well) for at least 10 min.
4. Aspirate the media off the cells, and add the transfection cocktail (linearized baculoviral DNA, recombinant transfer vector, and Superfect) to the adherent cells.

5. Allow the cells to transfect for 2–3 h in a humidified 27°C chamber, and then add 70 µL of SF900II media containing 10% FBS to each well.
6. Incubate the cells for 4 d in a humidified 27°C chamber for viral cultivation.
7. After 4 d, plate fresh *Sf9* insect cells onto a new 96-well tissue-culture plate at a density of 2×10^4 cells/well in 70 µL of SF900II media, and allow the cells to adhere for 30 min.
8. After the cells adhere, add 30 µL of supernatant (containing the recombinant baculoviral particles that had been successfully created from the original transfection plate) to each well of newly plated cells.
9. Continue viral amplification for 4 d.
10. Repeat amplification **steps 7–9** in 96-well format two to four more times.

3.4. Deep-Well Viral Amplification and Protein Expression

The final round of viral amplification is performed in a 96-deep-well plate (2 mL) to generate a larger volume of virus for protein production. A Carousel Levitation Magnetic Stirrer is used to culture up to 12 96-deep-well plates at once, for a total of 1152 clones to be produced simultaneously.

1. Add a 2.38-mm steel ball to each well in the 96-deep-well plate, and then add 1.5 mL of *Sf9* insect cells at a density of 1.5×10^6 cells/mL in SF900II media containing1% Pluronic F68 to each well.
2. Add virus at 5–10% v/v to the cells and cover the 96-deep-well plate with a gas-permeable seal.
3. Incubate the cells for 4 d on a carousel stirrer at a speed setting of 50 at 27°C.
4. Harvest the cells by centrifugation at 3000*g* on a Sorvall RT-6000.
5. Retain the supernatant containing the recombinant virus and discard the cell pellet.
6. For protein production, repeat **steps 1–3**, but only incubate the cells for 48 h rather than 4 d.
7. Harvest the cells by centrifugation at 3000*g* on a Sorvall RT-6000.
8. Aspirate the supernatant and freeze the cell pellet overnight at –80°C.

3.5. Protein Purification and Analysis

Protein purification from insect cells proceeds in a similar fashion to that of other cell types. Various affinity chromatographic techniques are available for protein purification. This method employs immunoaffinity chromatography by use of an antibody conjugated to a Sepharose matrix. The antibody was generated against the Glu peptide epitope tag ***(16)***.

1. Thaw the frozen cell pellets and add 0.5 mL lysis buffer to each well of the 96-deep-well plate, leaving the stainless steel balls in the wells to aid in mechanical lysis.
2. Shake the plate on a Microplate mixer MT-360 (TOMY) for 10 min at room temperature to resuspend and lyse the cells.
3. Add NaCl to each sample to a final concentration of 100 m*M*, and shake the plate for an additional 5 min.
4. Centrifuge the lysate at 3000*g* for 20 min.
5. Place 100 µL of the immunoaffinity column matrix in a 96-deep-well plate.
6. To equilibrate the matrix, wash two times by adding 500 µL wash buffer, gently agitate, and centrifuge at 1000*g* for 10 min.
7. Transfer the supernatants containing the soluble protein onto the immunoaffinity matrix, and save the insoluble cell pellets for future examination.

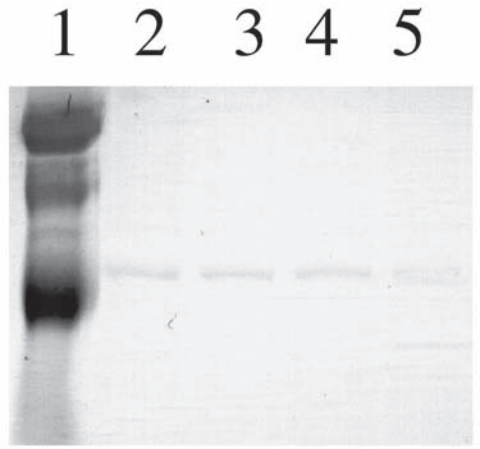

Fig. 3. Coomassie blue staining of purified Gus separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). *Lane 1*: Kaleidoscope markers (Bio-Rad). *Lanes 2–5*: 30 μL of purified Gus from four individual clones. (*See* **Note 2**.)

8. Bind proteins to the matrix for 10 min with gentle agitation by pipet.
9. Centrifuge the matrix at 1000*g* for 10 min, and carefully remove the supernatant.
10. Wash the matrix two times by adding 500 μL wash buffer, gently agitate, and centrifuge at 1000*g* for 10 min.
11. After discarding the supernatant, centrifuge at 1200 *g* for 5 min.
12. Discard any remaining supernatant.
13. Resuspend the matrix in 100 μL elution buffer and transfer to a 96-well filter plate (Whatman, 0.45 μ*M* cellulose acetate filter).
14. Allow the elution buffer to interact with the beads for 5 min.
15. Apply light vacuum to collect the supernatant containing the eluted protein in a fresh 96-well collection plate.
16. Analyze the soluble and insoluble protein fractions by gel electrophoresis and Western blot analysis.
17. Detect protein with an enhanced chemiluminescence (ECL) Plus kit.
18. Estimate protein purity by gel electrophoresis followed by Coomassie blue staining.

4. Notes

1. Because the procedures are performed in a 96-well format, many of the processes described can be automated using standard liquid-handling robots. A robust database is critical to track each cDNA clone through the many processes to produce a purified protein. Future iterations of the protocols will be implemented as modules for (a) PCR production of cDNA clone inserts; (b) ligation of cDNA inserts; (c) transfection and viral amplification; (d) viral amplification and protein expression; and (e) protein purification and analysis on these robots with Web-based graphic interface to access the database.
2. Throughout production, the gene for β-glucoronidase was used as a control. The efficacy of transfection, infection, and protein production can be measured by examining the abil-

ity of this enzyme to break down its substrate X-Glucuronide, which results in a blue-colored product that can be quantified by spectrophotometric analysis at 630 nm. An example of this purified protein is shown in **Fig. 3**.

3. Originally, the authors anticipated that the ligation reaction could be directly transfected into the insect cells along with linearized baculoviral DNA, to avoid the *E. coli* transformation step. However, it was determined that the low probability of ligation (approx 100 clones per transformation) resulted in decreased transfection efficiency. Therefore, the subcloning into *E. coli* was necessary to increase the probability of a productive homologous recombination event.
4. Although the transformation and DNA isolation were performed offline and not in 96-well format, kits and plates do exist to perform these steps in an automatable, 96-well format (Promega, Qiagen).

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3

Generation of Protein *In Situ* Arrays by DiscernArray™ Technology

Mingyue He

Summary

A cell-free DiscernArray™ technology is described for making protein arrays directly from polymerase chain reaction deoxyribonucleic acid (PCR DNA) fragments. In this method, individual tagged proteins are synthesized in a cell-free system on a tag-binding surface, such that the tagged proteins are immobilized on the surface as they are produced. This technology is particularly useful for arraying proteins/domains that cannot be functionally produced by heterologous expression or for which the cloned DNA is not available. Protein arrays of single-chain antibodies, ligand-binding domains, and enzymes have been successfully generated by this method.

Key Words:

Protein arrays; cell-free protein synthesis.

1. Introduction

Protein arrays are powerful tools for studying protein function/complexity in a global manner. Arrays are produced by immobilizing many hundreds of individual proteins in a defined pattern onto a solid surface ***(1)***. In the array format, large numbers of proteins are analyzed simultaneously in parallel, providing valuable information on function, interaction, and expression levels of proteins ***(2)***. Currently, the main limitation to protein array technology is the production of a huge diversity of proteins that form the array elements. Many proteins, especially human proteins, are not expressed as functional molecules in heterologous hosts ***(3)***, and cloning of individual genes is also a time-consuming process. To overcome these problems, scientists in the author's laboratory developed a cell-free method, termed DiscernArray™, which creates functional protein arrays directly from PCR DNA by in vitro synthesis of individual tagged proteins on tag-binding surfaces, such that the tagged proteins are immobilized *in situ* as they are synthesized (*see* **Fig. 1**).

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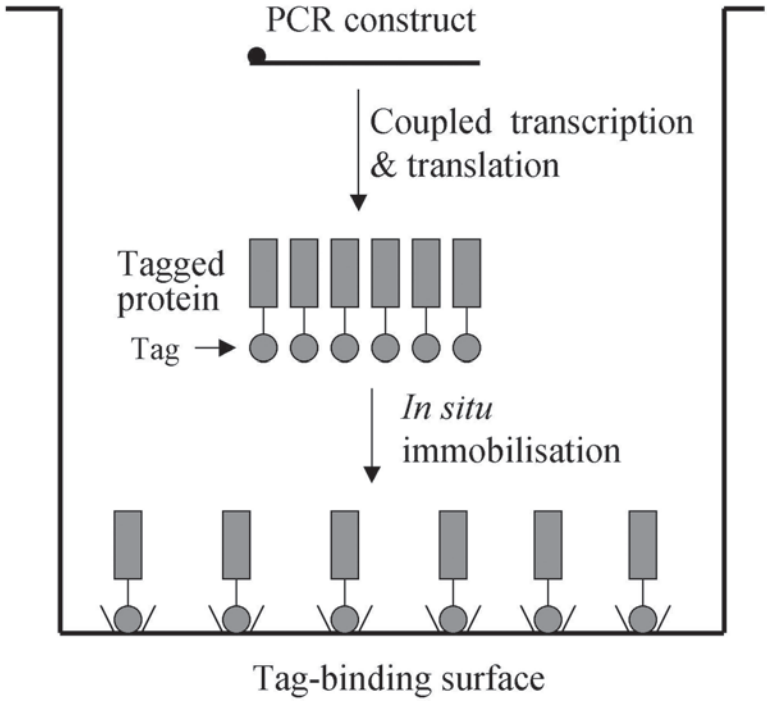

Fig. 1. DiscernArray™ procedure showing cell-free synthesis of tagged protein on the tag-binding surface.

DiscernArray™ avoids cloning and *Escherichia coli* expression processes, providing a rapid route for arraying proteins or domains for which DNA clones are not available. It is also particularly useful for proteins that cannot be functionally produced in heterologous hosts. With the recent improvements in cell-free expression systems ***(4,5)*** and with sensitive detection or readout technologies, this method has the potential to be adapted for high-throughput application and automation. This technology has been used to generate arrays of different proteins and protein fragments and have demonstrated their use for rapid functional analysis ***(6)***. Details of this method for general applicability are described here.

2. Materials

2.1. Primers

1. T7: 5'-GCAGC*TAATACGACTCACTATAG*GAACAGACCACC**ATG**-3'—an upstream primer containing T7 promoter (italics) and Kozak sequence (underlined) for translation in eukaryotic cell-free systems. The start codon ATG is indicated in bold.
2. G/back: 5'-TAGGAACAGACCACCATG$(N)_{15–25}$-3'—an upstream primer designed for amplification of the gene of interest. It contains a sequence overlapping with T7 (underlined) and 15–25 nucleotides from the 5' sequence of the gene of interest. $(N)_{15–25}$ indicates the number of nucleotides.

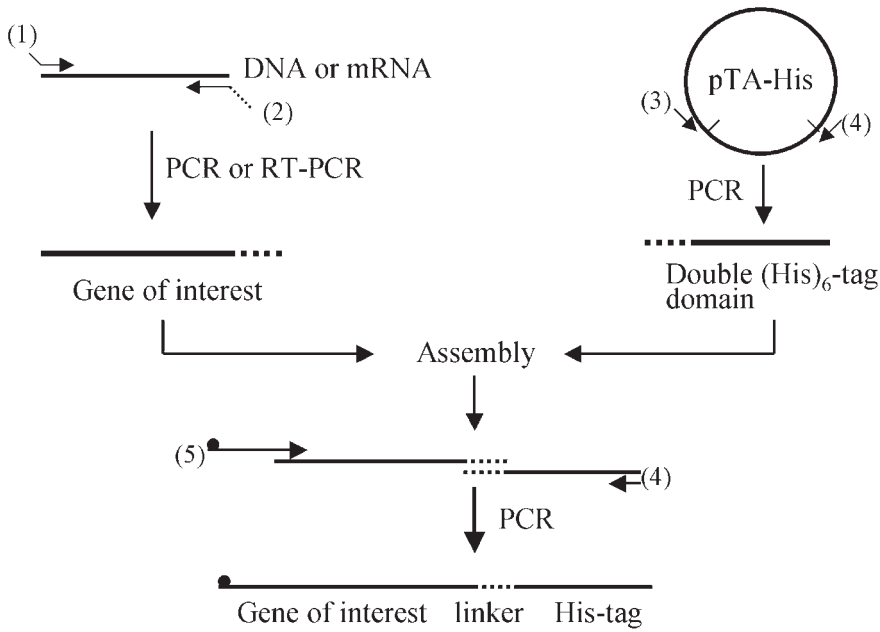

Fig. 2. A PCR strategy for DNA construction. The primers are: **(1)** G/back, **(2)** G/for, **(3)** Linker-tag/back, **(4)** T-term/for, **(5)** T7. Broken lines indicate the linker sequence.

3. G/for: 5'-CACCGCCTCTAGAGCG$(N)_{15–25}$-3'—a downstream primer designed for amplification of the gene of interest. It contains a sequence overlapping with a tag domain (underlined) and 15–25 nucleotides complementary to the 3' region of the gene of interest. In this chapter, a double $(His)_6$ tag domain is described (*see* **Subheading 2.2.**).
4. Linker tag/back: 5'-GCTCTAGAGGCGGTGGC-3'—an upstream primer for PCR generation of the double $(His)_6$ domain in combination with T-term/for (*see* **Subheading 2.2.**).
5. T-term/for: 5'-TCCGGATATAGTTCCTCC-3'—a downstream primer for PCR generation of either the double $(His)_6$ tag domain in combination with the linker tag/back or the full-length construct in combination with T7 (*see* **Fig. 2**).

2.2. Plasmid pTA-His Encoding a Double $(His)_6$-Tag Domain

Plasmid pTA-His contains a DNA fragment encoding (in order) a flexible linker and a double $(His)_6$ tag, followed by two stop codons, a poly (A) tail, and a transcription termination region ***(6)***. The DNA sequence is GCTCTAGAggcggtggctctggtg gcggttctggcggtggcaccggtggcggttctggcggtggcAAACGGGCTGATGC TGCACATCACCATCACCATCACTCTAGAGCTTGGCGTCACCCG CAGTTCGGTGGTCACCACCACCACCACCAC**TAATAA**$(A)_{28}$CCGCTGAGCAA T*AACTAGCATAACCCCTTGGGGCCTCTAAACGGGTCTTGAGGGGTTTTT TG*CTGAAAGGAGGAACTATATCCGGA-3'. The lower case indicates the linker encoding 19 amino acids ***(7)***; the double $(His)_6$ tag is underlined. Stop codons are in bold and $(A)_{28}$ is a poly (A) tail comprising 28 × A.The transcription termination region is shown in italics.

2.3. Cell-Free System and Molecular Biology Reagents and Kits

1. TNT T7 Quick for PCR DNA (Promega, UK).
2. Nucleotides (Sigma, UK).
3. Agarose (Sigma, UK).
4. *Taq* DNA polymerase (Qiagen, UK).
5. Gel elution kit, QIAEX II (Qiagen, UK).
6. Ni-NTA-coated HisSorb strip/plates (Qiagen, UK).
7. Ni-NTA-coated magnetic agarose beads (Qiagen, UK).
8. Titan™ one-tube reverse transcriptase PCR (RT-PCR) system (Roche Molecular Biochemicals, UK).

2.4. Solutions

1. Superblock (Pierce, UK).
2. Wash buffer: 50 m*M* NaH_2PO_4, 300 m*M* NaCl, 20 m*M* imidazole pH 8.0.
3. Stripping buffer: 1 *M* $(NH_4)_2SO_4$, 1 *M* urea.
4. Phosphate-buffered saline (PBS): pH 7.4

3. Methods

3.1. PCR Construction

DiscernArray™ uses PCR fragment as the template for protein expression in a cell-free system. The construct must have a T7 promoter, protein initiation site, and termination region of transcription and translation. A tag sequence is included at the C-terminus for protein immobilization (*see* **Fig. 3** and **Note 1**). In this chapter, a novel double His tag is described ***(6)***. To reduce any possible interference of the tag sequence on the folding of the attached protein, a flexible linker is placed between the protein to be arrayed and the tag sequence (*see* **Fig. 3**). A poly (A) tail is also added after the stop codon for promoting protein expression. To facilitate the PCR construction, a DNA fragment can be generated to encode the common elements, such as the flexible linker, the tag sequence, poly (A), and termination regions of transcription and translation for assembly with the gene of interest (*see* **Figs. 2** and **3** and **Subheading 2.2.**; *see* **Note 2**).

1. Generate target DNA by PCR or RT-PCR (if messenger RNA is used as template) using the primers G/back and G/for (*see* **Fig. 2** and **Subheading 2.1.**)
2. Generate the double His-tag fragment by PCR using the template plamsid pTA-His and primers linker tag/back and T-term/for (*see* **Fig. 2** and **Subheading 2.1.**)
3. Analyze the resultant PCR products by agarose gel, and elute the fragments using QIAEX II (Qiagen).
4. Assemble the target DNA with the double His-tag fragment by overlapping PCR: Mix the two fragments in equimolar ratios (total DNA 50–100 ng) into a PCR solution containing 2.5 μL 10X PCR buffer, 1 μL deoxynucleotide-triphosphates containing 2.5 m*M* of each, 1 U *Taq* DNA polymerase, and H_2O to a final volume of 25 μL. Place the mixture in a thermal cycler for eight cycles (94°C for 30 s, 54°C for 1 min, and 72°C for 1 min) to assemble the two fragments. Then amplify the assembled product by transferring 2 μL to a second PCR mixture in a final volume of 50 μL for 30 further cycles (94°C for 30 s, 54°C for 1 min, and 72°C for 1 min) using primers T7 and T-term/for (*see* **Subheading 2.1.**)

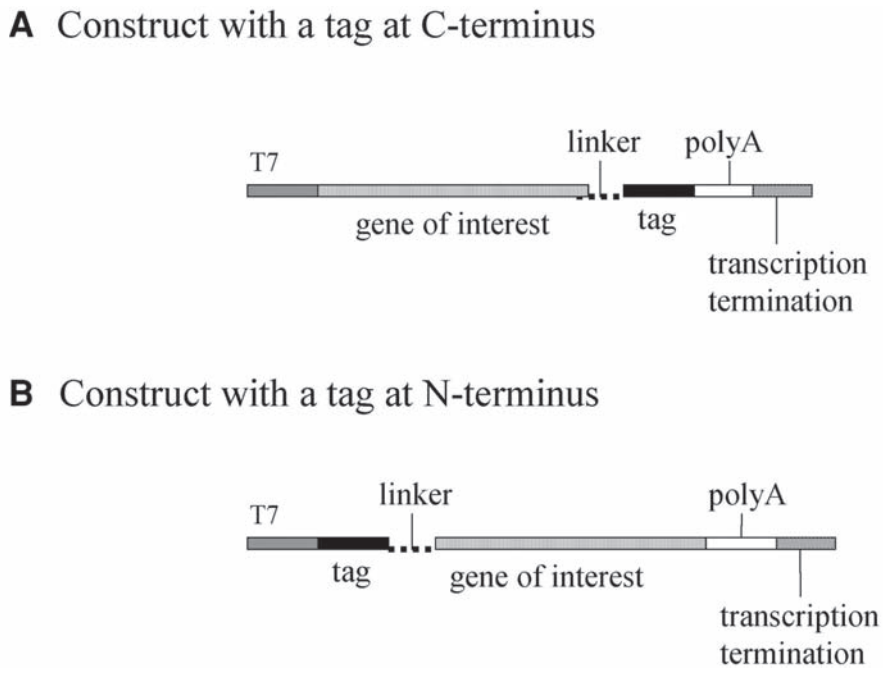

Fig. 3. PCR constructs for DiscernArray™. **(A)** Construct with a tag at C-terminus; **(B)** construct with a tag at N-terminus. T7, T7 promoter; linker, peptide linker.

5. Analyze the PCR product by agarose (1%) gel electrophoresis.
6. Confirm the identity of the construct by PCR mapping using primers at various positions (*see* **Note 3**).

3.2. Generation of Protein In Situ *Array*

The PCR construct generated above is used as the template for the generation of protein *in situ* array by simultaneous cell-free expression and immobilization of the synthesized protein through a tag onto the tag-binding surface. In this chapter, rabbit reticulocyte lysate system is used (*see* **Note 4**) to produce the His-tagged protein on two Ni-coated surfaces, namely Ni-NTA-coated microtiter plates and Ni-NTA magnetic agarose beads (*see* **Note 5**).

1. Set up TNT translation mixture as follows (25 μL; *see* **Note 6**): 20 μL TNT T7 Quick for PCR DNA; 0.5 μL 1 m*M* methionine; 0.25 μL 100 m*M* magnesium acetate (*see* **Note 7**); 0.25–0.5 μg PCR DNA; H_2O to 25 μL.
2. Add the TNT mixture to either of following surfaces: (a) Ni-NTA-coated HisSorb strips or plates and (b) 5–10 μL Ni-NTA-coated magnetic beads. Incubate the mixture at 30°C for 2 h with gentle shaking.
3. Remove the mixture and wash three times with 100 μL wash buffer (*see* **Note 8**), followed by a final wash with 100 μL PBS, pH 7.4. The array can be used directly for functional analysis (*see* **Subheading 3.3.**) or stored at 4°C (*see* **Note 9**).

3.3. Functional Analysis of Arrayed Proteins

The array can be used for detection of interaction, ligand-binding, or enzyme activity *(6)*.The method used will depend on the activity of arrayed proteins to be tested. This step may take less than 30 min or more than a few hours.

3.4. Re-use of Arrays After Exposure to Detection Reagents

1. Wash the array wells or beads three times with 100 µL PBS containing 0.05% Tween-20.
2. Incubate with 50 µL freshly prepared stripping buffer at room temperature for 2 h.
3. Wash three times with 100 µL PBS containing 0.05% Tween-20, followed by a final wash with PBS, pH 7.4. The arrays are ready for re-exposure to detection reagents.

4. Notes

1. Alternatively, a tag sequence can be placed at N-terminus for protein immobilization (*see* **Fig. 3**), especially when the C-terminus tag is not accessible *(8)* or it affects protein function.
2. A simpler approach to array construction is to generate a plasmid DNA fragment encoding the common elements, such as the tag sequence, linker, poly $(A)_n$, and termination region of transcription and translation. This DNA fragment can be assembled with the gene of interest through overlapping PCR (*see* **Fig. 2**).
3. PCR mapping is carried out by using a combination of primers that anneal at different positions in the construct. The construct may be assumed to be correct if all the PCR products are of the expected size.
4. Apart from the TNT rabbit reticulocyte lysate system described here, other systems such as wheat germ and *E. coli* S30 extract can also be used.
5. The use of Ni-coated beads to capture His-tagged proteins offers advantages over Ni-coated microtiter wells in that the immobilized protein can be analyzed in different tubes as well as by using different amounts.
6. The volume of TNT mixture used for cell-free expression can be scaled up 100 µL without significant reduction in protein expression.
7. Magnesium acetate concentration added to TNT mixture during translation improves protein expression. It has shown that single-chain antibodies can be produced more efficiently with the addition of magnesium concentrations ranging from 0.5 to 2 m*M*.
8. TNT lysate contains large amounts of hemoglobin that sometimes stick to Ni-coated magnetic beads. More washes may be required to remove hemoglobin from the beads.
9. The arrays can be stored in 50 µL PBS at 4°C for 2 wk.

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4

Multiplexed Protein Analysis Using Spotted Antibody Microarrays

Brian B. Haab and Heping Zhou

Summary

This chapter describes methods for the production and use of antibody microarrays. The methods are divided into (a) antibody handling and microarray production, (b) sample preparation, and (c) microarray use. Two types of detection methods are described: direct labeling and a fluorescence-linked immunosorbent assay (FLISA). In the direct labeling method, all proteins in a complex mixture are labeled with either a fluorophore or a hapten that allows subsequent detection. In FLISA detection, a capture antibody on the microarray captures the unlabeled protein target, which is detected by a detection antibody and a fluorophore-labeled secondary antibody. Each method has particular optimal uses, which are discussed in the text.

Key Words:

Antibody microarray; serum profiling; multiplexed immunoassays.

1. Introduction

Antibody microarrays continue to be developed as a useful tool for multiplexed protein analysis. The benefits of the technology include highly parallel protein measurements, rapid experiments and analysis, quantitative and sensitive detection, and low-volume assays. Recent publications have demonstrated the application of the technology to the study of proteins from serum ***(1,2)***, cell culture ***(3)***, tissue ***(4)***, and culture media ***(1,5)***. To facilitate the broad dissemination and more routine use of antibody microarray methods, this chapter describes practical and validated techniques that can be implemented by most laboratories. All aspects of the experimental process are described, including antibody handling, sample handling, and microarray production and use (*see* **Note 1**). Information on data analysis is not presented here but can be found in the previously referenced citations.

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2. Materials

1. Robotic microarrayer (several commercial models available).
2. Microarray scanner (several commercial models available).
3. Clinical centrifuge with flat swinging buckets for holding slide racks (Beckman Coulter, among others).
4. HydroGel-coated glass microscope slides (PerkinElmer Life Sciences).
5. *N*-hydroxysuccinimide (NHS)-linked Cy3 and Cy5 protein-labeling reagents (Amersham, PA23001 and PA25001).
6. Microscope slide-staining chambers with slide racks (Shandon Lipshaw, 121).
7. Polypropylene 384-well microtiter plates (Genetix or MJ Research).
8. Diamond Scriber (VWR, 52865-005).
9. Hydrophobic marker (PAP pen, Sigma, Z37782-1).
10. Cover slips (Lifterslip, Erie Scientific, 18x18I-2-4746).
11. Aluminum foil tape (R. S. Hughes, 425-3).
12. Wafer-handling tweezers (Technitool, 758TW178 style 4WF).
13. Gel-filtration columns for protein cleanup (Bio-Rad Micro Bio-Spin P-6, 732-6222).
14. Kit for Protein A clean up of antibodies (Bio-Rad Affigel Protein A MAPS kit, 153-6159).
15. Bicinchoninic acid (BCA) protein assay kit (Pierce, 23226).
16. Microcon YM-50 (Millipore, 42423).
17. Phosphate-buffered saline (PBS), pH 7.4: 137 m*M* NaCl, 2.7 m*M* KCl, 4.3 m*M* Na_2HPO_4, 1.4 m*M* KH_2PO_4.
18. Carbonate buffer: 50 m*M* Na_2CO_3, pH 8.5.
19. PBST0.5: PBS + 0.5% Tween-20.
20. Nonidet P-40 (NP40) lysis buffer: 50 m*M* HEPES, pH 7.0, 5 m*M* ethylenediaminetetraacetic acid, 50 m*M* NaCl, 10 m*M* sodium pyrophosphate, 50 m*M* NaF, 1% NP-40, 10 m*M* sodium vanadate, and complete protease inhibitors (Roche 1 696 498).

3. Methods

The methods are divided into three sections: (a) antibody handling and microarray production, (b) sample preparation, and (c) microarray processing.

3.1. Antibody Handling and Microarray Production

The success of this method depends, in part, on the quality of the antibodies used on the microarrays. Each antibody has different performance characteristics in the microarray assay, and each needs to be evaluated independently. Antibody performance can be evaluated using standard immunological methods, which will not be discussed here.

3.1.1. Antibody Selection and Preparation

3.1.1.1. Choosing the Targets and Antibodies

The first step in the project preparation is to determine the protein targets, which depend on the goals of the research. Not all proteins are suitable for measurement in this assay; the size of the target proteins and their estimated abundances in the samples need to be considered. If a protein is very small, it may not be compatible with direct

labeling detection methods (discussed in **Subheading 3.3.**) that use a size-based separation of labeled products from unincorporated labeling reagents. If a protein is in very low abundance (*see* **Note 1**), it may fall outside the detection limit of the assay. The authors recommend choosing monoclonal antibodies that work in enzyme-linked immunosorbent assays, but polyclonal antibodies can also work well.

3.1.1.2. Purity of Antibodies

Antibodies work best in the microarray assay when they are highly purified. A high concentration of other proteins in the antibody solution usually results in a weakened or nonspecific signal because many binding sites on the microarray are occupied by the other proteins. Antibodies that are provided in antisera or ascites fluid should be further purified. The simplest method is to isolate the IgG fraction of the sample using a kit such as the Bio-Rad Affigel Protein A MAPS kit. Some antibodies come in a high concentration (up to 50%) of glycerol to improve stability. Although glycerol does not interfere with the assay, the added viscosity may negatively affect the printing process. Glycerol concentrations above approx 20% should be avoided. To change the buffer of an antibody, the authors recommend the Bio-Rad Micro Bio-Spin P30 column (*see* **Note 2**). If the antibody is to be labeled subsequently, do not put the antibody in a Tris-HCl or amine-containing buffer, which will interfere with primary amine-based labeling reaction.

3.1.1.3. Buffer, Concentration, and Storage

Antibodies are stable refrigerated in a standard buffer such as PBS. The optimal spotting concentration is 300–500 μg/mL. Higher concentrations could yield higher signal intensities and lower detection limits and may be desirable if consumption of antibody is not a concern. Most antibodies can be stored refrigerated for up to a year. New antibodies should be divided into aliquots, using one as a refrigerated working stock and freezing the others at –70°C, to avoid repeated freeze/thaw cycles that can damage proteins. When retrieving antibodies from a freezer stock, thaw the solution slowly on ice to reduce damage from the thawing process.

3.1.2. Preparation of HydroGel-Coated Slides

Various substrates for antibody microarrays have been demonstrated, such as poly-L-lysine-coated glass ***(6)***, aldehyde-coated glass ***(7)***, nitrocellulose ***(4)***, and a polyacrylamide-based HydroGel ***(8,9)***. The authors prefer a HydroGel coating on a glass slide, such as that supplied by PerkinElmer Life Sciences. HydroGels should be stored dry at room temperature. They must be used within 2 d after this procedure, so do not prepare the HydroGels until ready to print microarrays.

1. Load the HydroGel slides into a slide rack, briefly rinse in purified H_2O, and wash three times at room temperature with gentle rocking for 10 min each in purified H_2O (*see* **Note 3**).
2. Centrifuge slides to dry (*see* **Note 4**).
3. Place HydroGel slides in a 40°C incubator for 20 min.
4. Remove the slides from the incubator and allow slides to cool to room temperature. The slides are ready for printing.

3.1.3. Printing Microarrays

After the antibodies have been prepared at the proper purity and concentration, they are assembled into a *print plate*—a microtiter plate used in the robotic printing of the microarrays. Polypropylene microtiter plates are preferable to polystyrene because of lower protein adsorption. The plate should be rigid and precisely machined for optimal functioning with printing robots. Load about 6–10 μL of each antibody solution into each well of a 384-well print plate (*see* **Note 5**). If printing is sometimes inconsistent or variable between printing pins, it is desirable to fill multiple wells with the same antibody solution so that different printing pins spot the same antibody. Store the 384-well print plates sealed in the refrigerator until ready to use. Aluminum foil tape provides a good seal. Long-term evaporation-free storage is ensured by enclosing the covered plate in a sealed plastic bag. Prepare a spreadsheet containing the well identities for use in downstream data processing applications.

The details of the printing process depend on the type of printing robot used, but the authors give some general notes here. Minimize the time that the print plates are unsealed and exposed to keep evaporation of the antibody solutions low. Maintaining a moderately high humidity in the printing environment (around 45%) will minimize evaporation and may also improve spot quality. The proper printing of the robot should be confirmed with test prints on dummy slides before starting the microarray production. Use 500 μg/mL bovine serum albumin (BSA) in 1X PBS for the test prints. Make sure the H_2O in the tip wash bath is changed regularly to prevent contamination of the tips. It is desirable to confirm sufficient washing of the pins between loads. This test can be done by loading labeled protein into one of the print-plate wells in a dummy print, followed by scanning the slide. If fluorescence is seen in spots after the spots containing fluorescently labeled material, the pins need to be washed more stringently. Most microarrayers will allow the printing of replicate spots on each array, which are useful to obtain more precise data through averaging and to ensure the acquisition of data if a portion of the array is unusable; 6–10 spots per array per antibody are usually sufficient.

3.1.4. Postprint Processing of Microarrays

Follow the procedure below after printing on HydroGels. Microarrays printed on highly absorptive surfaces such as nitrocellulose will not require such a long incubation before blocking.

1. Prepare staining chambers with a wet paper towel soaked in saturated NaCl on the bottom.
2. Place the slides in slide racks in the staining chambers. Seal the chambers.
3. Incubate at room temperature overnight to allow adsorption to the HydroGel matrix.
4. The next day, circumscribe the array boundaries on each slide with a hydrophobic marker (e.g., a PAP pen). Leave at least 3–4 mm between the array boundary and the marker line. Allow the hydrophobic marker lines to fully dry (2–3 min).
5. Rinse the slides.
   a. Rinse briefly (for 20 s) in PBST0.5.
   b. Wash in PBST0.5 for 3 min with gentle rocking.
   c. Wash in PBST0.5 for 30 min with gentle rocking.

6. Block the slides. If the arrays are not to be used for 1 d or more, they can be left in the BSA blocking solution until ready for use. Add sodium azide (0.05%) to the blocker if storing for more than 1 d. Begin at **step 6b.** when ready to use.
   a. Place the slide racks in 1% BSA and PBST0.5 for 1 h at room temperature with constant shaking.
   b. Briefly rinse twice with PBST0.5.
7. Dry slides by centrifugation (*see* **Note 4**) immediately prior to incubation with samples.

3.2. Sample Preparation

Here, the authors describe the preparation of proteins for use in the microarray assay from either clinical specimens or cell culture. **Subheading 3.2.1.** concentrates on the use of serum or plasma (also applicable to other bodily fluids), and **Subheading 3.2.2.** describes the preparation of proteins from tissue specimens or cell culture.

3.2.1. Using Serum or Plasma Samples

The analysis of proteins from serum or plasma is convenient because all the proteins are soluble and only need to be diluted in the proper buffer (described in **Subheading 3.3.**). Clinical samples should be handled as biohazards because they can be carriers of infectious agents. Tips and tubes that contact clinical samples should be discarded in a biohazard bag. Samples should be aliquoted so that no more than three thaws are necessary for any experiment, as some researchers have observed measurable breakdown in proteins after three thaws. Samples should be stored at –80°C.

3.2.2. Preparing Proteins From Cell Culture or Tissue

3.2.2.1. Preparation of Protein Extracts From Cell Culture

1. Wash cells cultured in a 10-cm Petri dish at 80% confluency with ice-cold PBS three times.
2. Add 1 mL of NP-40 lysis buffer and keep on ice for 15 min.
3. Scrape the lysate with a rubber policeman and transfer into a 1.5-mL Eppendorf tube.
4. Centrifuge at 15,000*g* for 10 min.
5. Transfer the supernatant into a fresh 1.5-mL tube.
6. Measure protein concentration using a Pierce BCA™ protein assay kit.
7. Bring the cellular extracts to the same concentration (approx 2 mg/mL) with NP-40 lysis buffer.
8. Aliquot into working stocks and freeze at –80°C.

3.2.2.2. Preparation of Protein Extracts From Tissue

Tissue specimens should be handled as biohazards. Tissue samples fixed with formaldehyde and embedded in paraffin are not suitable for protein extraction for microarrays. Tissue samples fresh frozen in liquid nitrogen or frozen embedded in optimal cutting temperature (OCT) compound are suitable for this process. To optimally make use of the specimen, one may cut sections with a cryostat as needed for protein extraction, saving the rest of the specimen for later experiments. A 50-μ*M*-thick section of a 1–2 cm^2 tissue sample yields approx 100–200 μg of protein (depending on the tissue type), which is plenty for several microarray experiments because about 20 μg is used per experiment.

1. Prepare 1.5-mL Eppendorf tubes with 70 µL of NP-40 lysis buffer on ice.
2. Collect 50-µ*M* tissue sections, and put each section into a different tube.
3. Homogenize the tissue sections with a pellet pestle immediately. Keep on ice for 15 min.
4. Centrifuge at 15,000*g* for 10 min.
5. Transfer the supernatant into a fresh 1.5-mL tube.
6. Measure protein concentration using a Pierce BCA™ protein assay kit.
7. Bring the cellular extracts to the same concentration (approx 2 mg/mL) with NP-40 lysis buffer.
8. Aliquot into working stocks and freeze at –80°C.

3.3. Microarray Use

Figure 1 presents the types of detection methods described here: direct labeling (either with a fluorophore or a hapten), and a FLISA. Discussed below are the advantages, disadvantages, and the types of experiments suitable for each.

3.3.1. Direct Labeling

In the direct labeling method, all proteins in a complex mixture are labeled with either a fluorophore or a hapten (e.g., biotin) that allows subsequent detection. Advantages of this method are simplicity and the requirement for only one antibody per target, as compared to two for a sandwich assay. Another advantage is multicolor detection, allowing multiple samples to be labeled with different-color fluorophores, mixed and incubated on the same microarrays. That capability allows the use of a reference mixture, which provides an internal normalization standard to account for concentration differences between spots. A good choice of reference is a pool of equal aliquots from each sample to be measured ***(2)***, thus ensuring that all proteins from the samples are represented in the reference.

A disadvantage of the direct labeling method is increased background resulting from the labeling of all proteins, especially high-concentration proteins, such as albumin in serum. Detection sensitivity using the direct labeling method is limited by the concentration of the background proteins relative to the target protein ***(6)*** and is typically around 100 ng/mL for proteins in blood serum.

3.3.2. Fluorescence-Linked Immunosorbent Assays

In FLISA detection, a capture antibody on the microarray captures the unlabeled protein target, which is detected by a hapten-labeled detection antibody and a fluorophore-labeled anti-hapten antibody. The sandwich assay is usually more sensitive than the direct labeling method because background is reduced through the specific detection of two antibodies instead of one. This method is not as easily scalable as the direct labeling method, because it is more difficult to find high-quality matched pairs than single antibodies against particular targets. Also, antibody consumption is higher, and the optimization of assays measuring many targets is difficult. Nevertheless, multiplexed sandwich assays can be very powerful for certain applications. Microarray-based sandwich immunoassays have been demonstrated using enhanced chemiluminescence detection ***(1)*** and rolling-circle amplification ***(5)***.

Based on these considerations, FLISA detection should be used for a limited number of targets that are below the detection limit of the direct labeling method. Direct

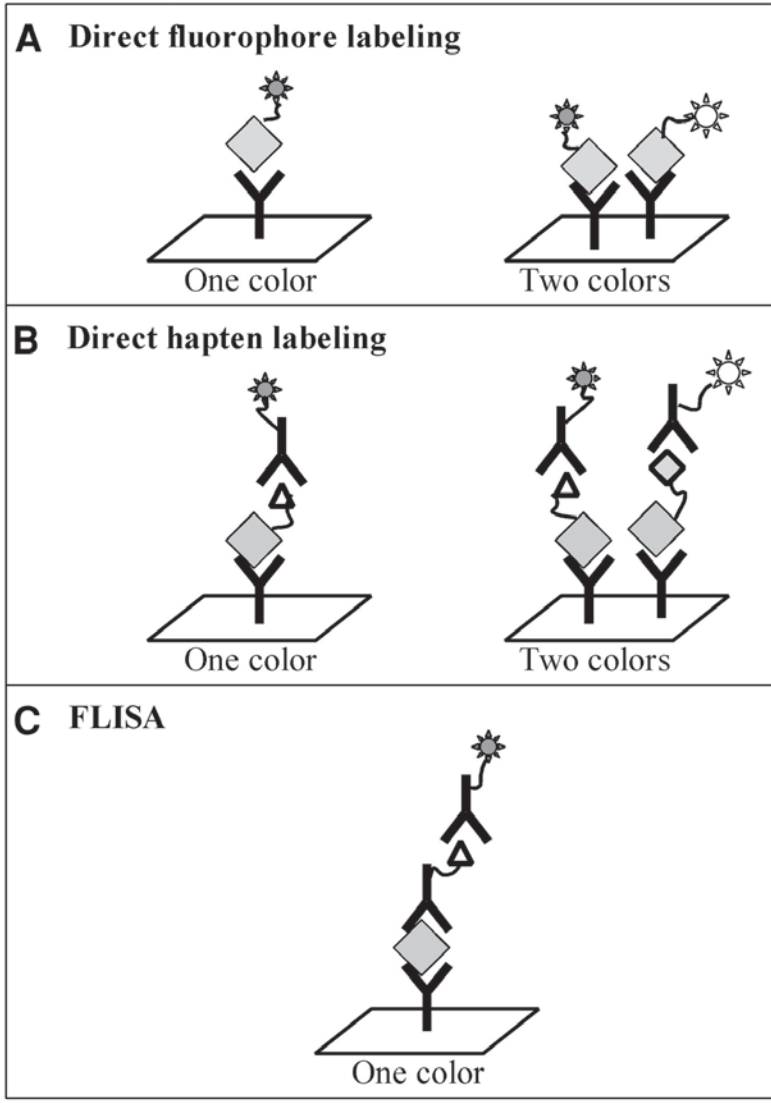

Fig. 1. Schematic representation of the described detection methods. (**A**) One-color and two-color direct fluorescent labeling. Proteins are directly labeled with a fluorescent tag. In the two-color case, two pools of proteins are labeled with distinct fluorescent tags and incubated together on an antibody array. (**B**) One- and two-color detection of direct hapten labeling. Proteins are directly labeled with a hapten (such as biotin, represented by the *triangle*). In the two-color case, two pools of proteins are labeled with distinct haptens (represented by the *triangle* and the *diamond*). Fluorescently labeled antibodies that target the haptens are then incubated on the array. (**C**) Sandwich FLISA detection. Proteins are incubated on the array, followed by incubation of a detection antibody labeled with a hapten (such as biotin, represented by the *triangle*). Fluorescently labeled antibodies that target the hapten are then incubated on the array.

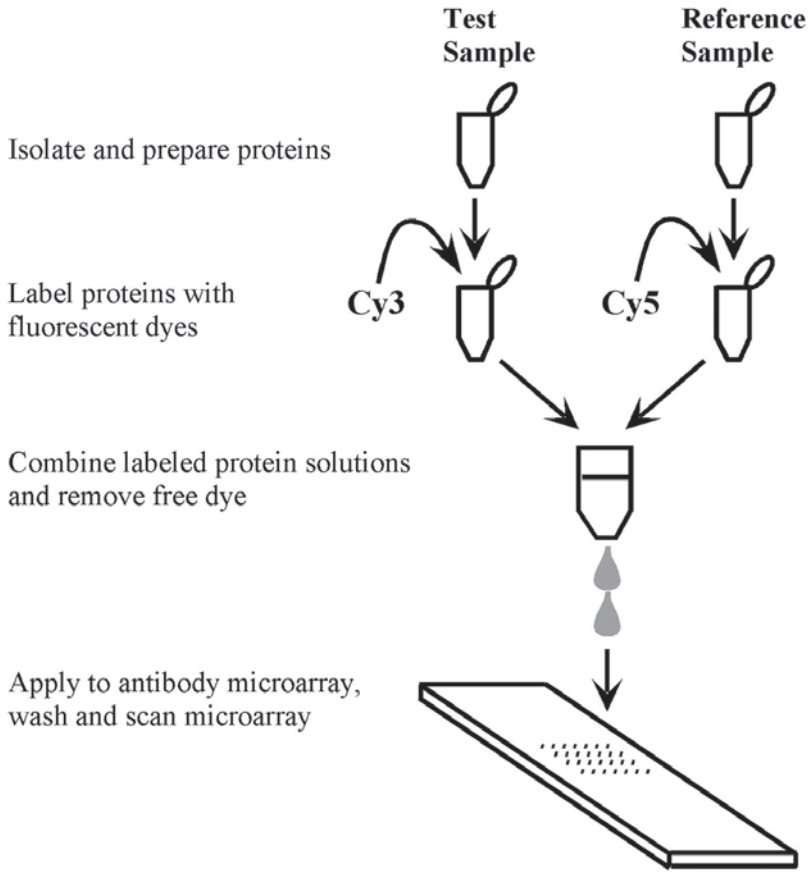

Fig. 2. Two-color detection of directly labeled samples. Proteins from a test sample and a reference sample are isolated and labeled with distinct fluorophores. The two samples are mixed, passed through a column to remove unincorporated dye, and incubated on an antibody microarray. After washing and scanning the array, the relative sample-specific to reference-specific fluorescence at each antibody spot provides a measure of the protein binding from each sample.

labeling should be used for the higher abundance proteins and can be expanded easily to target a large number of proteins.

3.4. Sample Labeling

Figure 2 presents a schematic representation of the steps in two-color detection of directly labeled samples. The method described below uses commercially available reagents that react with primary amine groups. The protocol refers to labeling with Cy3 or Cy5 but also applies to labeling with haptens, such as biotin, digoxigenin, or dinitrophenol (DNP).

1. Determine the volume to be used on each array. The volume should be enough to easily cover the array without the possibility of drying at the edges. (Drying will cause a severe increase in background.) A 12 × 12-mm HydroGel pad with a hydrophobic marker boundary 1 mm beyond the edge of the pad requires about 50 μL of sample. The use of a cover slip over the pad reduces the required volume to about 20 μL.
2. Determine the volume to label of each sample.
   a. Serum samples: The amount to label of each sample is equal to $(V_a*A)/D$, where V_a is the volume per array determined above, A is the number of arrays on which the sample will be used, and D is the desired final dilution of the sample. A 50X final dilution usually works well for serum samples. Thus if the volume on the array is 50 μL with a dilution of 50X and two arrays per sample, the volume to use of each serum sample is 2.0 μL.
   b. Proteins extracted from cells: Use enough to give about 500 μg/mL on the array, according to the formula $(500\ \mu g/mL*V_a*A)/C_s$, where V_a and A are as defined previously, and C_s is the starting concentration of the sample. Thus if a protein extract solution is 10 mg/mL, and the volume on the array will be 50 μL, use 2.5 μL of sample per array.
   c. Samples to be used in a pooled reference: The amount to be labeled of each component of the reference is $(V_a*A)/(D*N_r)$, where V_a, A, and D are as defined previously, and N_r is the number of samples pooled in the reference. For example, if a pool of 10 samples at a 50X dilution will be used as the reference for 20 arrays of 50 μL each, the volume to be used of each sample in the reference will be (50*20)/(50*10) = 2.0 μL.
3. Dilute the samples with carbonate buffer spiked with a normalization standard (*see* **Note 6**). For serum samples, dilute by about 15X (e.g., 14 μL buffer per 1 μL sample). For cell lysates, dilute 2X, or to about 1 mg/mL.
4. Using approx 7 m*M* dye stock in dimethyl sulfoxide (DMSO), add a 20th volume of dye to each sample (e.g., 0.75 μL for a 15 μL solution). The dye stock is prepared by dissolving the Cy3 or Cy5 reagent in 30 μL DMSO (the Amersham dyes come in tubes of 200 nmoles each), followed by aliquoting and freezing at –80°C. Use a different dye for the reference samples and the test samples.
5. Mix well, spin the tubes briefly to collect the solutions, and let the reactions proceed on ice in the dark for 2 h.
6. Add a 20th volume 1 *M* pH 7.5 Tris-HCl to quench the reaction. Let the solutions sit for 30 min.
7. Mix each labeled sample with an appropriately labeled reference sample. If a pooled reference is being used, collect all the reference samples into one tube, mix, and distribute among the samples. Keep these solutions on ice and in the dark.
8. Prepare a Bio-Rad Bio-Spin 6 microcolumn for each sample (*see* **Note 2**).
9. Load each sample onto a Bio-Rad Bio-Spin 6 microcolumn, spin at 1000*g* for 2 min and collect the flowthrough.
10. Make 10X blocking solution: 30% nonfat milk in PBS and 1% Tween-20. (e.g., add 3 g milk to 10 mL buffer.)
11. Spin the blocking solution at 10,000*g* for 10 min. Use the supernatant in the next step.
12. To each labeled sample-reference mixture, add the 10X blocking solution. Use the volume of 10X blocking solution that will give 1X in the final volume. For example, for a final volume on the array of 50 μL, add 5 μL 10X blocking solution.
13. Add 1X PBS to bring each mixture to the final volume. The labeled samples may be stored overnight in the refrigerator.

3.5. Microarray Use—Direct Labeling Detection

3.5.1. Fluorophore-Labeled Samples

1. Carefully pipet the appropriate volume of each fluorophore-labeled sample (prepared according to **Subheading 3.4.**) onto each microarray. If using a cover slip, cover immediately (*see* **Note 7**).
2. Place the slides in a covered, humidified box (*see* **Note 8**). Place the box on an orbital shaker rotating about once per second.
3. Incubate the arrays for 2 h at room temperature with constant shaking.
4. Using wafer-handling tweezers to hold each slide, dunk each slide briefly in PBST0.5 to remove the sample and cover slip (if used). Load each slide in a slide rack in a staining chamber filled with PBST0.1.
5. Wash the slides for 10 min at room temperature in the PBST0.1.
6. Wash the slides twice more for 10 min each in fresh changes of PBST0.1.
7. Dry the slides by centrifugation (*see* **Note 4**). Keep the slides in the dark until ready to scan.

3.5.2. Hapten-Labeled Samples to be Detected by Fluorophore-Labeled Antibody

1. Prepare Cy3- and/or Cy5-labeled antihapten antibodies.
   a. Prior to labeling, the antibodies should be relatively pure (not in ascites fluid or antisera), at a concentration of at least 1 mg/mL, and not in a Tris-HCl or amine-containing buffer. See **Subheading 3.1.** if the antibodies need to be purified or buffer exchanged.
   b. Determine the amount of labeled antibody needed: equal to the volume per array (*see* **Subheading 3.4.**) times the final concentration times the number of arrays.
   c. Use the labeling and cleanup method described in **Subheading 3.4.**
2. Prepare the detection antibody solution.
   a. Mix the appropriate amounts of Cy3-labeled and Cy5-labeled antihapten antibodies, based on the solution volume per array times the concentration of each antibody times the number of arrays. The optimal concentration of the antibodies can be determined by titration (often around 10 μg/mL).
   b. Prepare a 10X blocker solution as described in **Subheading 3.4.**
   c. Add 10X blocker. Use 1/10 of the intended final volume of the antibody mix.
   d. Add 1X PBS to the final volume.
3. Incubate hapten-labeled samples (prepared according to **Subheading 3.4.**) on the arrays, and wash and dry the arrays according to **steps 1–7** above.
4. Incubate the detection antibody solution on the arrays for 1 h and wash and dry the arrays according to **steps 1–7** above.
5. Keep the slides in the dark until ready to scan.

3.6. Microarray Use—FLISA Detection

Variations on this strategy exist, such as in the choice of hapten for the detection antibody or the choice of dye for the secondary antibody. Enzyme-linked amplification methods that produce soluble products may not be compatible with the microarray assay because soluble products can diffuse away from the spot of origin. Amplification methods that are compatible with a planar multiplexed assay include tyramide signal amplification and rolling-circle amplification ***(10)***. The authors describe here

the use of a dye-labeled secondary antibody, and the extension to related techniques is straightforward.

1. Prepare a 10X blocker solution as in **Subheading 3.4.**
2. Prepare the detection antibody mix. This is a mix of detection antibodies corresponding to all the capture antibodies on the arrays.
   a. Each detection antibody has a different optimal concentration, which can be determined by titration. Each detection antibody should be biotinylated (*see* **Note 9**). If an antibody is not available biotinylated, biotin-labeling kits are commercially available.
   b. Determine the volume needed of the detection antibody mix: equal to the volume per each array (*see* **Subheading 3.4.** for the determination of the volume per array) multiplied by the number of arrays.
   c. Pipet 1/10 of the volume determined in **substep b.** of the 10X blocker into a tube and add to it the appropriate amounts of each detection antibody.
   d. Add 1X PBS to make up the appropriate final volume.
3. Prepare the secondary antibody mix. This is generally fluorophore-labeled anti-biotin antibody, but variations exist as noted above. The optimal concentration of the secondary antibody can be determined by titration (it is usually around 10 μg/mL). Refer to the second protocol from **Subheading 3.5.** for information on preparing dye-labeled antibodies in 1X blocker.
4. Prepare the samples. Prepare serum samples at a 10X to 50X dilution in 1X blocker, and prepare cell lysates at 200–500 μg/mL in 1X blocker.
5. Incubate each sample on a microarray, and wash the slides as described in the first protocol of **Subheading 3.5.**
6. Incubate the detection antibody mix on the arrays, and wash as described in the first protocol of **Subheading 3.5.**
7. Incubate the secondary antibody mix on the arrays, and wash as described in the first protocol of **Subheading 3.5.**
8. Keep the slides in the dark until ready to scan.

4. Notes

1. Detection limits for the assay depend on the antibodies used, the protein background in the sample, and the detection conditions. In general, the direct labeling method (described in **Subheading 3.3.**) can give detection limits in the low-ng/mL range for targets in a serum background. The FLISA method (described in **Subheading 3.3.**) can give detection limits in the low pg/mL range.
2. The Bio-Spin columns come prepacked with two types of buffers: sodium saline citrate (SSC) and Tris-HCl buffer. The filtrate will come through in the packing buffer. The packing buffer can be changed by running a different buffer through the column three times. The P30 column removes solution components smaller than 30 kDa, and the P6 column removes components smaller than 6 kDa. Thus the P30 column is better for purification of antibodies, and the P6 column is better for the purification of complex mixtures in which low molecular weight species should be preserved.
3. A microscope slide-staining chamber is useful for the washing steps (*see* **Section 2.**) The staining chambers come with slide racks that hold 10–30 slides. The racks can be transferred between staining chambers containing different washes and to a clinical centrifuge for drying the slides.

4. A clinical centrifuge with flat swinging bucket holders works well for this task. Place a paper towel layer on the bottom of the swinging bucket to absorb H_2O that is removed from the slides. Place the slide rack on the paper towel and centrifuge at approx 300*g* for about 3 min.
5. The volume may depend on the shape of the well and how far the print tips descend into the well. Too much volume can lead to droplets of antibody solution sticking to the outside of the print tip. The volume may also need to be optimized for particular applications, such as multiple draws from each well, which would require a greater volume.
6. As part of the analysis of each array, a normalization factor is calculated for each array that sets data from normalization antibodies to known values. A normalization antibody could detect a spiked-in standard such as flag-labeled BSA. A normalization standard could also be a protein normally found in the samples, such as IgG in serum.
7. Use wafer-handling tweezers to hold the cover slip. Lower it onto the array at an angle so one edge of the cover slip first touches the solution. Lower the rest of the cover slip onto the array so that no bubbles are trapped. The authors recommend using the Lifterslip-style cover slip, which has thin spacers attached to two edges of the cover slip. The spacers slightly elevate the cover slip above the array and allow more movement of the liquid under the cover slip. The use of a cover slip reduces the volume required per array and is useful if sample is limited. However, a cover slip greatly limits diffusion in the sample and can reduce signal strengths, so the authors normally recommend not using a cover slip.
8. A microscope slide box that holds 100 slides works well for this purpose. A paper towel soaked with 1X PBS can be placed in the bottom of the box, and the slides can be placed flat over the slide-holding slots.
9. Other haptens besides biotin would work well, such as DNP or flag peptide. Kits to label antibodies with these tags are available.

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5

Target-Assisted Iterative Screening of Phage Surface Display cDNA Libraries

Alexei Kurakin, Susan Wu, and Dale E. Bredesen

Summary

The novel screening format, target-assisted iterative screening (TAIS), comprises a simple and rapid two-step procedure for in vitro affinity selection of specific binders from enormous molecular diversities to the target molecule of interest. This detailed protocol describes the application of TAIS to a T7 phage-displayed complementary DNA (cDNA) library with a protein domain as a target. Protocols for purification of the target as glutathione-*S*-transferase (GST) fusion protein and modifications of the purified target that are required for the screening complement the TAIS protocol. The described application is a method of choice for the researchers interested in the identification and characterization of novel protein–protein interactions mediated by peptide recognition domains.

Key Words:

TAIS; phage surface display; cDNA library; screening format; molecular repertoires; protein–protein interactions.

1. Introduction

This chapter describes a novel screening format for large molecular repertoires. It is often desirable to find molecules that bind tightly and specifically to the experimenter's molecule of interest, which is referred to henceforth as a "target." The target can be a small drug-like molecule, protein, nucleic acid, polysaccharide, or other organic or nonorganic chemical. One of the approaches to achieve this goal is to create large repertoires of chemically similar entities—for example, combinatorial peptide, chemical, aptamer, or other libraries, and to apply a selection procedure, or screening format, that is specifically designed to isolate binders from a particular repertoire to the target of interest. This chapter is concerned with a novel screening method, TAIS, and its application to the selection of polypeptides from phage-displayed cDNA libraries. The protocols describing construction of various peptide and cDNA libraries

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as well as classical phage-display biopanning procedure are not discussed in this chapter and can be found in other laboratory manuals and publications ***(1–5)***.

The TAIS flowchart is shown in **Fig. 1**. In a first, preselection step, a protein target is immobilized on a solid support, such as Sepharose beads, and incubated with a phage-displayed cDNA library in solution. This step aims to retain phages displaying on their surfaces interacting partners of the target on the solid support. After washing, the retained phages are eluted and plated on a bacterial lawn. In the second step, the preselected cDNA library plaques are transferred onto a nitrocellulose membrane by blotting the bacterial lawn with the membrane. The protein target tagged with a reporter such as alkaline phosphatase is used then, as a detection reagent, to screen for interacting plaques on the membrane. The identities of the displayed polypeptides that interact with the target are deduced by sequencing of the corresponding cDNA inserts of the phages from individual positive plaques. Therefore, TAIS allows rapid identification of virtually all polypeptides present in a given library that interact with the target in a simple two-step procedure (*see* **Note 1**).

2. Materials

2.1. Protein Target Expression Construct

2.1.1. pGEX Family of Expression Vectors

1. pGEX-2TK vector (Amersham Pharmacia Biotech).

2.1.2. Construction of Expression Plasmid

1. Expand High Fidelity polymerase chain reaction (PCR) system (Roche).
2. SeaPlaque agarose (FMC BioProducts).
3. *Bam*HI restriction enzyme (e.g., Promega).
4. *Eco*RI restriction enzyme (e.g., Promega).
5. Deoxyribonucleic acid (DNA) gel purification kit (e.g., "JetSorb" from Genomed).
6. T4 DNA ligase (Promega).
7. *Escherichia coli* DH5α competent cells (Invitrogen).
8. *E. coli* BL21(DE3)pLysS competent cells (Novagen).
9. pGEX (5') forward sequencing primer:
   5'-GGGCTGGCAAGCCACGTTTGGTG-3' (Integrated DNA Technologies, Inc. [IDT]).
10. pGEX (3') reverse sequencing primer:
    5'-CCGGGAGCTGCATGTGTCAGAGG-3' (IDT).

2.2. GST-Fusion Protein Purification and Biotinylation

2.2.1. Protein Production

1. 2X YT medium: 16 g tryptone, 10 g yeast extract, 5 g NaCl per 1 L of H_2O, pH 7.0.
2. Ampicillin stock solution (100 mg/mL in H_2O, filter sterilized).
3. Glycerol.
4. Isopropyl-β-D-thiogalactopyranoside (IPTG) stock solution (100 m*M*, filter sterilized).

2.2.2. Cell Lysis

1. CelLytic B II reagent (Sigma).
2. Deoxyribonuclease I (DNase I) (Sigma).

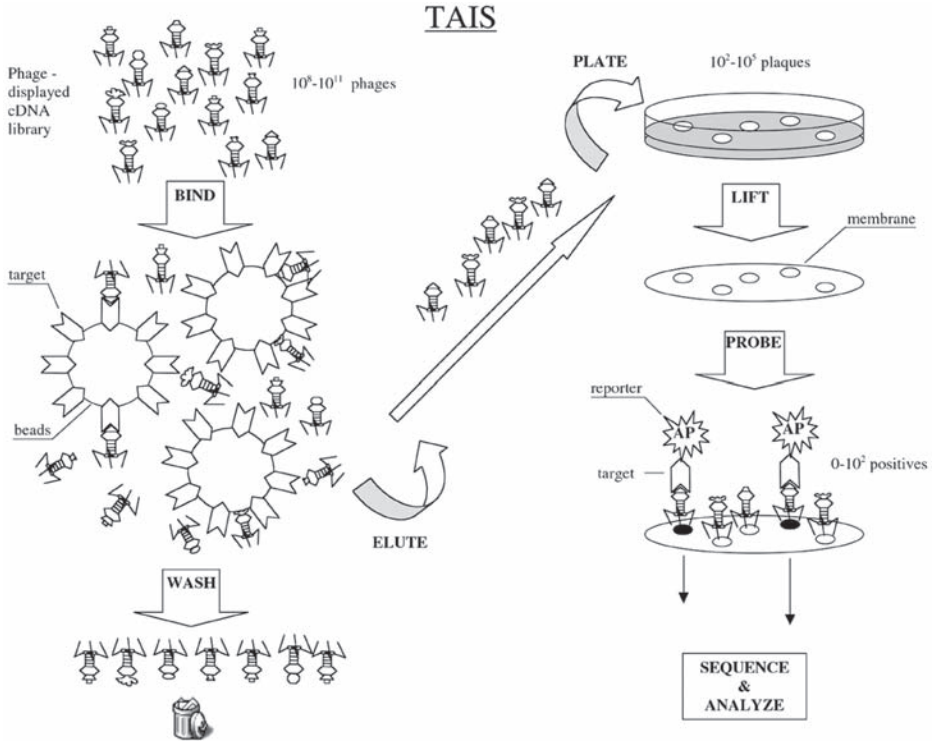

Fig. 1. Flowchart of TAIS. (Reproduced from **ref. *13*** with permission from the Adenine Press.)

2.2.3. Immobilization and Washing of GST-Fusion Protein

1. Glutathione Sepharose 4B (Amersham Pharmacia Biotech).
2. Phosphate-buffered saline (PBS), pH 8.0, sterile.
3. Siliconized 1.5-mL Eppendorf tubes (Ambion).

2.2.4. Cleavage of Target Protein

1. Thrombin protease solution (1 U/μL in PBS) (Amersham Pharmacia Biotech).
2. Coomassie Plus Protein Assay Reagent (Pierce).

2.2.5. Biotinylation of Target Protein

1. EZ-Link Sulfo-*N*-hydroxysuccinimide (NHS)-LC-LC-biotin (Pierce).
2. Tris-buffered saline (TBS), pH 7.4, sterile.
3. Microspin G-25 columns (Amersham Pharmacia Biotech).
4. Mini Dialysis Units, 3500 MWCO, 10–100 μL (Pierce).
5. [2-(4'-hydroxyazobenzene)] benzoic acid (HABA) (Pierce).
6. Avidin (Pierce).
7. Biotin (Pierce).

2.3. Target-Assisted Iterative Screening

2.3.1. Preselection of Specific Binders in Solution

1. Tris-buffered saline with 0.1% Tween-20 (TBST), pH 7.4.
2. 1% bovine serum albumin (BSA) in TBS, pH 7.4.
3. T7 phage-display library (Novagen).
4. 1% sodium dodecyl sulfate (SDS).

2.3.2. Plating Phage

1. 150-mm agar plates.
2. 0.6% top agarose (6 g of electrophoresis-grade agarose in 1 L of 2X YT medium).
3. Ampicillin stock solution (100 mg/mL in H_2O, filter sterilized).
4. 100 m*M* IPTG stock solution (filter sterilized).
5. *E. coli* BLT5615 overnight culture.

2.3.3. Probing Preselected Library Subset on Membranes

1. TBST, pH 7.4.
2. 132-mm, 0.45-μm pore-sized nitrocellulose membranes (Schleicher&Schuell).
3. Syringe needle.
4. 1% BSA (in TBS, pH 7.4).
5. Biotinylated target (*see* **Subheading 3.2.5.**).
6. Streptavidin-alkaline phosphatase conjugate (STRAP) (Sigma).
7. 5-Bromo-4-Chloro-3-Indolylphosphate/Nitro Blue Tetrazolium (BCIP/NBT) alkaline phosphatase substrate (Sigma).
8. 150-mm cell culture Petri dishes.

2.3.4. Preparation of Phage DNA From Positive Plaques

1. Luria Bertani (LB) medium: 10 g tryptone, 5 g yeast extract, 10 g NaCl per 1 L of H_2O, pH 7.5.
2. Sterile toothpicks.
3. 50-mL Falcon tubes (Corning).
4. *E. coli* BLT5615 cells (Novagen).
5. Wizard Lambda Preps DNA Purification System (Promega).
6. T7 forward-sequencing primer: 5'-ACTGTTAAGCTGCGTGACTTG-3'.
7. T7 reverse-sequencing primer: 5'-GTTCATATCGTATGAGCGCATATAG-3'.

3. Methods

3.1. Protein Target Expression Construct

In the application presented here, the TAIS method uses a purified protein as a target. Quality of the target and the library are major determinants of success or failure of a particular screen (*see* and **Note 2**).

There is a vast literature describing different systems for heterologous protein expression ***(6,7)***. Discussion and comparison of idiosyncrasies of multiple purification schemes are beyond the scope of this chapter and can be found elsewhere ***(8–10)***. A general strategy for construction of plasmid vector for bacterial expression of the target protein as a C-terminal fusion to the GST as well as GST-fusion protein puri-

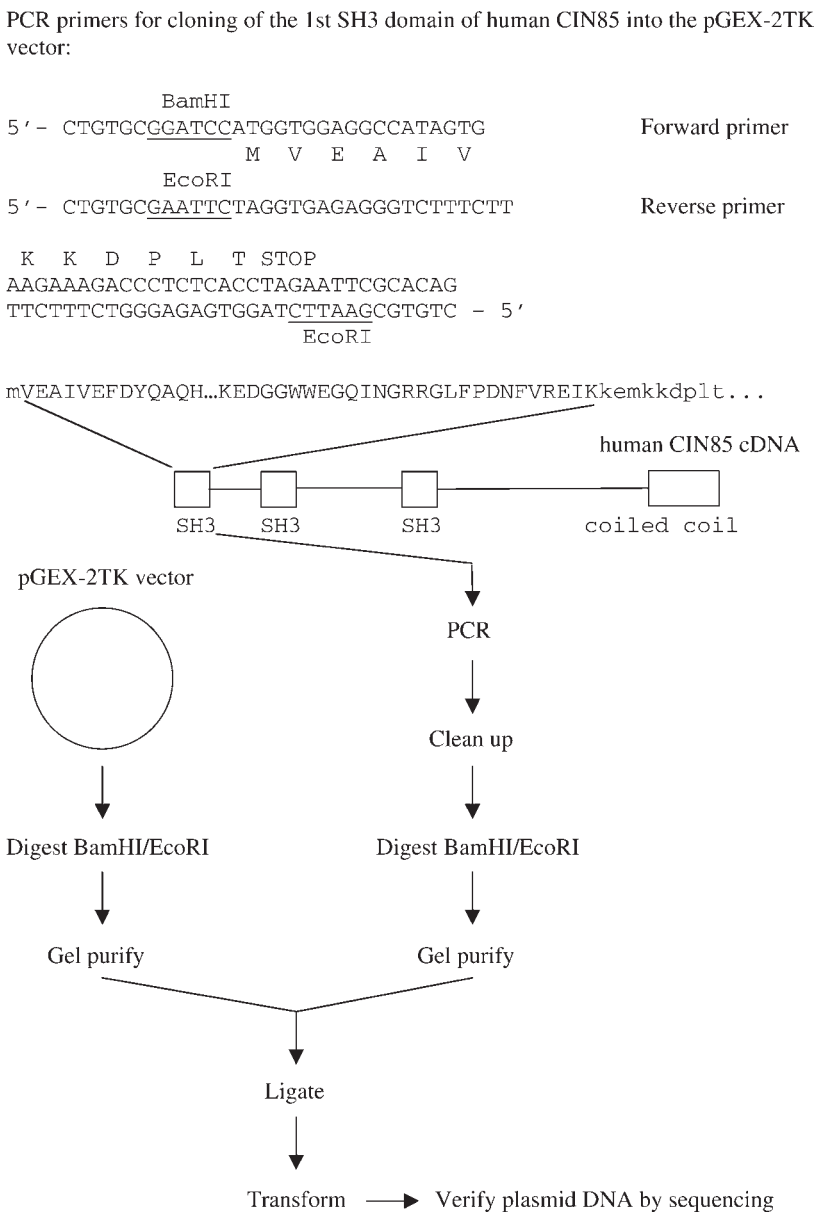

Fig. 2. Flow diagram of expression vector construction (*see* **Subheading 3.1.2.**). PCR primers designed for the first SH3 domain of human CIN85 are shown here as an illustration. The domain structure of CIN85 and borders of the domains were defined by the SMART software (http://www.smart.embl-heidelberg.de). The beginning and the end of the amino acid sequence of CIN85 SH3 domain are shown in capital letters.

fication and biotinylation of the purified target are described under **Subheadings 3.1.2.** and **3.2.**

3.1.1. pGEX Family of Expression Vectors

The pGEX family of plasmid vectors (Amersham Pharmacia Biotech) has been designed for the cloning of DNA coding sequences of proteins or protein fragments in frame with the *Schistosoma japonicum* GST coding sequence as well as for the expression, purification, and detection of resulting GST-fusion proteins produced in bacteria. The relevant features of this family of plasmid vectors are (a) GST gene under IPTG-inducible *tac* promoter, (b) an internal *lac* I^Q gene for use in any *E.coli* host, (c) Amp^r selection marker, (d) multiple cloning site at the C-terminus of the GST gene, and (e) PreScission, thrombin, or factor Xa protease recognition site for cleaving off the target protein from GST tag ***(11)***.

3.1.2. Construction of Expression Plasmid (see ***Fig. 2***)

The construction involves basic recombinant DNA techniques that are described in ***(12)***.

1. Obtain cDNA for the desired protein target.A growing number of sources of full-length and partial cDNAs from different organisms are available. Examples include the Mammalian Gene Collection, the American Type Culture Collection, the Research Genetics (Invitrogen Life Technologies), the Open Biosystems, and the Guthrie cDNA Resource Center.
2. Design and synthesize PCR primers flanking coding sequence of the protein or protein fragment of interest. Ensure that the coding sequence of the target is preserved in frame with the GST gene in the pGEX expression vector on ligation. Primers should include "overhangs" featuring recognition sequences of the endonucleases chosen for cloning plus additional 3–6 base pairs, as some of the common enzymes perform poorly on the very ends of a linear DNA duplex (see example of primers designed for the cloning of the CIN85-SH3 domain between *Bam*HI and *Eco*RI cloning sites in the pGEX-2TK vector in **Fig. 2**).
3. Amplify the coding sequence of the protein target of interest by PCR.
4. Check the presence, quantity, and correct size of the PCR product by agarose gel electrophoresis.
5. Clean up the PCR product. Phenol/chloroform extraction followed by ethanol precipitation ***(12)***, or commercial kits for PCR cleanup, such as ones supplied by Qiagen or Agencourt, are preferable methods in the authors' laboratory.
6. Digest the PCR product overnight by the restriction enzymes chosen for cloning.
7. Purify the DNA fragment to be cloned from other reaction products and components. The authors usually separate products of the reaction in 1% SeaPlaque agarose gel, excise the DNA fragment, and extract it from the gel using the JetSorb gel purification kit purchased from Genomed. As alternatives, GENECLEAN kit (Bio 101) or Agencourt's magnetic beads can be used.
8. Ligate the purified DNA fragment coding for your target of interest into the appropriately digested and purified pGEX expression vector.
9. Transform DH5α competent cells with the ligation product and plate transformants on ampicillin-containing agar plates (*see* **Note 3**).

10. Purify plasmid DNA from an individual ampicillin-resistant colony.
11. Check the construct by sequencing. Ensure that the coding sequence of your target is intact and cloned in the correct reading frame.

3.2. GST-Fusion Protein Purification and Biotinylation

3.2.1. Protein Production

The BL21 strain is recommended for production and purification of the GST-fusion proteins, as it is deficient in both *omp*T and *lon* proteases. Alternatively, DH5α cells can be used.

1. Inoculate 6 mL of 2X YT medium containing 100 μg/mL ampicillin with a single colony of *E. coli* BL21(DE3)pLysS cells harboring the expression construct. Shake overnight at 37°C and 230 rpm.
2. Freeze 1 mL of overnight culture in 10% glycerol and keep the aliquot at –80°C.
3. Inoculate 500 mL of prewarmed 2X YT medium containing ampicillin with 5 mL of overnight culture. Shake at 37°C for 3–4 h until the optical density $(OD)_{600}$ reaches 1.0–1.5.
4. Induce protein expression with 0.1 m*M* IPTG. Continue shaking at 37°C for 3 h.

3.2.2. Cell Lysis

GST-fusion proteins are extracted from bacterial cells using CelLytic B II reagent (Sigma). For each gram of cell paste use 5 mL of CelLytic B II. DNase I (up to 5–10 μg/mL) is recommended to reduce viscosity of the lysate. To minimize protein degradation, keep the cell extract on ice whenever possible.

1. Harvest cells by centrifugation at 6000*g* for 15 min at 4°C.
2. Discard supernatant and weigh cell mass.
3. Lyse cells completely by adding CelLytic B II (5 mL per 1 g of cell paste) and DNase I (5 μg/mL) to the pellet and vigorously pipetting the mixture up and down until no cell clumps are detectable.
4. Transfer cell extract to a fresh tube and shake at room temperature for 10–15 min.
5. Centrifuge extract at 25,000*g* for 15 min. Trying not to disturb the pellet, transfer lysate to a new tube.

3.2.3. Immobilization and Washing of GST-Fusion Protein

GST-fusion proteins are purified from the lysate by immobilizing them on glutathione Sepharose beads. Beads should be washed and reconstituted as 50% slurry in PBS before use as outlined in the protocol provided by the supplier (Amersham Pharmacia Biotech). When aspirating the supernatant, care should be taken to not disturb the beads. Once the target protein is immobilized and washed, it can be stored at 4°C for a few months. Always check the quality of the protein preparation by performing SDS-PAGE (*see* **Fig. 3**).

1. Add 600 μL of glutathione Sepharose 4B (50% slurry) to the lysate from **Subheading 3.2.2.**, **step 5**.
2. Incubate with gentle rotation at room temperature for 30 min.
3. Centrifuge at 500*g* for 5 min to sediment the beads. Carefully aspirate the supernatant.
4. Wash beads with 20 mL of ice-cold PBS. Centrifuge at 500*g* for 5 min to sediment beads. Carefully aspirate the wash solution.

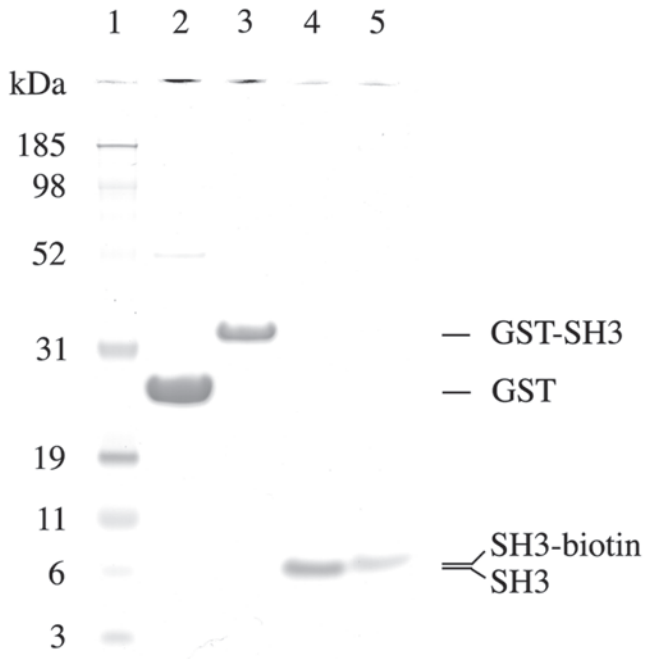

Fig. 3. A typical SDS-PAGE protein gel used to control for quality of the target protein preparation. Proteins were separated using Novex precast gels system (10% *Bis*-Tris gel with Z-[N-morpholino]ethane sulfonic acid buffer). *Lanes*: (1) molecular weight marker (MultiMark, Invitrogen), (2) GST (4 μg); (3) GST-SH3 domain fusion (2 μg); (4) SH3 domain cleaved off from the Sepharose beads by thrombin protease (1 μg) (*see* **Subheading 3.2.4.**), (4) biotinylated SH3 domain (*see* **Subheading 3.2.5.**). Proteins were stained with the GelCode Blue Stain Reagent (Pierce).

5. Wash beads two more times.
6. Transfer beads into a siliconized Eppendorf tube.
7. Sediment beads on a benchtop minicentrifuge, aspirate the residual buffer, and add 300 μL of PBS to generate 50% slurry. Store at 4°C.

3.2.4. Cleavage of Target Protein

Proteins tend to precipitate over time when kept in a soluble form. Therefore, the authors recommend releasing the target protein into solution just before use. Correspondingly scale all the amounts indicated below if a different fraction of the protein preparation is used. When transferring beads, make sure that the 50% slurry is thoroughly mixed.

1. Transfer 300 μL of 50% slurry to a new Eppendorf tube. Spin down beads using a minicentrifuge.

2. Remove 30 µL of PBS and replace with 20 µL of thrombin solution. Shake for 2–3 h at room temperature. If cleavage efficiency is poor, extend incubation overnight.
3. Spin down beads and transfer soluble protein to a fresh siliconized tube.
4. Measure protein concentration using the Bradford assay.

3.2.5. Biotinylation of Target Protein

The biotinylation reagent is unstable in H_2O and should be prepared fresh immediately before use. To prevent overbiotinylation, do not allow the reaction to proceed for more than 30 min. Always remove unconjugated biotin either by passing the reaction mixture through G-25 minicolumns or by dialysis.

1. Mix the target protein and the freshly prepared biotinylation reagent, EZ-Link Sulfo-NHS-LC-LC-biotin at a 1:6 molar ratio in a siliconized Eppendorf tube.
2. Incubate the reaction at room temperature for 30 min. Place on ice immediately when the incubation is over. As an option, the reaction can be quenched by concentrated Tris-HCl, pH 7.4.
3. Remove excess of unreacted biotin by passing the reaction mixture through a G-25 minicolumn (Amersham Pharmacia) or by dialysis.
4. Measure protein concentration by Bradford assay.
5. Control the extent of biotinylation by performing HABA assay (Pierce).
6. *See* **Note 4**.

3.3. Target-Assisted Iterative Screening

3.3.1. Preselection of Specific Binders in Solution

Blocking, washing, and binding steps are performed in a 1.5-mL siliconized Eppendorf tube. Aerosol-resistant tips are highly recommended during all manipulations involving bacteriophage-containing liquids.

1. Block 20 µL of beads (50% slurry) covered by immobilized protein target (approx 30 to 50 µg) (from **Subheading 3.2.3.**, **step 7**) in 250 µL of TBST and 750 µL of 1% BSA (in TBS, pH 7.4). Tumble at room temperature for 1 h.
2. Spin down the beads and aspirate the supernatant.
3. Wash with 1 mL TBST. Spin down the beads and aspirate excess of TBST. Repeat wash two more times.
4. Add to the washed beads 250 µL of TBST, 750 µL of 1% BSA, and 10 µL of T7 phage-displayed cDNA library (approx 10^8 plaque-forming units [pfu]). Mix well. Tumble at room temperature for 2 h.
5. Spin down the beads and aspirate the supernatant.
6. Wash with 1 mL TBST. Spin down the beads and remove excess of TBST. Repeat wash four more times. Take care to remove all the residual TBST after the last wash.
7. Elute phages that remained bound to the beads with 200 µL of 1% SDS. Incubate at room temperature for 10 min (*see* **Note 5**).
8. Spin down the beads and plate 100 µL of the supernatant on a 150-mm agar plate immediately.
9. Mix the beads with the rest of the SDS solution and plate the remainder on a second plate (*see* **Note 6**).

3.3.2. Plating Phage

1. Place agar plates in a 37°C incubator and the molten 0.6% top agarose in a 50°C water bath at least 1 h before plating to equilibrate the plates and the agarose at the indicated temperatures.
2. Immediately before plating, add to the molten top agarose: (a) *E. coli* BLT5615 host cells from overnight culture to 10%; (b) IPTG to a final concentration of 0.5 m*M*; (c) ampicillin to 100 μg/mL. Mix well and keep at 50°C.
3. For each 150-mm agar plate quickly mix 8 mL of the top agarose from **step 2** with the eluted phage from **Subheading 3.3.1.**, **step 8**.
4. Pour the mixture on a prewarmed agar plate. Avoid creating bubbles. Distribute the top agarose evenly by tilting the plate gently in different directions.
5. Let the top agarose solidify on the bench for 5–10 min.
6. Cover the plate and place it in an inverted position at 37°C for 2–3 h until phage plaques are well developed.

3.3.3. Probing Preselected Library Subset on Membrane

The following outlines a protocol for development of one 132-mm membrane. Scale the amounts proportionally if additional or alternatively sized plates and membranes are used.

1. Chill a plate with well-developed plaques for 30 min at 4°C to prevent top agarose from sticking to the nitrocellulose membrane.
2. Place nitrocellulose membrane on the plate. Slightly bend the membrane and begin contact at its center. Avoid trapping air bubbles. Leave membrane on the plate for 5 min at room temperature.
3. Using a red-hot syringe needle, make three deep holes on the membrane perimeter to introduce a coordinate system. Label the membrane.
4. Block the membrane in 1% BSA for 1 h at room temperature with gentle rocking in a 150-mm cell culture Petri dish.
5. Mix 5 μg of the biotinylated protein target with STRAP at a molar ratio of 4:1 in 100 μL of TBST. Incubate 10–15 min at room temperature (*see* **Note 7**).
6. Dilute target–STRAP conjugate in 30 mL of TBST containing 0.5% of BSA.
7. Incubate the blocked membrane in 30 mL of target–STRAP solution at 4°C overnight with gentle rocking.
8. Transfer membrane to a fresh dish with at least 50 mL of TBST. Place on rocker for 15 min.
9. Wash the membrane with TBST three more times.
10. Prepare 15 mL of BCIP/NBT solution. Gently rock the washed membrane in substrate solution until positive plaques become visible, usually 2 min to 2 h (*see* example in **Fig. 4**). The developed membrane can be washed with and stored in TBST at 4°C.

3.3.4. Preparation of Individual Phage Lysates and DNA for Sequencing

The authors recommend using 15 mL of individual phage lysates for purification of T7 phage DNA for sequence analysis. An aliquot of each lysate should be additionally saved in 10% glycerol and kept at –80°C for future reference.

1. Dispense 1 mL of LB medium into a number of 50-mL Falcon tubes. The number of Falcon tubes equals the number of positive plaques chosen for analysis.

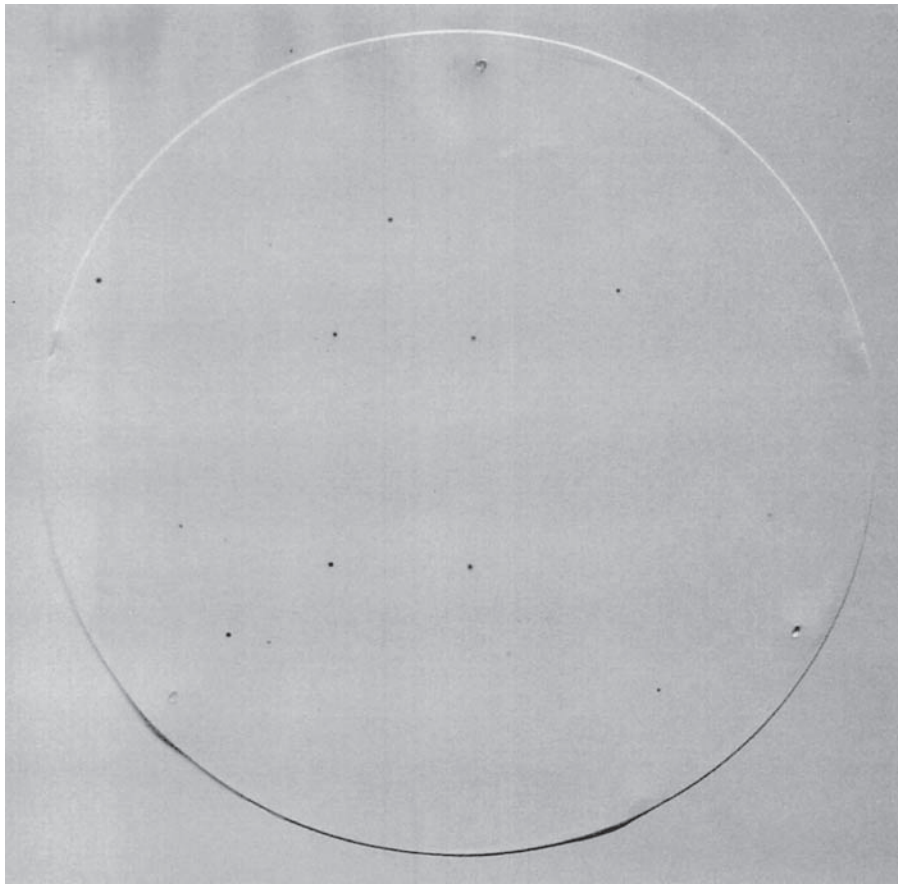

Fig. 4. A typical membrane with positive plaques. Positive plaques were visualized with BCIP/NBT alkaline phosphatase substrate (*see* **Subheading 3.3.3.**).

2. Define positions of positive plaques on the developed membrane and identities of the respective plaques on the agar plate using the coordinate system introduced in **Subheading 3.3.3.**, **step 3**. Pick up positive phages on the agar plate by gently touching them with a sterile toothpick. Place the toothpick into a Falcon tube with LB medium. Choose well-separated plaques for pick up. Avoid cross-contamination.
3. Prepare a logarithmically growing culture of *E. coli* BLT5615 host cells. Calculate total volume of the culture, reserving 20 mL per each individual phage to be analyzed. Shake the culture in a 37°C incubator at 230 rpm until OD_{600} reaches 0.3.
4. Add IPTG to a final concentration of 0.5 m*M*.
5. Shake the culture for an additional 20–30 min until OD_{600} reaches 0.6–0.7.
6. Immediately add 20 mL of the logarithmic culture to Falcon tubes with toothpicks from **step 2**. Shake at 37°C for 2–3 h until cells are completely lysed.

7. Spin down cell debris by centrifugation at 10,000*g* for 15 min at 4°C.
8. Transfer the cleared lysate to a fresh 50-mL Falcon tube.
9. Prepare phage DNA using Wizard Lambda Preps DNA Purification System (Promega).
10. Sequence cDNA inserts in phages from individual positive plaques using T7 forward- and reverse-sequencing primers (*see* **Subheading 2.3.4.**).
11. Deduce and analyze sequences of polypeptides displayed on the surface of the phages that bound to your protein target ***(13,14)***.

4. Notes

1. Omitting competition between phages altogether, TAIS eliminates a loss of weaker binders and propagation biases inherent in classical phage-display panning as a result of competition between individual phages during repetitive selection–amplification cycles. At the same time, the method permits the screening of significantly larger libraries than ones routinely used in cDNA-expression library screening. If a practical limit of the cDNA-expression library screening assay is 10^6–10^7 phages, the upper limit on the size of the library used in TAIS is limited by existing technologies of phage-display library preparation, that is, on the order of 10^8–10^{11} phages.
2. As a high-quality target the authors consider a protein of >90% purity as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (*see* example in **Fig. 3**). As little as 100 μg of the target protein is usually enough to accomplish an individual screen. The T7 phage-displayed cDNA libraries derived from different normal and disease tissues are available from Novagen. The same provider supplies a kit for phage library construction. The cDNA and peptide libraries displayed on another lytic bacteriophage, λ, have also been reported ***(15,16)***. Traditional peptide libraries displayed on the surface of nonlytic filamentous phages, such as M13 and fd, as well as recently reported cDNA libraries on filamentous phage ***(17)*** are readily amenable for the TAIS.
3. Though it is advisable to produce and purify the target protein from BL21 strain (*see* **Subheading 3.2.**), the authors avoid direct transformation of the BL21 cells with the ligation product because of their poor transformation efficiency. Instead, they prefer to use DH5α-competent cells for the initial preparation of plasmid DNA. Following verification of the ligation product by sequencing, they move the verified expression construct into BL21(DE3)pLysS cells for protein production.
4. It is important to keep the extent of biotinylation at 1 to 2 biotins per protein molecule. Overbiotinylation often results in inactivation of the target and should be carefully avoided. HABA displacement from avidin by free biotin can be quantitatively monitored by spectrophotometry. Therefore, the unknown amount of biotin present in a solution can be determined by preparing a standard curve using known amounts of biotin to displace the HABA bound to avidin. The detailed protocol can be found in instructions provided by the suppliers of the HABA reagent (e.g., Pierce, cat. no. 28010).
5. 1% SDS will disrupt most of the protein–protein interactions. Avoid prolonged incubation of T7 phages in 1% SDS because of their significant inactivation rate in this solution. It is recommended to try different agents for elution of T7 phages, such as 5 *M* NaCl, 4 *M* urea, and 2 *M* guanidine-HCl ***(18)***. If using this protocol with phages other than T7, for example, λ or M13, keep in mind that different phages may vary greatly in their resistance to inactivation by the same solution. As an example, M13 filamentous phage is relatively stable at pH extremes, such as pH 2.0 or pH 12.0, whereas T7 is almost instantly inactivated under those conditions.

6. The authors usually start with plating the entire sample on two 150-mm agar plates. Only phage solution and no beads are plated on the first plate. The second plate has all the beads and the rest of the solution. Equal density of plaques on both plates is indicative of efficient elution. If densities of plaques are too high to pick up the individual ones without risk of cross-contamination, it is recommended to divide and plate the sample on 5, 10, 20, or more plates. Alternatively, positive plaques can be excised and their phages eluted in 1 mL of PBS by shaking the excised agarose stubs at 37°C for 3 h. The eluted phages then are replated on fresh agar plates, and a secondary screening is performed as described in **Subheadings 3.3.2.** and **3.3.3.** at lower plaque densities.
7. Use a target-to-STRAP ratio of 4:1 or higher for multivalent target presentation if detection of weak interactions and low stringency conditions are desired, and the respective ratio of 1:4 or lower in case of strong interactions and high-stringency conditions.

Acknowledgments

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6

Design, Construction, and Use of Tissue Microarrays

Stephen M. Hewitt

Summary

Tissue microarrays are a platform for high-throughput analysis of tissue specimens in research. They are used for target verification of results from complementary deoxyribonucleic acid (cDNA) microarrays, expression profiling of tumors and tissues, as well as in epidemiology-based investigations. Tissue microarrays are a simple reagent that can be constructed from archival paraffin-embedded tissue for immunohistochemical staining, *in situ* hybridization, and other methodologies. The design and precision in execution of a tissue microarray is essential to obtain the desired results. Tissue microarrays are a powerful proteomic platform for research.

Key Words:

Tissue microarray; immunohistochemistry; *in situ* hybridization; high-throughput screening; paraffin-embedded tissue; formalin-fixed tissue.

1. Introduction

Tissue microarrays are simply a platform for high throughput of analysis of tissue. Any methodology that can be applied to a tissue section on a slide can be applied to a tissue microarray ***(1,2)***. A well-designed tissue array can replace the need to perform the same experiment over and over, and it reduces the variability of experiments performed in multibatch mode. A tissue microarray does not replace the basic underpinning of histology and pathology, that is, microscopic examination of the tissue. The limits of a tissue microarray are based on the quality of the tissue and on the person interpreting the tissue.

Tissue microarrays constitute an alternative proteomic platform with limitations and advantages compared to protein arrays. Advantages include the use of archival material and histologic and cytologic detail that is not possible with conventional protein arrays. Limitations include the artifacts and restrictions of working with fixed tissue and the inability to titrate and quantitate the proteins detected.

This chapter covers the design, construction, and use of tissue microarrays with emphasis on design and use. The actual method of arraying is rather simple and easily

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mastered with a little practice. However, maximizing the benefit of tissue microarrays requires extensive planning and attention to detail prior to construction of the array, as well as well-planned and well-executed experiments afterward. The construction of a tissue microarray is functionally the preparation of a reagent for an experiment. The quality of the reagents will be reflected in the quality of the results. A tissue microarray is limited in quality by the poorest quality tissue on the array.

The field of tissue arraying is rapidly changing. This chapter deals primarily with the design and preparation of tissue microarrays from paraffin-embedded tissue with a manual tissue arrayer ***(1,2)***. Recent advances, not covered in this chapter, include methods for building frozen tissue arrays ***(3)*** as well as methods for constructing frozen protein lysate arrays using similar methodology ***(4)***. Recently, an automated tissue arrayer has come to market (Beecher Instruments), but its use is largely limited to industrial and high-volume production facilities. The fundamentals of design and use of frozen and automated tissue arrays are not much different from those covered in this chapter.

2. Materials

2.1. Tissue Arraying

1. Beecher Instruments Manual Tissue Arrayer (www.beecherinstruments.com) with accessories including recipient block holder, bridge holder for donor block, and matched pair of donor and recipient needles, available as either 0.6-, 1.0-, 1.5-, or 2.0-mm needles.
2. Blank recipient block of low-melt paraffin.
3. Donor blocks of paraffin-embedded tissue (*see* **Note 1**).

2.2. Embedding Cells Grown in Culture

1. SeaPlaque Low-Melt Agarose.
2. Phosphate-buffered saline (PBS), 1X.
3. Round-bottom tubes or 15-mL conical tubes.
4. Vortex mixer.
5. Pippetmen and pippetors.
6. 42°C water bath.
7. Fixative: 70% ethanol or 10% formalin.

3. Methods

3.1. Collection of Tissue

Construction of a tissue microarray requires a collection of tissue. It is essential that appropriate ethical approval be obtained for the collection and use of the tissue prior to its use. Every institution, public or private, should have guidelines for the use of human and animal tissue in research. It is not necessary that the tissue come from the same source or be of the same age. It is desirable, at a minimum, to know what the fixative was for the tissue, as different fixatives will result in different experimental methods. Although one can construct arrays of mixed fixatives, interpretation requires knowledge of the fixative (*see* **Note 2**).

In collecting the tissue, the first determination is if the tissue will be arrayed *to depletion*, or sampled and returned to the source or archive. Donor blocks are readily

Table 1
Practical Array Densities

Needle size	Suggested center	Maximum practical array size
0.6 mm	0.7–1.0 mm	500 cores
1.0 mm	1.2–1.5 mm	200 cores
1.5 mm	1.75–2.0 mm	150 cores
2.0 mm	2.25–2.5 mm	75 cores

sectioned after arraying and, depending on the volume of tissue arrayed from them, have little/no loss of pathologic information. The number of different samples planned for the array impacts the volume of tissue removed from each donor block. Donor blocks can be material gathered for research only or, with appropriate approval, archival diagnostic material. The age or paraffin type of the blocks has less effect on array quality than the original quality of the processing of the tissue and the environment that the tissue has been stored in. If the blocks show excessive shrinkage or swelling of the tissue within the paraffin, the processing and/or storage of the blocks may be inadequate, as this is an indication that water has infiltrated the material. Older paraffins, frequently yellow in color and with higher melting points, are not a hindrance to arraying if well processed.

3.2. Array Design

The essentials of array design are matching the core size to the number of cores and putting this into a design that is user friendly. The needles for making the cores of tissue are available in four sizes (**Table 1**). Obviously, the larger the core, the fewer the samples that can fit on the array. Although tissue microarrays can be constructed to fit nearly any size paraffin block, there are important justifications for limiting the surface area of an array. The chief limitation on size is the area that can be efficiently and uniformly stained by immunohistochemistry, manually or automated. Many tissue microarrays are sectioned by a tape transfer methodology (discussed later), which introduces additional array surface-area limits. A practical array area is 18 × 22 mm, which will easily hold up to five hundred 0.6-mm cores. As the diameter of the needle increases, the spacing, or center-to-center distance between cores must increase. Although, in theory, no space is required between cores, the elasticity of paraffin necessitates some empty paraffin between cores to maintain the alignment of the array.

The optimal design of a tissue microarray will take the end user into consideration. To this end, placing the cores in *subarrays* will significantly ease the work of analyzing the array, as the reader will not get lost as easily. Subarrays of 10 × 10 or larger are very laborious, requiring the reader to constantly switch between low- and high-power objectives and count to determine where they are. Optimal subarray sizes include 3 × 3, 4 × 4, and 5 × 5. A 5 × 5 subarray with 0.6-mm cores, on a 0.7- or 0.8-mm center can be viewed in its entirety with a ×4 objective on most microscopes. The tissue on the array should be organized by subarray, rather than randomly placed on the array. If the centers between cores are too large, the reader may easily become lost. Optimal design

is based on subarrays with empty rows and columns of unit size between subarrays. The spacing (center) in both the *x*- and *y*-axes should be the same. The last two points are essential for most automated microscopes and software collections of tissue microarray data. The intentional inclusion of an offset in the alignment of the subarrays so that the array is asymmetric in both the *x*- and *y*-axes is of benefit. The asymmetry will assure proper orientation of the array when data is collected from it. Examples of such designs are available at www.cancer.gov/tarp.

The ultimate choice of what tissue to place where on an array has no definitive answer. There are considerations that always must be balanced in the design of an effective tissue microarray; these include the inclusion of appropriate control/normal tissues, core size, oversampling, and matched disease state and normal tissue pairing. The statistical concerns of sampling are beyond the scope of this chapter. A simple and practical guide is to *fill the array*; do not leave empty spaces—add samples or oversample. In general, the tissue available for construction of a tissue array is one of two types: (a), a limited number of cases, or (b), statistical concerns are paramount, and the sample size will be predetermined.

The inclusion of normal/control tissue is essential on all tissue arrays. The most common experiment performed on a tissue microarray is immunohistochemistry. Lacking appropriate controls, it is impossible to determine the quality of the staining. At a minimum, a normal sample of the tissue used in the array should be included. The inclusion of kidney and liver are excellent controls, as they contain endogenous biotins and frequently reveal false-positive background staining patterns. The more complex the design of the array, the greater the number of normal tissues required, and they should be between 5 and 10% of total number of cores. Some investigators advocate placing control/normal tissue in a distributed manner across the array ***(5)***. This can assist in verifying that an immunohistochemical stain is well performed and even across the entire array. An alternative approach is the inclusion of *mixed cell line cores* (*see* **Subheadings 3.13.** and **3.14.**) at regular intervals around the array.

3.3. Sampling

The subjects of oversampling and core size are inseparable. Oversampling attempts to deal with tissue heterogeneity by taking samples from different regions or portions of the donor tissue. In contrast, increasing core diameter presents a greater histoarchitectural view of the tissue. Both approaches result in a greater number of cells from a donor block on the array. It remains inconclusive, in direct comparison, which is more effective—oversampling or larger cores—in accurately representing a sample. It has been demonstrated that accurate representation of some tissues to match the published rates of staining for preselected markers requires oversampling with the 0.6-mm needles ***(6)***. No hard-and-fast guidelines are available for oversampling because they are based on predetermined and previously published results of previously studied markers. However, as the number of cases included in the array increases, the effects of tumor heterogeneity decrease. As a result of this quandary, the best advice remains, *fill the array*, either by using larger needles, oversampling, or both. Examples include 350 donor blocks—an array with 0.6-mm cores, 50 normal

cores, and no oversampling compared to 75 donor blocks—an array with 1.0-mm needles, 3X oversampling, and 25 normal cores.

Certain tissues and situations merit noting. There are tissues and disease states not readily amenable to representation on tissue arrays. Among normal tissues, the glomeruli of the kidney and portal tracts of liver are not representable on tissue microarrays using normal methodologies. *In situ* lesions of most epithelial surfaces cannot be adequately represented on tissue arrays, and flat- or thin-surface epithelial cancers are challenging to array. Bony tissues, such as osteosarcomas, are very difficult to array, and best results are obtained with 1.0-mm needles *(7)*.

3.4. Mapping of Slides

The goal of a tissue array is to present the pertinent tissue on the array. As a result, removing cores from the donor at random, without reference to the underlying histology results in limited arrays at best. Different tissue types and disease states require different levels of accuracy in selecting the appropriate tissue from a donor block. Many normal tissues, and some disease states can be cored from unmapped blocks. Examples include normal liver and spleen, whereas others require extreme precision, such as *in situ* lesions of the breast. The optimal method to select the tissue for arraying from the donor block is to *map* an Hematoxylin and Eosin (H&E) slide from the donor block.

During arraying, the map slide is overlaid with the donor block and the target region is aligned under the needle of the arrayer. The blocks can be directly drawn on with some markers, based on comparison to the mapping slide, but this still lacks the precision of a mapping slide. Methods of viewing the slide and block separately are inadequate for precision mapping of cores. A pathologist or someone well versed in the histologic features of interest is essential to map the donor block slides. The slide should be a recently cut section from the block, without intervening sections taken or reembedding, if possible. Mapping is best accomplished under a microscope or dissecting microscope, and it is performed easiest at a total magnification of ×40 (×4 objective and ×10 eye pieces), but higher power objectives are useful to examine diagnostic details. Green ink provides the best contrast for mapping the slides. If multiple tissue types (normal and tumor) are to be taken from the same block, two colors can be used, or use the designations "N" and "T." When mapping, it is essential to pay attention to the size of the lesion targeted and the number of cores desired. Targetting a small lesion, the precision of matching a needle to the desired target is approx 0.5 mm, or, simply put, if you want to make sure you get the target, it should be two 0.6-mm needles in diameter. With larger needles, the entire lesion can be cored out. It is essential to remember that the target is three-dimensional and that the depth of the target is just as important as the width, as this will affect how many slides can be generated that contain the target. A first approximation that works well is that all lesions are seen at maximal diameter and that the depth of the lesion is equal to half the diameter of the lesion. If multiple cores of the same lesion are required for oversampling or multiple blocks, one must have sufficient tissue. A convenient rule of thumb is that five 0.6-mm cores can be taken from a lesion approx 7.0 mm in diameter, or the size of the head of a pencil.

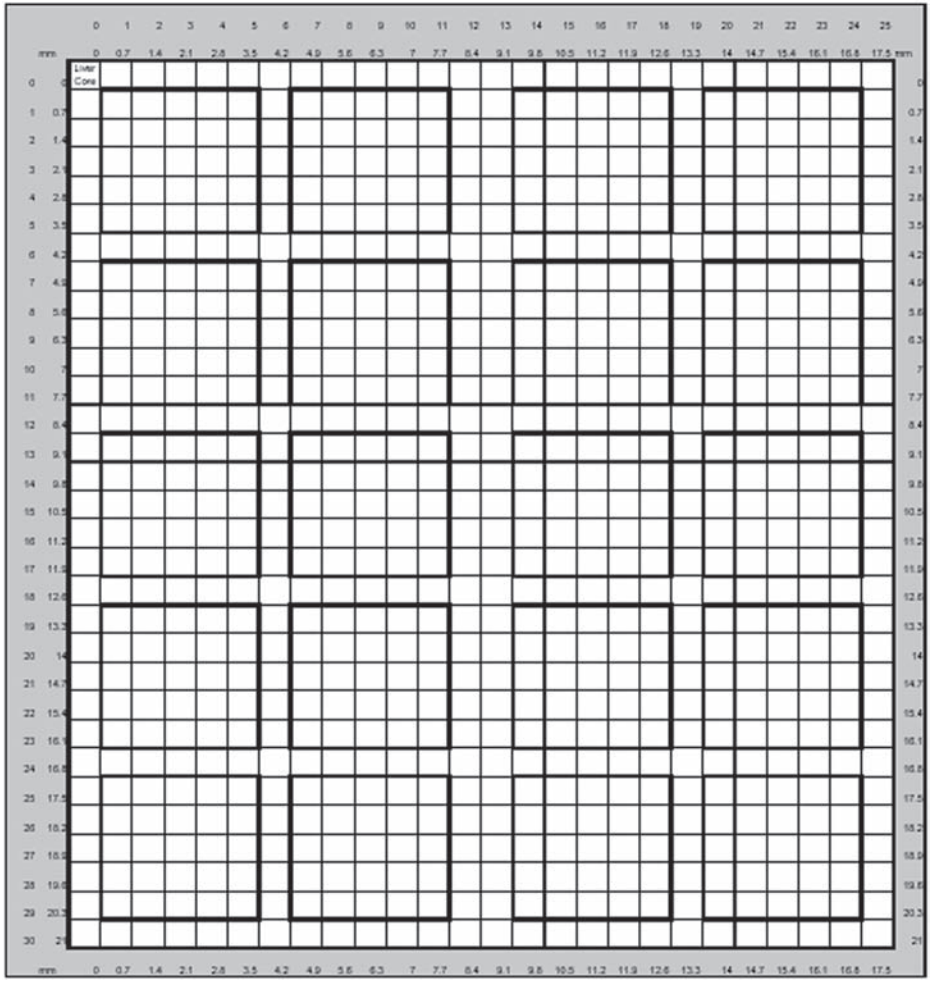

Fig. 1. Sample template for a 500-core array with 0.6-mm cores on 0.7-mm centers.

3.5. Finalization of Design of Array

Although a preliminary design or *schema* of the array should be designed prior to mapping of the donor blocks, the final design cannot be completed until all the donor blocks are mapped and a final list of blocks is available. It should be expected that approx 15% of blocks designated for arraying will be excluded for quality issues. Common exclusions include wrong tissue, inadequate tissue for completion of array, or poor tissue quality.

It is easiest to organize the donor blocks in a spreadsheet such as Excel. Once this list is completed, the array can be finalized. This is accomplished by constructing a map of the array as it should be laid out in the spreadsheet (*see* **Fig. 1**). This spread-

sheet can then be printed in a large format for arraying and as a single page for scoring the array.

3.6. Arraying

The exact procedure for arraying is well described in the instruction manual provided with the instrument from Beecher Instruments. The following instructions pertaining to arraying are specific points on the method and should not be considered all-inclusive directions on how to array.

The donor blocks and accompanying slides should be organized. A recipient block should be cast from low-melt paraffin (*see* **Note 3**). The recipient block should be cast in a mold as large as possible to provide the largest face for arraying onto. Great care should be exercised to avoid bubbles from forming underneath the plastic chuck. Deeper-than-standard recipient blocks are frequently constructed with paper molds but are at higher risk for air bubbles. The recipient block should then be *faced* on the same microtome that will be used to section the completed array. The recipient block is then placed in the block holder.

The choice of which needle goes on which side of the pivoting needle holder is arbitrary and left for the user to decide. Cleaning of the needles with solvent is discouraged, as it impairs the ability of the needles to extract the cores. Needles can be cleaned by repetitive insertion of the stylet. A bright light on an adjustable arm, with or without magnifying lens, will greatly assist in arraying. The 0 set points on the micrometers on the arrayer should be set at a location that is approx 2 mm from the left-forward corner of the recipient block as viewed when in place in the tissue arrayer. There is some variability between block holders, and this position will allow for this variability when constructing multiple blocks at the same time, as well as prevent the blocks from cracking during arraying.

Arraying is accomplished as outlined in the instruction manual provided with the arrayer. The choice of making the hole in the recipient block before or after removing the core from the donor block is left to the user. It is *not* advisable to make a series of holes in the recipient block and then attempt to come back and place the donor cores in them. Paraffin has a great deal of elasticity, and the holes will deform as additional holes are made.

If more than one recipient block is to be constructed on the same design, it is advisable that they be constructed simultaneously, with the core placed in each block, before moving the micrometers, rather than arraying one block (or portion thereof) and coming back and repeating the process.

Frequently, there is significant variability in the thickness of the donor blocks and the tissue within these blocks. Some users choose to deal with this by only limited sectioning of the recipient block, whereas others will *stack* multiple cores from a donor block within a single hole in the recipient block ***(5)***. This is best accomplished with a pair of fine-pointed forceps to break off the empty paraffin from the cores as they are extruded from the needle. Some cores can be difficult to remove from the donor block. This can be facilitated by either turning the needle handle while the needle is in the donor block or *tamping*, which is to lightly press down on the core with the stylet while it is still in the block. Nothing is better than practice.

3.7. Finishing and Sectioning of Recipient Array Block

Once arraying is complete, the surface of the recipient block should be flattened. This is best accomplished by warming the recipient block in an incubator at 37°C for 10 min and pressing the surface, subarray by subarray, with a clean glass slide. Then, the entire surface should be pressed at one time. Use no more pressure than a firm finger. This will flatten the surface and should improve the number of usable slides obtainable from the array block. The block should then be *tempered.* Tempering allows the paraffin to soften and then recrystallize, better adhering the cores to the surrounding paraffin (*see* **Note 4**). Tempering is accomplished by heating the recipient block to 35°C for 1–8 h in an oven and then cooling on a cooling plate (or in a –4°C freezer) for 15 min. This cycle should be repeated three to four times. These temperatures are based on paraffin with a 58–65°C melting temperature. Tempering dramatically improves the quality of the sections that are cut from the recipient block as well as the appearance of cores on the slides, with less cracking and fewer crescent defects.

There are two options for sectioning the array: the use of regular-charged (+) slides and the tape transfer slides from Instrumedics, Inc. (www.instrumedics.com). Sectioning to regular-charged (+) slides is straightforward and is not different from sectioning any other tissue block. The tape transfer system from Instrumedics works well for high-density tissue microarray slides, and it maximizes the number of cores that are retained on the slide and assures a perfectly aligned array without stretch. The ×4 slides (cat. no. PSA-4X) are preferred. The tape transfer system comes with instructions, but users should be aware that if the slides are sticky after exposure to the lamp (prior to or after removal of the tape), they should be exposed to the lamp for additional time to complete crosslinking of the acrylic; frequently this is more than 3 min of exposure to the lamp. The ultraviolet (UV) energy of the light is insufficient to damage the nucleic acids or proteins in the tissue.

The recipient blocks should be sectioned on the same microtome used for preparation of the block prior to arraying. Section thickness can be adjusted to preference; however, sections thinner than 5 μ*M* by the tape transfer method are frequently uneven in thickness. It is recommended to cut the entire block at one time, or at least in groups of 20 slides or more, rather than cut as needed. This maximizes the number of high-quality slides available from each block. Each time the recipient block is removed and replaced from the microtome, the subsequent realignment of the block to the blade results in the loss of more than 10 potential slides.

3.8. Storage of Recipient Array Blocks and Array Slides

Recipient array blocks should be stored at room temperature, in a dry "office-like" environment, the same as is used for donor blocks. Temperature and humidity changes should be avoided. There is controversy regarding the best storage for paraffin sections of tissue on glass, be it whole sections of tissue or tissue microarrays. The simplest recommendation is that humidity and oxygen are more important than temperature. Slides should be stored in a dry environment. Many laboratories find a desiccation chamber sufficient, whereas others will dip the sections in molten paraffin or store them under nitrogen gas.

3.9. Quality Control Issues

The simplest manner in which to check the quality of an array is to perform an H&E. It is important to determine the core retention as well as verify the correct tissue in each core at different depths of the block. To accomplish this, H&E should be performed once every 50 slides, or, if the block is removed and replaced on the microtome, the first slide should have an H&E performed on it. These H&Es should then be reviewed for accuracy of tissue within a core and the number of cores. Working with *normal surgical pathology material*, it is reasonable to expect to get between 100 and 200 sections from a single recipient block containing more than 50% of cores. This is largely dependent on the sectioning method and skill of the person making the sections. Using the tape transfer method, the first 50 sections from a block should have approx 95% of core present on the slide. It is suggested that the sections from the top of the block be reserved for final experiments, and that the deepest sections be used to work out the conditions of stains, which, when perfected, are applied to sections from the top of the block.

Additional quality control steps should be considered. Immunohistochemical staining conditions can vary by tissue preparation, and, in a tissue array, where each individual core of tissue has its own processing conditions, optimization of immunohistochemical staining conditions is essential. It is frequently beneficial to perform a stain with antibodies that may not be of scientific interest but verify the tissue is not "immuno-dead"—in other words, the tissue is processed correctly and antigens can be detected. Examples of antibodies that can be useful are vimentin, CD2O, pan-cytokeratins, and factor VIII. The array can then be reviewed quickly for tissue quality considerations. This method is not foolproof, but it provides reassurance that tissue antigenicity is retained. Similarly, a control probe for a housekeeping gene such as glyceraldehyde-3-phosphate dehydrogenase, β-actin, or Histone H3 should be performed with all *in situ* experiments to test RNA integrity.

3.10. Considerations for Immunohistochemistry and In Situ Hybridization

The basic protocols for immunohistochemistry and *in situ* hybridization of tissue microarrays are the same as those for regular tissue sections. However, tissue microarrays are susceptible to poorly optimized staining protocols, especially when donor blocks come from many sources. There are some special considerations that can improve the process. A common problem encountered in immunohistochemistry is inadequate deparaffinization. This is worse when dealing with tissue arrays and can be avoided with deparaffinization in xylene for 5 min times three changes. Tissue microarrays that are sectioned onto regular-charged (+) slides should be baked at 65°C for a minimum of 15 min. This improves adhesion of the tissue to the slide and reduces the chances of loss during staining. Cores are exceptionally at risk for loss during antigen retrieval by microwaving methodologies. The loss of cores is worse for smaller cores. Loss of cores can be minimized or avoided by using other antigen-retrieval methods (autoclaves, pressure cookers, rice cookers, or vegetable steamers) or by using the tape transfer method for sectioning slides.

With the use of Instrumedic slides, minor adjustments in the deparaffinization and dehydration steps provide more consistent staining conditions. The UV-sensitive acrylic adhesive becomes a trap for both H_2O and solvents. As a result, the slides and tissue on them come to equilibrium slower than regular slides. The key change is to increase the time in each step of the titration from xylene to buffer (deparaffinization/hydration) and buffer to xylene (dehydration) from the typical 2–3 min to 5 min/step. If the adhesive shows some background staining (check when the slides are in 90 or 70% ethanol), let the slides destain in 70% ethanol. This background can occur with eosin, hematoxylin, or the chromagens used in immunohistochemical staining. Antigen retrieval can be accomplished with any of the common methodologies (including microwaving) using adhesive slides without significant loss of cores. With prolonged microwave antigen retrieval (over 40 min) core loss has been observed.

In situ hybridization is becoming increasing popular with tissue microarrays. *In situ* hybridization is a challenging and laborious method, and, until the advent of tissue microarrays, most investigators have found it too difficult and too expensive to use on large numbers of specimens. The best results are obtained with radioactive probes, which can easily detect basal-level expression of single copy genes. Other detection methods are available, but they lack the sensitivity of radioactive probes. As stated previously, a control slide testing for RNA integrity is essential with all experiments. The suggestions made for immunohistochemical staining are the same as for *in situ* hybridization.

3.11. Data Collection

The greatest challenge in collecting and interpreting the data is finding qualified reviewers, but this challenge is the same as for immunohistochemistry. There are systems on the market from various vendors that can automate collection and archiving of the data. Some of these instruments provide tools for analysis as well. These instruments are expensive and require extensive training to use. No instrument replaces the interpretive skills of a pathologist or well-trained researcher; rather, they automate collection of the data and speed analysis. Most laboratories will find review of the tissue microarray with a standard microscope and qualitative (+/–) or semiquantitative (0,1,2,3,4) interpretation of the data more than adequate for data collection. Recording the results is easiest when using a blank template from which the array was designed and then inputting the data into a spreadsheet.

3.12. Data Analysis

Currently, the methods for tissue microarray analysis are varied and fluid and beyond the scope of this review. Consultation with a biostatistician is strongly recommended. It should be noted that the tools for analysis of cDNA microarrays are not necessarily valid for tissue arrays. In general, the data is not ratio-based or continuous (quantitative), and the choice of antibodies is directed.

3.13. Embedding Cells Grown in Culture for Tissue Microarraying

It is not uncommon to incorporate cell lines within a tissue microarray. Entire arrays of cell lines can be constructed. The method in **Subheading 3.14.** is one protocol for

embedding cells for this purpose. This protocol can be applied to cells from culture, aspirates, scrapings, or other nontissue sources (including magnetic beads). Protocol will work with as few as 5×10^6 cells, but the more cells the better. Determining cell numbers is not required. 5×10^6 cells will result in approx 75 cells/0.6 mm "spot" in a tissue array.

3.14. Method for Embedding Cells Grown in Culture for Tissue Microarraying

1. Make a solution of 3% SeaPlaque Agarose with PBS as the buffer. Cool in water bath to 42°C.
2. Collect cells and spin down into a pellet.
3. Remove supernatant so that a volume of supernatant equal to the volume of the cell pellet is present (i.e., 1:1 packed cells to supernatant).
4. Resuspend cells in residual supernatant by pipetting or vortexing.
5. Add equal volume of agarose.
6. Vortex.
7. Allow to cool on bench or in ice bucket.
8. Add fixative, or "knock out" of tube and place directly in histology cassettes.

Scrape to collect the adherent cells. Trypsin digestion use will alter the proteins expressed on the surface of the cells. The agarose should be cooled to 42°C prior to use. When added to cells at room temperature or cooler, it will solidify rapidly. Tubes should be vortexed individually as soon as agarose is added. Vortexing produces a more homogenous mixture than pipetting or other methods of mixing. Vortexing should be done with care so as not to add excessive air. Best results are obtained with cooling on ice, then banging tube on counter to loosen pellet.

4. Notes

1. Paraffin-embedded tissue refers to any tissue that has been processed through dehydration, with replacement of the H_2O in the tissue with paraffin. Prior to processing, tissue must be fixed. The most common fixative is 10% formalin; however, other fixatives work well. The most popular alternative fixative is 70% ethanol. Fixatives that include acids should be avoided, as they result in brittle tissue that will crack when cored.
2. The same is true for decalcifying agents.
3. The recipient block should be cast in a paraffin of equal or lower melting-temperature paraffin compared to the donor blocks. The use of harder, higher melting-temperature paraffin in the donor blocks is not an obstacle to array preparation.
4. This is similar to the process of embedding, when the tissue is always warmed and mixed with liquid paraffin. If cold tissue is used, the blocks crack and do not cut as well.

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7

A Streptavidin–Biotin-Based Microarray Platform for Immunoassays

Petra Pavlickova and Hubert Hug

Summary

The authors describe a microarray system for disease diagnosis based on antibody–antigen interactions. Either biotinylated antibodies or antigens are coupled via streptavidin linkers onto a gold surface. This platform has been used to establish recombinant antibody–antigen interactions and to detect specific IgM antibodies in sera of patients suspected of Lyme borreliosis. Therefore, this microarray system can be adapted for further applications.

Key Words:

Biotin; streptavidin; gold layer; immunoassay; *Lyme borreliosis*; protein microarray; biochip; recombinant antibodies.

1. Introduction

Unlike DNA biochips, which provide a measure for gene expression or mutation analysis, there is a need to implement protein microarray strategies that address the many different features of proteins that can be altered in disease. In the past few years, several strategies and technologies for study of protein–protein interactions, posttranslational protein modifications, and protein functions in cells on a proteomic scale were described ***(1–6)***.

The biochip platform reported here is based on gold-coated glass in a microscope slide format. Chip-to-chip reproducibility and the prevention of crosstalk between adjacent spots are ensured by a hydrophobic Teflon matrix ***(7)***. A streptavidin monolayer provides the interface for coupling biotinylated recombinant antibodies or antigens. This technology was used for the detection of IgM antibodies in the human sera of patients suspected of Lyme borreliosis ***(8)*** and for testing recombinant antibody–peptide (protein) antigen interactions ***(9)***. Recombinant antibody fragments are likely to be the major components in the construction of antibody chips for high-throughput screening of protein expression in human cells. They provide an excellent target-bind-

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ing specificity and can be produced in an automated fashion ***(10)***. Significant progress has been made in the use of recombinant antibodies in proteomic research ***(11)***. Recently, a recombinant antibody microarray with single-chain Fv fragments has been used to detect differences in cytokine expression in dendritic cells ***(12)***. Here, three different strategies of peptide and recombinant antibody immunoassays performed on a biochip platform based on the interaction between a streptavidin chip surface and a biotinylated probe are described in detail.

2. Materials

1. Protein chip platform XNA on Gold™ (ThermoHybaid, Ulm, Germany).
2. Fluorescent scanner (XNA ScanPro 20 microarray scanner) (ThermoHybaid, Ashford, UK).
3. Microarray analysis software AIDA 2.11 (Raytest; Straubenhard, Germany).
4. Synthetic peptide AA outer surface protein C8 (AAOspC8) (Biotin-AAVAESPKKP) (Staten Serum Institute, Copenhagen, Denmark) was from previous study ***(13)***. The 8-mer peptide is derived from C-terminus of OspC in the bacteria *Borrelia burgdorferi.*
5. Patient sera ($n = 5$) of Lyme borreliosis suspect patients containing IgG against the *Borrelia* flagellum and healthy controls ($n = 2$) were kindly donated by Dr. Michael Theisen (Staten Serum Institute, Copenhagen, Denmark).
6. Fluorescein isothiocyanate (FITC)-conjugated rabbit antihuman IgM antibody (DAKO, Copenhagen, Denmark).
7. Biotinylated recombinant antibodies Ab27 (scFv) and Ab28 (Fab) against human adhesion protein intracellular adhesion molecule (ICAM)-1, nonbiotinylated recombinant antibody Ab23 (scFv) recognizing synthetic peptide M42 (NH-FTFKEFQNNPNPRSLVK-COOH) (MorphoSys AG, Martinsried/Planegg, Germany). All recombinant antibody fragments were derived from the Human Combinatorial Antibody Library (HuCAL) system ***(14)***. The antibodies are epitope-tagged at their C-terminus with the FLAG tag (DYKDDDDK) and can be detected using the anti-FLAG antibody M2 ***(15)***. They are stored at –18°C.
8. Human recombinant ICAM-1 (R&D Systems, Wiesbaden, Germany).
9. Synthetic peptide M42 (Biotin-NH-FTFKEFQNNPNPRSLVK-COOH) with a purity >93%, derived from the α-chain of human integrin CD11b (ThermoHybaid, Ulm, Germany). It is stored at 4°C.
10. Goat monoclonal antimouse IgG-Cy5 antibody (Amersham Pharmacia Biotech AB, Uppsala, Sweden). It is stored in a dark environment.
11. Cy5 monofunctional dye (Amersham Pharmacia Biotech AB, Uppsala, Sweden).
12. Mouse IgG anti-FLAG (M2) antibody (Sigma-Aldrich, St. Louis, Missouri).
13. Bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, Missouri).
14. Wash phosphate-buffered saline-Tween-20 (PBST) buffer: 120 m*M* NaCl, 2.7 m*M* KCl, 10 m*M* phosphate buffer pH 7.4, 0.1% Tween-20 (v/v); store at 4°C.
15. PBS-BSA-T buffer: PBST buffer containing 1% BSA (w/v) prepared on the day of experiment.

3. Methods

Three types of immunoassays were performed on biochips. Either the recombinant antibody or the peptide antigen was immobilized on the streptavidin surface of the chip and detected directly or indirectly with labeled antigen or antibody, respectively. The first assay is based on immobilization of peptide antigens followed by incubation with human sera. The IgM–peptide interaction can be detected by an anti-IgM-FITC

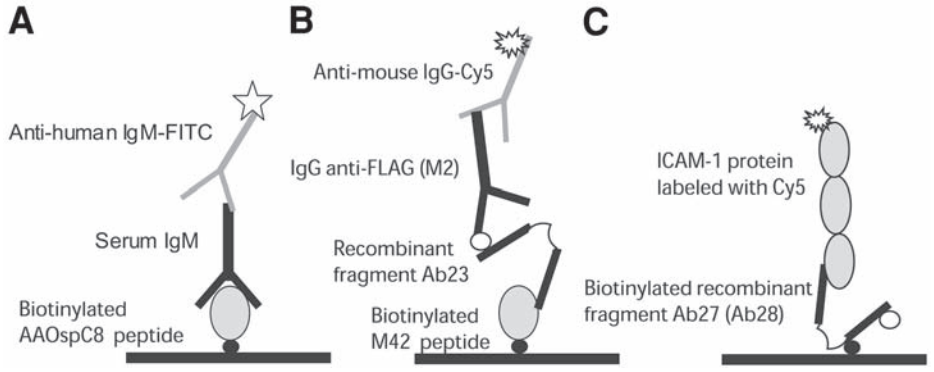

Fig. 1. Scheme of three immunoassays performed on biochip surface using biotin–streptavidin interaction. (**A**) Immobilization of peptide antigen AAOspC8 followed by incubation with human sera and subsequently with anti-IgM-FITC conjugate. (**B**) Immobilization of peptide antigen M42, interaction with recombinant antibody Ab23 containing a FLAG-tag, followed by incubation with an anti-FLAG (M2) antibody and detection with a secondary antibody conjugated to the fluorescent dye Cy5. (**C**) Immobilization of recombinant antibodies Ab27 and Ab28 detected with protein antigen ICAM-1 labeled with Cy5.

conjugate (*see* **Fig. 1A**). In the second assay, the immobilized peptide antigen was verified with a specific recombinant antibody containing a FLAG-tag followed by incubation with an anti-FLAG (M2) antibody. The reaction was detected with a secondary antibody conjugated to the fluorescent dye Cy5 (*see* **Fig. 1B**). In the third immunoassay, the recombinant antibody was immobilized on the chip surface and detected with protein antigen labeled with Cy5 (*see* **Fig. 1C**).

The following methods outline construction of the biochip platform (*see* **Subheading 3.1.**), immobilization of peptide and antibody probes on a streptavidin chip surface (*see* **Subheading 3.2.**), immunodetection by fluorescence-labeled target using both direct and indirect strategies (*see* **Subheading 3.3.**), reproducibility of the chip (*see* **Subheading 3.4.**), and kinetic studies using recombinant antibodies (*see* **Subheading 3.5.**).

3.1. Biochip Platform

The biochip platform is based on a nanoscale biocompatible sensor architecture constructed by using the principles of self-assembly techniques on gold ***(5,16)***. The glass slide is coated with a 0.1 μm 24-carat gold layer. Layered onto this is a self-assembly monolayer (SAM), a long chain of thiol alkanes (16-mercaptohexadecanoic acid), to which biotin is covalently coupled via linker molecules [*N*-hydroxysuccinimide and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide]. Biotin-terminated SAMs are used to immobilize a streptavidin layer with high-packing density. The interaction between biotin and streptavidin is strong (kDa approx 10^{-15} *M*), ensuring the integrity of the protein chip surface under a wide range of experimental conditions. The streptavidin sensor surface can be used to couple any biomolecule (nucleic acid,

protein, lectin, saccharide, lipid, etc.) that is conjugated to biotin (*see* **Fig. 2**). The protein biochip design is based on a standard microscope slide format. Two sets of 96 or 384 spots (i.e., 192 or 768 total spots) are available for conducting biomolecular interactions. The individual gold spots with an area of 1.8 mm^2 are separated from each other by a Teflon layer. The hydrophobic Teflon surface prevents crosstalk between the adjacent spots and virtually eliminates microarray smearing. The chips are compatible with fluorescence, mass spectrometry, and radio-imaging detection devices.

3.2. Immobilization of Biotinylated Probes onto Streptavidin-Coated Chip Surfaces

Different probes were coupled onto the chip surface to test their binding properties and dynamic range.

3.2.1. Immobilization of Synthetic Peptide AAOspC8

1. Dissolve AAOspC8 in PBST buffer to obtain a series of concentrations in the range of 0.1–25 ng/mL.
2. Pipet 1 µL of biotinylated sample onto each chip in duplicate and incubate at room temperature for 30 min (*see* **Note 1**).
3. After the immobilization of AAOspC8 probes, rinse the chip with sterile H_2O and subsequently wash three times for 5 min in PBST with gentle agitation.
4. Rinse the chip with sterile H_2O and dry under nitrogen gas stream (*see* **Note 2**).

3.2.2. Immobilization of the Synthetic Peptide M42

1. Dissolve the biotinylated peptide M42 probe in PBST buffer to obtain a series of concentrations in the range of 0.5–15 µg/mL.
2. Spot 1 µL of the biotinylated sample onto each chip in duplicate (*see* **Note 1**), and incubate at room temperature for 60 min.
3. After immobilization, rinse the chip with sterile H_2O and wash three times for 5 min in PBST with gentle agitation.
4. Rinse the chips with sterile H_2O and dry under nitrogen (*see* **Note 2**).

3.2.3. Immobilization of Recombinant Antibody Fragments Ab27 (scFv) and Ab28 (Fab)

1. Dissolve biotinylated recombinant antibodies Ab27 and Ab28 probes in PBS-BSA-T buffer to obtain a series of concentrations in the range of 0.1–5 µg/mL (*see* **Note 3**).
2. Spot 1 µL of biotinylated sample onto each array in duplicate (*see* **Note 1**), and incubate at room temperature for 60 min in all experiments except the kinetic study, where incubation is for 40 min (*see* **Subheading 3.5.**).
3. After immobilization, rinse the chip with sterile H_2O and wash three times for 5 min in PBST with gentle agitation.
4. Rinse the chips with sterile H_2O and dry under nitrogen (*see* **Note 2**).

3.3. Immunodetection

The dried chips with the immobilized peptide (*see* **Subheadings 3.2.1.** and **3.2.2.**) or antibody (*see* **Subheading 3.2.3.**) probe were incubated with an appropriate target and detected using a fluorescence scanner.

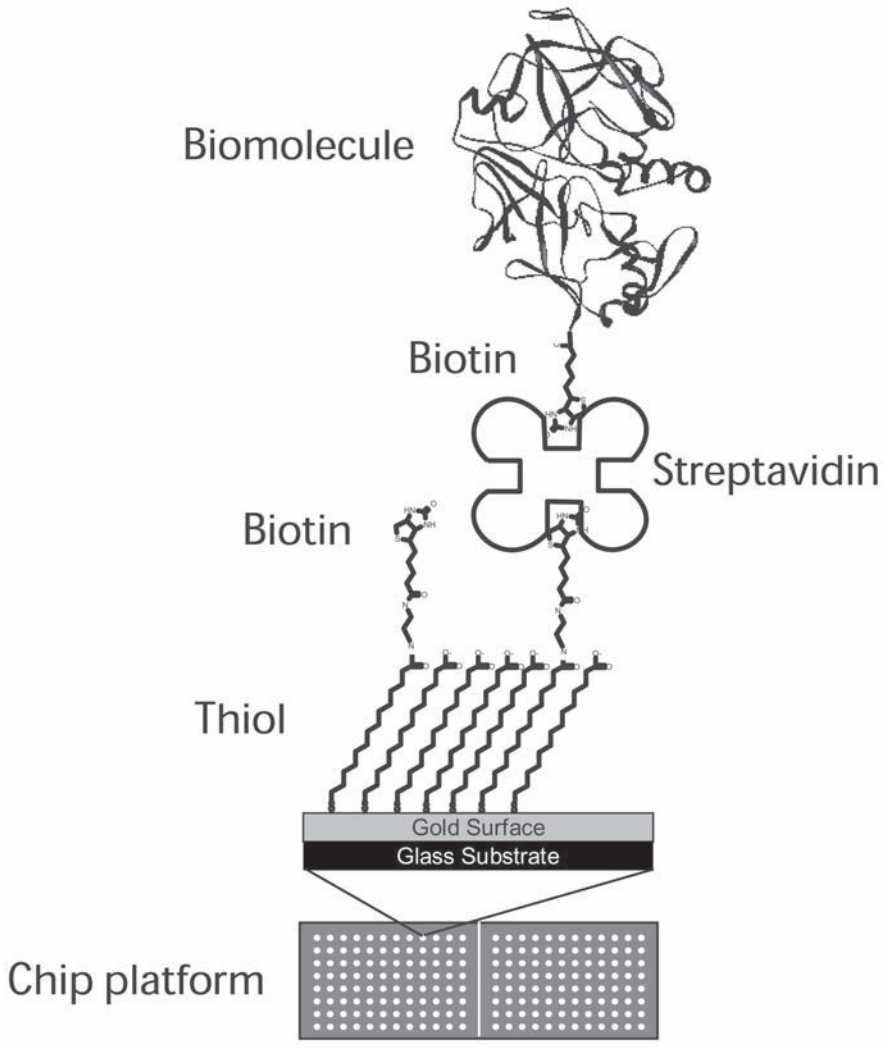

Fig. 2. Schematic of the biochip platform. Biotinylated architectures are self-assembled onto gold surface to couple streptavidin with high-packing density. Many kinds of biotinylated probes can then be immobilized onto this surface. Teflon matrix (regions around the gold spots) prevents crosstalk between adjacent spots.

3.3.1. Immunodetection of IgM in Sera of Lyme Borreliosis Suspect and Healthy Patients

1. Dilute the human sera in 1/10 PBST buffer and pipet it onto the spots of the chip containing the immobilized AAOspC8 probes (*see* **Subheading 3.2.1.**). Incubate at room temperature for 90 min in a humidified chamber (*see* **Note 1** and **Fig. 3**).

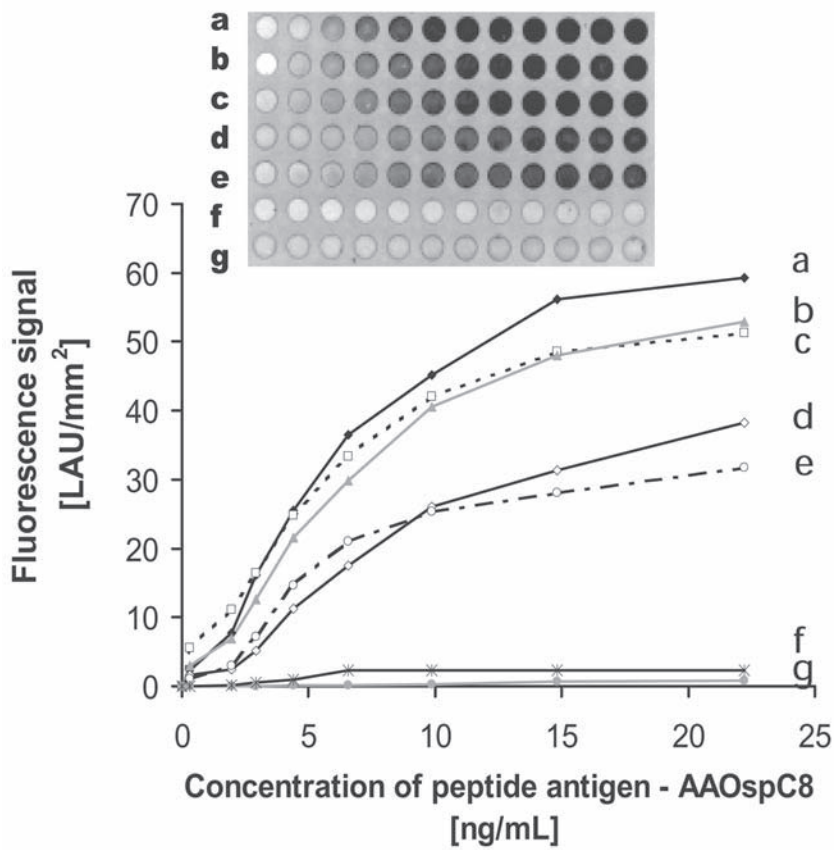

Fig. 3. Quantification of biotinylated peptide antigen AAOspC8 using the biochip platform. The AAOspC8 antigen probe was coupled on the streptavidin chip surface at different concentrations (0–25 ng/mL) at room temperature for 30 min, followed by incubation with five different sera from Lyme borreliosis suspect patients (**a–e**) and two sera from healthy donors (**f**,**g**) (diluted 1/10 in PBS-T). Human IgM antibodies were detected by rabbit antihuman IgM antibody labeled with FITC (diluted 1/40 in PBS-T). The AAOspC8 antigen was coated onto the chip vertically in columns, whereas the sera were spotted in horizontal directions (**a–g**).

2. Rinse the chips with sterile H_2O and wash three times for 5 min in PBST with gentle agitation.
3. Rinse the chips with sterile H_2O and dry under nitrogen.
4. Dilute FITC-conjugated rabbit antihuman IgM antibody in 1/40 PBST immediately after the drying step, and incubate at room temperature for 60 min in a dark environment (*see* **Note 1**).
5. Rinse the chips with sterile H_2O and wash three times for 5 min in PBST.
6. Drop PBST buffer onto the chip surface, cover with a glass slide, and immediately scan using a fluorescent scanner (*see* **Note 4**).

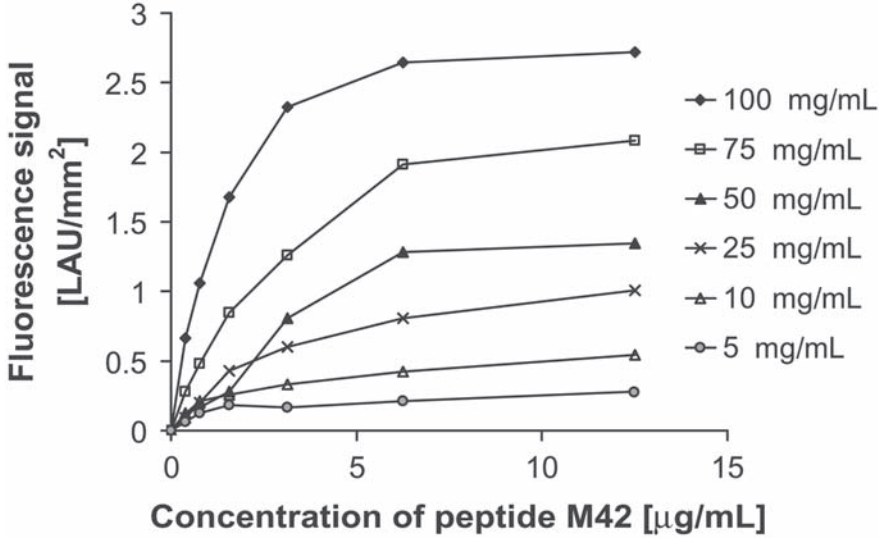

Fig. 4. Indirect immunoassay based on immobilized peptide antigen M42 interaction with a specific recombinant antibody fragment Ab23. The M42 probe was coated onto the chip surface at different concentrations (0.5–15 μg/mL) and subsequently incubated with Ab23 antibody (scFv fragment containing FLAG tag) at concentrations ranging from 5–100 μg/mL. A monoclonal anti-FLAG antibody was used for reaction with the secondary anti-IgG antibody conjugated to Cy5 to detect the immunoreaction.

3.3.2. Indirect Assay: Interaction of Immobilized M42 Peptide Probe With Recombinant Fragment Ab23 Target Using Anti-FLAG Antibody

1. Dissolve recombinant antibody fragment Ab23 (*see* **Note 3**) in PBS-BSA-T at concentrations ranging between 5 and 100 μg/mL and spot 1-μL aliquots onto the dried chip with immobilized M42 peptide (*see* **Subheading 3.2.2.** and **Fig. 4**). Incubate at room temperature for 60 min in a humid case.
2. Rinse the chips with sterile H_2O and subsequently wash three times for 5 min in PBST with gentle agitation.
3. Rinse the chips with sterile H_2O and dry under a nitrogen gas stream.
4. After drying, incubate the chips with mouse IgG anti-FLAG (M2) antibody at room temperature for 30 min (c = 20 μg/mL, diluted with PBS-BSA-T, 1 μL/spot) (*see* **Note 1**).
5. Rinse the chips with sterile H_2O and wash three times for 5 min in PBST with gentle agitation.
6. Rinse the chips with sterile H_2O and dry under nitrogen.
7. Incubate the chips with a goat antimouse IgG-Cy5 conjugate (diluted 1:500 with PBS-BSA-T, 1 μL/spot) at room temperature for 30 min in a dark environment (*see* **Note 1**).
8. Rinse the chips with sterile H_2O and wash three times for 5 min in PBST with gentle agitation.
9. Drop PBST buffer onto the chip surface, cover with a glass slide, and immediately scan using a fluorescent scanner (*see* **Note 4**).

3.3.3. Direct Assay: Detection of Immobilized Recombinant Antibody Fragments Ab27 (Ab28) With Cy5-Labeled Protein Antigen ICAM-1

1. Dilute ICAM-1–Cy5 in PBST buffer to obtain seven different antigen target concentrations: a = 100, b = 50, c = 25, d = 12.5, e = 6.1, f = 3.1, g = 1.6 μg/mL (*see* **Note 5**).
2. Incubate the dried antibody-immobilized chips (*see* **Subheading 3.2.3.**) with the ICAM-1 antigen (1 μL/spot) at room temperature for 60 min in a dark environment (*see* **Fig. 5** and **Notes 1** and **2**).
3. Rinse the chips with sterile H_2O and subsequently wash three times for 5 min in PBST with gentle agitation.
4. Drop PBST buffer onto the chip surface, cover with a glass slide and immediately scan using a fluorescent scanner (*see* **Note 4**).

3.4. Chip-to-Chip Variability Test

The use of biotin–streptavidin technology enables highly reproducible data by its specific interactions. In a model system, the variation coefficient can be as low as 6% in reproducibility tests of biochips using the immobilized recombinant antibody fragment Ab27 (*see* **Note 6**).

1. Couple Ab27 onto the chip surface (*see* **Subheading 3.2.3.**).
2. Incubate the protein antigen ICAM-1 (c = 20 μg/mL) with surface-immobilized Ab27 antibody probe as described above (*see* **Subheading 3.3.3.**).
3. Repeat the same experiment five times on 5 different days.

3.5. Kinetic Study of Recombinant Antibody–Protein Antigen Interactions on the Chip Surface

Kinetic analysis can be performed on chip surfaces containing the recombinant antibody Ab27 by incubation with an appropriate antigen target at different times (*see* **Note 7**).

1. Immobilize Ab27 onto the chip surface for 40 min as described previously (*see* **Subheading 3.2.3.**).
2. Incubate the dried antibody-immobilized chip with protein antigen ICAM-1 (c = 20 μg/mL) labeled with Cy5 for 1, 2, 5, 10, 20, 30, 40, 50, 60, 75, and 90 min at conditions described previously (*see* **Subheading 3.3.3.**).

4. Notes

1. The surface immobilization step and immobilized probe-target interaction should be conducted within a humidified plastic chamber with moistened tissue to minimize evaporation effects. Cover the humidified chamber with a tight lid immediately after spotting. Incubation of the immobilized probe with a corresponding target at 37°C is not recommended.
2. The dried chips should be immediately used for immunodetection. Storage of the dry chips with immobilized peptide or recombinant antibody probes for longer than 24 h (at 4°C) results in a significant decline of the final fluorescence signal. No blocking reagent is needed either during antigen immobilization or prior to target incubation.
3. Purified and biotinylated antibody fragments Ab27 (scFv), Ab28 (Fab), and purified nonbiotinylated antibody fragment Ab23 (scFv) were generated against the respective

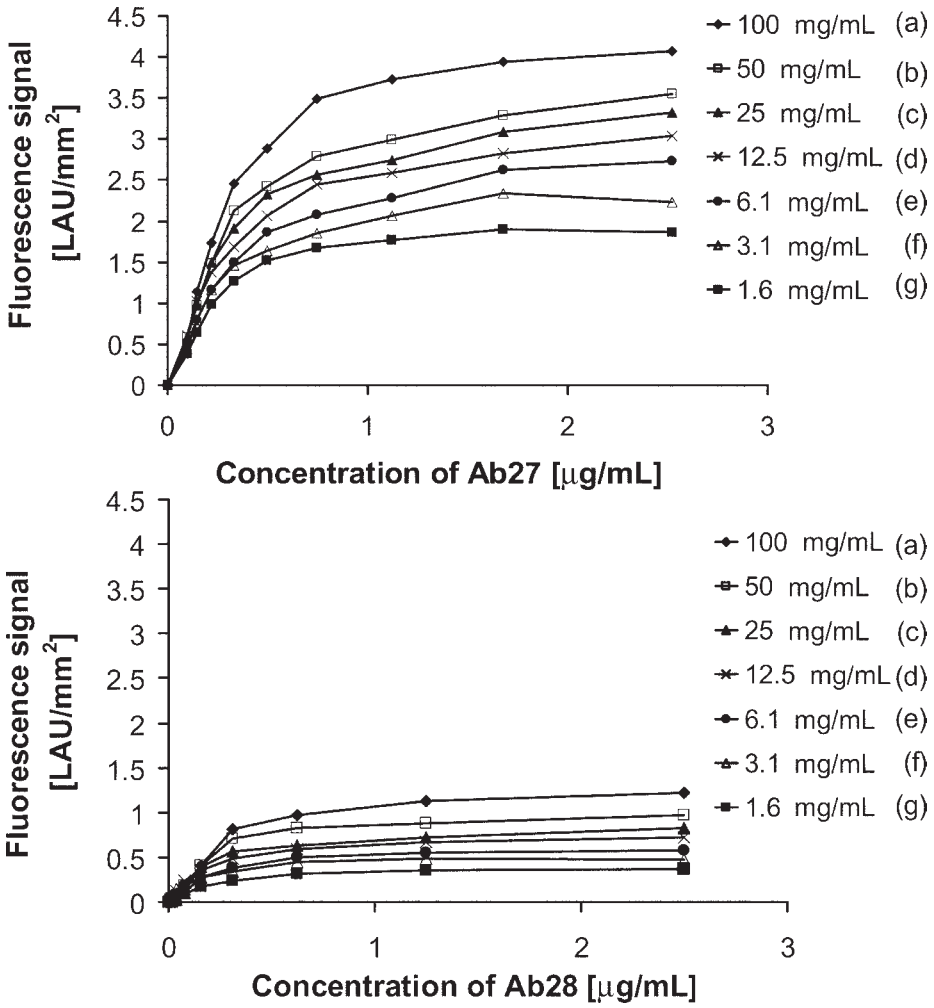

Fig. 5. Direct immunoassay based on immobilized recombinant fragment Ab27 (scFv) or Ab28 (Fab) recognizing ICAM-1 protein antigen labeled with Cy5. The biotinylated Ab27 (Ab28) were coupled onto the streptavidin chip surface at concentrations ranging from 0.1–2.5 μg/mL. The antiboidies were incubated with ICAM-1 antigen labeled with Cy5 at seven different concentrations (**a–g**).

antigens by solid phage-display panning and were selected for a recently published study ***(9)*** that compared the antibody types (scFv and Fab) and to compare their affinities (kDa of 10–100 n*M*). The detection limit for the immobilized antibody (Ab27, Ab28) probes was found to be 0.1 μg/mL (3–30 fmol/spot) and 0.5 μg/mL (200 fmol/spot), respectively, for the M42 peptide antigen.

4. Scanning the chip in a dry state will lead to a dramatic reduction of the signals. Therefore, scanning with a thin glass cover slide in washing buffer is highly recommended. Washing buffer should be between the chip surface and the cover slide. Air bubbles can be removed by gently moving the cover slide on top of the chip to the left and right. The chip bottom (edges and surface of the cover slide) should be dried with a tissue prior to scanning. A He-Ne laser (excitation wavelength: 633 nm, absorption wavelength: 675 nm), with a resolution selections of 16 bits per pixel and 50 μm pixel size should be chosen for scanning of the chips. The fluorescence signal is expressed as linear arbitrary unit across 1-mm^2 area (LAU/mm^2).
5. Fluorescence labeling of protein ICAM-1 with Cy5 monofunctional dye was performed according to the instruction manual. The molecular ratio of the ICAM-1/dye was found to be 2:4. The stock solution was stored in a dark environment at 4°C for 2 wk or at –18°C for a longer time period.
6. All dilutions and sample solutions should be freshly prepared on the day of the experiment. All experiments should be performed at least in duplicate on the same chip surface. The coefficient of variation (CV) (%) can be calculated from the ratio of standard deviation (*SD*)/average value × 100. In the authors' experiments, the CV for the fluorescence signals obtained from the values on the different biochips was in the range between 1.5 and 5.8% for recombinant antibody chips with immobilized Ab27. The intensity of the Cy5 signal corresponding to the ICAM-1 target varies with the Ab27 concentrations immobilized on the chips.
7. The time-course of immunobinding of the ICAM-1 antigen target onto the immobilized Ab27 depends on the target concentration. A higher target concentration (above c = 20 μg/mL) results in a faster interaction: The half-maximal signal can be reached after 5 min, and the signal saturation can be reached after 40 min. Also, better signal-to-noise ratios can be obtained using 20 μg/mL ICAM-1 concentration. Target concentrations below 1 μg/mL lead to two times slower immunobinding ***(9)***.

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8

Site-Specific Immobilization of Biotinylated Proteins for Protein Microarray Analysis

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Summary

The postgenome era has led to a new frontier of proteomics that requires the development of protein microarray, which enables us to unravel the biological function of proteins in a massively parallel fashion. Several ways of immobilizing proteins onto surfaces have been reported, but many of these attachments are unspecific, resulting in the unfavorable orientation of the immobilized proteins. His_6 tag has been used to site-specifically immobilize proteins onto nickel-coated slides, which presumably oriented proteins uniformly on the surface of the slide. However, the binding between Ni-NTA and His tag proteins is not strong, causing the immobilized proteins to dissociate from the slide even under simple wash conditions. The authors have developed a novel strategy of using an intein-mediated expression system to generate biotinylated proteins suitable for immobilization onto avidin-functionalized glass slides. Array-scan results not only show successful immobilization of proteins onto slides by antibody detection method but also full retention of biological activities of the immobilized proteins. The strong and specific interaction between biotin and avidin also permits the use of stringent incubation and washing conditions on the protein microchip, thus making it a highly robust method for array studies.

Key Words:

Protein array; C-terminal biotinylation; biotin–avidin interaction; high-throughput; intein.

1. Introduction

With the completion of the Human Genome Project, one can estimate that the number of proteins in the human proteome could vary from approx 40,000 to as many as 1 million. The DNA microarray technology has allowed simultaneous expression profiling of thousands of genes from various tissues and organisms ***(1)***. However, it is well known now that at the cellular level the relative abundance of messenger RNA does not always correlate to its protein expression level. Therefore, to study the large

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number of proteins present in an organism, protein microarray, which adopts the same spotting technology used to fabricate DNA microarray, is being developed ***(1)***. It promises to provide a means for high-throughput identification and quantification of proteins from biological samples ***(2,3)***.

With a protein microarray, tens of thousands of proteins are immobilized on a solid surface, such as a glass slide ***(4)***. Currently, one of the main challenges in the fabrication of protein microarray is the ability to immobilize proteins in their native conformation on surfaces and to preserve their active sites for functional studies ***(4)***. Several approaches have been developed to immobilize proteins, as well as other biomolecules, onto glass surfaces ***(4–6)***. In most cases, however, these modes of attachments are nonspecific, causing the molecules to be immobilized in the wrong orientation. Numerous strategies have been reported recently that allow site-specific immobilization of molecules in the microarray format ***(7–10)***. To date, there has been only one report of site-specific attachment of proteins on glass slides using His tag ***(7)***. However, the binding between Ni-NTA and His-tag proteins is not very stable and is often susceptible to interference by many commonly used chemicals and salts ***(10,11)***.

The authors have recently exploited the high affinity of avidin for biotin in protein microarray applications ***(10)***. The tight and specific noncovalent interaction between biotin and avidin results in their extensive use in research for detection, immobilization, and purification. Each avidin molecule can bind rapidly and almost irreversibly up to four molecules of biotin. Historically, biotinylation has been carried out using chemical reagents that lack site specificity, which can lead to inactivation of some biological molecules. Biotin ligase is also commonly used to covalently attach biotin to a particular lysine residue in a unique protein sequence ***(12–15)***. This enzyme can site-specifically biotinylate chimeric proteins, both in vitro and in vivo ***(16,17)***. Unfortunately, this enzymatic method has its own limitations. Overexpression of fusion proteins in bacterial cells often results in the formation of inclusion bodies that greatly decrease the efficiency of biotinylation in vivo. Even with a high level of soluble protein expressed, an appreciable fraction of the chimeric protein is not biotinylated. Furthermore, a high level of the fusion protein expressed in *Escherichia coli* can also be toxic to the host cell. The toxicity results from decreased biotinylation of the essential *E. coli* lipid synthetic protein AccB resulting from competition for endogenous ligase. In vitro biotinylation is used when expression of the soluble fusion protein in *E. coli* is insufficiently biotinylated. However, the most common problem faced during in vitro biotinylation is the presence of proteases. In addition, numerous reagents (i.e., NaCl, glycerol, and ammonium sulfate) commonly present in biological buffer have also inhibited activity of biotin ligase, thus reducing the efficacy of in vitro biotinylation.

Intein-mediated protein expression was developed originally for purification of fusion proteins on chitin columns, and it has recently found wide applications in protein engineering where the expressed protein ligation strategy is used ***(18–21)***. Recently, this strategy was extended to the modification of proteins at their C-termini with numerous chemical tags ***(22)***. By taking advantage of a similar approach and using the

intein affinity tag, the authors recently developed a method to purify and site-specifically biotinylate recombinant proteins at their C-terminal end within a single column-purification step. With this approach, they were able to use intein-mediated expression of proteins to generate site-specifically biotinylated proteins with high efficiency ***(10)***. Subsequently, these biotin-labeled proteins can be immobilized onto avidin-functionalized glass slides for array studies (**Fig. 1**).

2. Materials

2.1. Chemical Synthesis of Cysteine-Biotin (Cys-Biotin)

1. Boc-protected or Fmoc-protected cysteine (Advanced ChemTech, Louisville, KY).
2. *N*-tetramethyluronium tetrafluorobonate (TBTU) (Advanced ChemTech).
3. *N*-hydroxybenzotriazole (HOBt) (Advanced ChemTech).
4. High-pressure (performance) liquid chromatography (HPLC)-grade dimethyl formamide (DMF).
5. Dichloromethane (CH_2Cl_2).
6. Magnesium sulphate ($MgSO_4$).
7. Sodium carbonate (Na_2CO_3).
8. Ethyl acetate.
9. Methanol (MeOH).
10. 4-methyl morpholine.
11. Biotinylethylenediamine.
12. Trifluoroacetic acid (TFA).
13. Triisopropylsilane.
14. Triethylamine.
15. Piperidine.
16. Ethanedithiol (EDT).
17. Dichloromethane (DCM).
18. HPLC.
19. C_{18} reverse-phase column.
20. Polypropylene vials.
21. Solvent-resistant vacuum pump.
22. Nitrogen tank.

2.2. Expression Plasmid Construction

1. pTVB1 expression vector (New England Biolabs, Beverly, MA).
2. *E. coli* strains DH5α and ER2566 (New England Biolabs, Beverly, MA).
3. Oligonucleotide primers.
4. Deoxynucleotide 5'-triphosphates (dNTPs) (Promega, Madison, WI).
5. Deep Vent polymerase (New England Biolabs, Beverly, MA).
6. Restriction enzymes (*Nde*I and *Sap*I) (New England Biolabs, Beverly, MA).
7. T4 DNA liagse (New England Biolabs, Beverly, MA).
8. Shrimp alkaline phosphatase (Promega, Madison, WI).
9. Agarose (Bio-Rad).
10. Thermal cycler (MJ Research, San Francisco, CA).
11. DNA gel electrophoresis equipment (Bio-Rad).
12. DNA sequencing reagents and equipment (Applied Biosystems, Foster City, CA).

2.3. Expression and Extraction of Fusion Protein

1. Luria Bertani (LB) media.
2. Ampicillin.
3. Isopropyl-β-D-thiogalactopyranoside (IPTG) (Bio-Rad).
4. Lysis buffer: 20 m*M* Tris-HCl, pH 8.0, 500 m*M* NaCl, 1 m*M* ethylenediaminetetraacetic acid (EDTA), 0.1% Triton X-100.
5. Incubator shaker.
6. Orbital shaker.
7. Ultrasonic liquid processor.

2.4. Affinity Purification and C-Terminal Biotinylation of Expressed Proteins

1. Chitin beads (New England Biolabs, Beverly, MA).
2. Disposable column (Bio-Rad).
3. Column buffer: 20 m*M* Tris-HCl pH 8.0, 500 m*M* NaCl, 1 m*M* EDTA.
4. Peristalic pump.
5. Protein assay (Bio-Rad).
6. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) reagents and equipment.
7. Silver nitrate (Sigma).
8. Polyvinylidene difluoride (PVDF) membrane (Bio-Rad).
9. Whatman filter paper no. 1.
10. Transblot reagents and equipment.
11. Nonfat dry milk powder.
12. Phosphate-buffered saline with Tween-20 (PBST): 0.1% Tween-20 in phosphate-buffered saline, pH 7.4.
13. Monoclonal antibody against target protein.
14. Horseradish peroxidase (HRP)-conjugated streptavidin (New England Biolabs, Beverly, MA).
15. Enhanced ChemiLuminescent kit (Amersham, UK).
16. Western blotting reagents and equipment.
17. Lyophilizer.

2.5. Spotting of Biotinylated Proteins onto Avidin Slides and Fluorescent Antibody-Based Detection

1. Microscope glass slides (Fisher or Sigma) and slide tray.
2. Sulfuric acid (H_2SO_4).
3. Hydrogen peroxide (H_2O_2).
4. Glycidoxypropyltrimethoxisilane (Sigma-Aldrich, St. Louis, MO).
5. Acetic acid.
6. Ethanol.
7. Cover slips.
8. Avidin (Pierce).
9. Sodium bicarbonate ($NaHCO_3$).
10. Aspartic acid.
11. Arrayer (ESI SMA™, Toronto, Canada).
12. 384-well polypropylene source plate.
13. Monoclonal antibody against target protein.
14. Fluorescent dye; FLUORESCEINISOTHIOCYANATE (Molecular Probe); Cy3 and Cy5 (Amersham , UK).

15. NAP5 column (Amersham, UK).
16. PBS buffer.
17. Tween-20.
18. Bovine serum albumin (BSA).
19. ArrayWoRx™ microarray scanner (Applied Precision).

3. Methods

The methods described in the following sections outline the chemical synthesis of the Cys-biotin, the construction of the expression plasmid, the expression of fusion protein, the affinity purification and C-terminal biotinylation of expressed proteins, and the spotting of biotinylated proteins on avidin slides and fluorescent antibody-based detection.

3.1. Chemical Synthesis of Cys-Biotin

Biotin-labeled cysteine can be synthesized with either Boc-protected or Fmoc-protected cysteine.

3.1.1. Chemical Synthesis of Cys-Biotin Using Boc-Protected Cysteine

1. Dissolve 1.2 g of *N*-α-*t*-Boc-*S*-Trityl-L-cysteine (2.6 mmol), 1.0 g of TBTU (3.10 mmol), and 0.60 g of HOBt (3.9 mmol) in 50 mL of dry DMF.
2. Stir the mixture under argon for 20 min at room temperature.
3. Add 0.75 g of 4-methyl morpholine (7.8 mmol) and 0.75 g of biotinylethylenediamine (2.6 mmol).
4. Stir the reaction mix for another 3 h, followed by evaporation *in vacuo.*
5. Dissolve the crude product in 200 mL of CH_2Cl_2.
6. Extract with 3X 200 mL of H_2O, dry over $MgSO_4$, and concentrate the crude product *in vacuo*.
7. Further purify the crude product by flash chromatography (4–8% MeOH in CH_2Cl_2) to give the protected form of Cys-biotin.
8. Deprotect the synthesized Cys-biotin by first stirring in a solution containing 50 mL TFA, 1.6 mL H_2O, and 1.2 g, tri-isopropylsilane (7.8 mmol) for 30 min before evaporation *in vacuo.*
9. Dissolve the resulting residue in a mixture of 1:1 H_2O/CH_2Cl_2 (200 mL).
10. Extract the aqueous layer with 3X 100 mL of CH_2Cl_2 before evaporation to dryness.

3.1.2. Chemical Synthesis of Cys-Biotin Using Fmoc-Protected Cysteine

1. Dissolve 0.996 g of *N*-Fmoc-*S*-Trityl-L-cysteine (1.7 mmol), 0.674 g of TBTU (2.1 mmol) and 0.3989 g of HOBt (2.6 mmol) in 17 mL of DMF.
2. Stir the mixture for 30 min at room temperature.
3. Add 0.5 g of biotinylethylenediamine (1.7 mmol) and 0.515 g of triethylamine (5.1 mmol).
4. Allow the reaction to be carried out under nitrogen for 3 h at room temperature, followed by concentration *in vacuo.*
5. Dissolve the resulting residue in 50 mL of ethyl acetate and extract with 1.0 *M* HC (50 mL), 10% Na_2CO_3 (50 mL), saturated NaCl (50 mL).
6. Dry over $MgSO_4$, and then evaporate to dryness.
7. Add 15 mL of 20% piperidine in DMF to the resulting residue and stir for 30 min at room temperature before evaporation.

8. Dissolve the residue in ethyl acetate and wash with 50 mL 2X 10% Na_2CO_3 and saturated 50 mL NaCl.
9. Dry over $MgSO_4$, and then evaporate to dryness.
10. Dissolve the residue in 15 mL of TFA/EDT/H_2O (9/0.5/0.5) and stir for 1 h before evaporation to dryness.
11. Dissolve the resulting residue in 100 mL of 1:1 DCM/H_2O and remove insoluble solid by filtration.

3.1.3. Purification of Synthesized Cys-Biotin

Final purification of the product from both syntheses was done using HPLC with a C_{18} reverse-phase column to give the final product as a white solid (69% and 39% overall yield for Boc and Fmoc synthesis, respectively). ^{1}H NMR (400 MHz, D_2O) δ 4.57 (dd, 1H, *J* = 7.8, 5.0), 4.39 (dd, 1H, *J* = 7.8, 5.0), 4.12 (t, 1H, *J* = 5.4), 3.45 (m, 1H), 3.33–3.24 (m, 4H), 3.03 (dd, 1H, *J* = 14.9, 5.4), 3.00–2.93 (m, 2H), 2.74 (d, 1H, *J* = 13.2), 2.22 (t, 2H, *J* = 7.3), 1.72–1.50 (m, 4H), 1.48–1.31 (m, 2H); ^{13}C NMR δ 179.62, 170.46, 64.53, 62.70, 57.79, 56.01, 42.16, 42.12, 41.45, 37.96, 30.39, 30.12, 27.50, 27.30; ESI MS 390.2 (MH^+).

3.2. Construction of the Expression Plasmid

The construction of the expression plasmid is described in **Subheadings 3.2.1.** and **3.2.2.** It includes the description of the pTVB1 expression vector and the cloning of target genes into pTVB1 expression vector.

3.2.1. pTVB1 Expression Vector

The pTVB1 (*see* **Fig. 2**) expression vector is a C-terminal fusion vector in which the C-terminus of the target protein is fused to the intein tag. Transcription of the fusion gene is initiated from the pTVB1 T7 promoter ***(23)*** under the tight control of a *lac* operon. Binding of the *lac* repressor (encoded by the *lac*I gene in pTVB1) to the *lac* operator sequences immediately downstream of the T7 promoter suppresses basal expression of the fusion gene in the absence of IPTG induction. pTVB1 vector also carries an ampicillin resistance gene (*Ampr*) for selection of transformed host strain. The ATG codon of the *Nde*I site in the multiple cloning region is used to initiate translation of the fusion protein, whereas the *Sap*I site is used to clone the 3' end of the target gene into pTVB1 to fuse the C-terminus of the target protein to the N-terminal cysteine of the intein tag. The use of *Sap*I allows the target protein to be purified with no extra vector-derived amino acid residues added to the native sequences of the target protein. *Sap*I also allows the addition of extra amino acid residues at the C-terminus of the target protein to either prevent in vivo cleavage or favor in vitro cleavage (*see* **Note 1**).

3.2.2. Cloning of Target Genes into pTVB1 Expression Vector

The expression plasmid was constructed using standard molecular biology techniques ***(24)*** and, thus, not described in detail in this section. The target gene fragment was first polymerase chain reaction (PCR)-amplified and cloned into the expression vector pTYB1. PCR amplification of target gene fragment was done using an upstream primer (5'-GC GGC GGT *CAT ATG* $N_{15–18}$-3') containing an *Nde*I site with a transla-

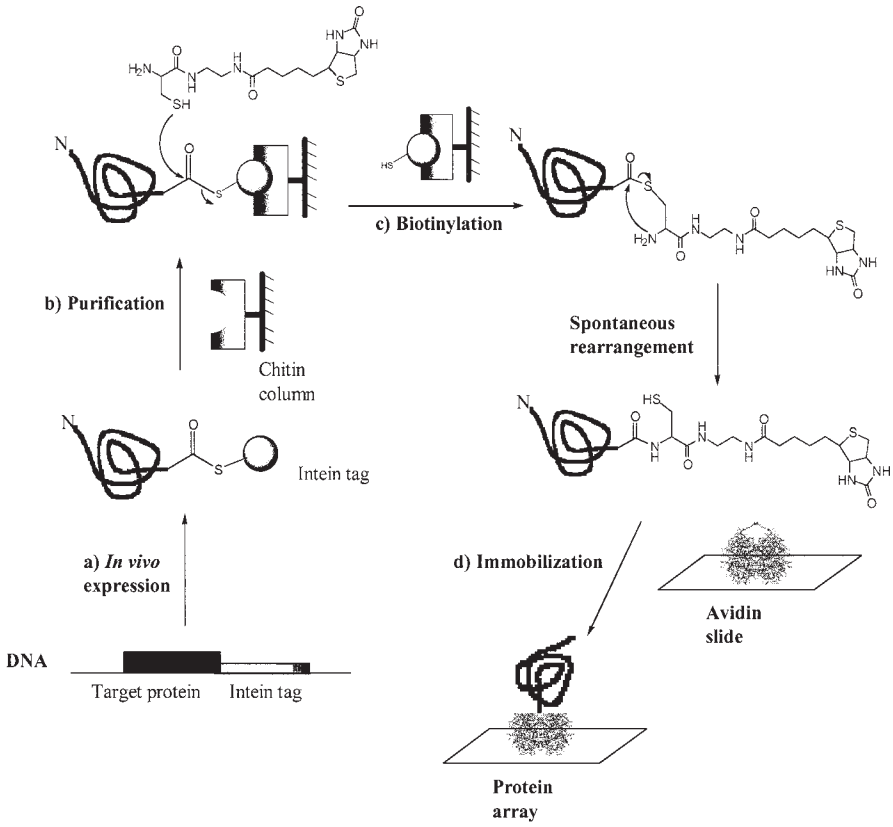

Fig. 1. Overview of the biotinylation and immobilization strategy.

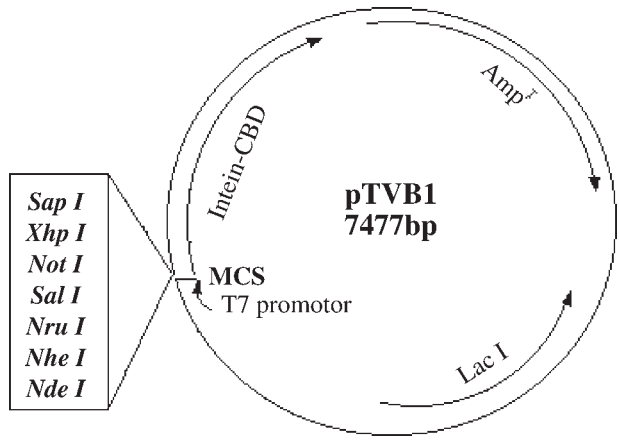

Fig. 2. Map and multiple cloning sites (MCSs) of pTVB1 expression vector.

tion initiation codon (ATG) and the sense strand sequence of the target gene. The downstream primer (5'-GGT GGT T*GC TCT TCC* GCA $N_{15–18}$-3') contains a *Sap*I site followed by the C-terminal antisense strand sequence of the target gene. PCR was performed in a 100-μL reaction scale, containing Deep Vent DNA polymerase buffer, 0.2 m*M* of each dNTP, 1 μ*M* of each primer, 100 ng of plasmid DNA template, and 2 U of Deep Vent DNA polymerase. Amplification was carried out with a thermal cycler at 94°C for 45 s, 65°C for 45 s, and 74°C for 1 min, for 29 cycles. The PCR product was purified before double digestion with *Nde*I and *Sap*I. The digested PCR fragment was ligated to the *Nde*I–*Sap*I digested pTVB1 vector to yield the intein fusion construct, which was then transformed into *E. coli* DH5α cells by the heat-shock method. The vector was dephosphorylated prior to the ligation reaction by shrimp alkaline phosphatase at 37°C for 15 min to prevent self-ligation. The transformed cells were plated on LB plates containing 100 μg/mL of ampicillin and incubated overnight at 37°C. Positive colonies carrying the desired expression plasmid were selected by colony PCR and grown overnight in LB broth with ampicillin. The resulting T7-driven expression plasmid shown to be free of mutation by DNA sequencing was then transformed into *E. coli* ER2566 host, which contained an IPTG-inducible T7 RNA polymerase gene, for protein expression. The overall cloning strategy is illustrated in **Fig. 3** (*see* **Note 2**).

3.3. Expression and Extraction of Fusion Proteins

This section outlines steps for the induction of protein expression, the cell harvest, and the preparation of crude cell extract.

3.3.1. Induction of Protein Expression (see ***Note 3***)

1. Inoculate 2 mL of freshly grown transformed *E. coli* cells into 200 mL of LB medium supplemented with 100 μg/mL ampicillin.
2. Incubate the culture at 37°C in a 250 rpm incubator shaker to an optical density $(OD)_{600}$ of about 0.5 (about 3 h).
3. Add IPTG to a final concentration of 0.5 m*M* to induce fusion protein expression.
4. Incubate the culture overnight at room temperature on an orbital shaker.

3.3.2. Cell Harvest (see ***Note 4***)

1. Cells are harvested by centrifugation (6000*g*, 15 min, 4°C).
2. After discarding the supernatant, the cell pellet is resuspended immediately in lysis buffer for protein extraction or stored at –20°C without any significant breakdown of the fusion protein.

3.3.3. Preparation of Crude Cell Extract (see ***Note 5***)

1. Resuspend cell pellet in 5 mL of lysis buffer.
2. Lyse cell by sonication on ice at 50% duty, 20% power, in three treatments of 60 s each with 60 s cooling interval. The cell debris is removed by centrifugation.
3. Centrifuge the cell lysate at 20,000*g*, 30 min, and 4°C.
4. Collect clarified supernatant for loading onto the chitin affinity column.

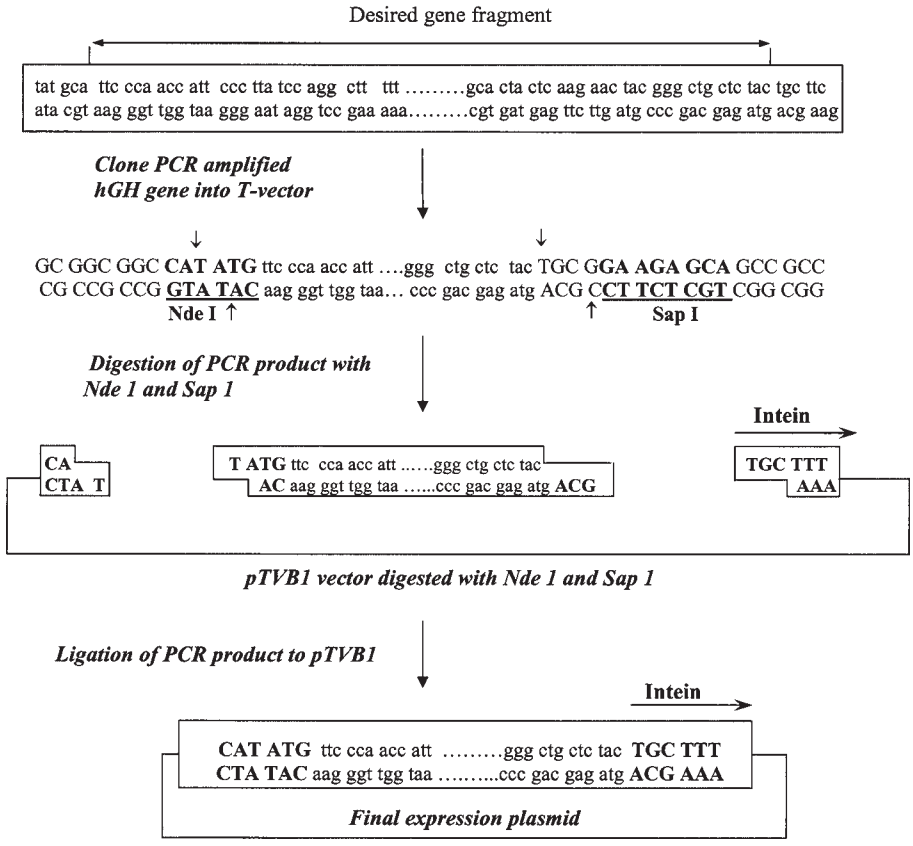

Fig. 3. Construction of the expression plasmid. After digestion with *Nde*I and *Sap*I, the restriction-digested PCR product and pTVB1 are ligated to regenerate the *Nde*I site with the translation initiation codon (ATG), and codon for *Cys*1 (TGC) at the intein N-terminus.

3.4. Affinity Purification and C-Terminal Biotinylation of Expressed Proteins

The following steps describe the purification and biotinylation procedures for the expressed fusion protein. The chitin-binding domain in the intein tag allows affinity purification of the fusion protein after which Cys-biotin is added to induce on-column cleavage such that the expressed protein can be separated from its affinity tag as it gets biotinylated. All purification procedures should be carried out at 4°C to ensure the stability of the fusion protein (*see* **Note 6**).

1. Pack 3 mL of chitin beads into a column for a 200-mL culture.
2. Preequilibrate the chitin column with 30 mL of column buffer.
3. Load clear cell lysate onto the column, and allow the column to empty by gravity flow.

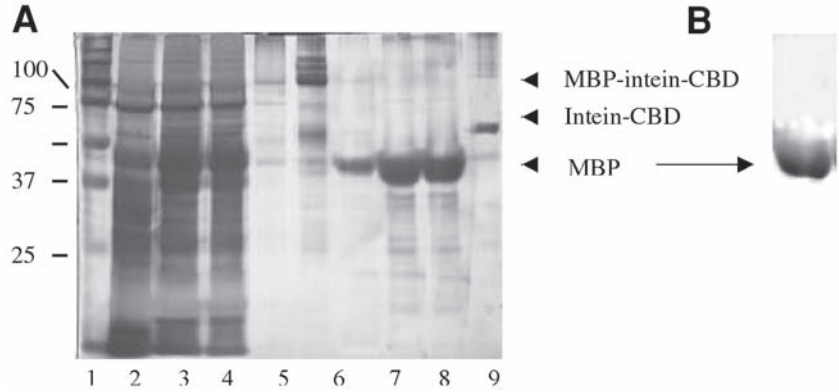

Fig. 4. MBP purification and biotinylation using chitin column. (**A**) (**1**) protein marker, (**2**) uninduced cell extract, (**3**) induced cell extract, (**4**) flowthrough from column loading, (**5**) flowthrough from column wash, (**6**) proteins bound to chitin column before cleavage, (**7**) flowthrough from quick flush of cleavage agent, (**8–9**) first two elution fractions after overnight incubation at 4°C with **1**, (**10**) remaining proteins bound to chitin column after cleavage. (**B**) Western blotting of biotinylated MBP using HRP-conjugated streptavidin.

4. Wash the loaded column with at least 30 mL of column buffer at a flow rate of 2 mL/min.
5. Quickly flush the column with 6 mL of the column buffer containing 30 m*M* of the cysteine-biotin.
6. Stop the column flow and incubate the column at 4°C overnight.
7. Elute the resulting biotinylated protein with 10 mL of column buffer.
8. Collect 1-mL fractions of the eluent.
9. Determine protein concentration for each of the fractions collected using Bio-Rad protein assay (biotinylated protein is normally eluted in the first few fractions).
10. Analyze purity of the column product with 10% SDS-PAGE gel.
11. Visualize the separated protein bands with silver staining (*see* **Fig. 4A**).
12. Confirm the presence of biotin label on the target protein by Western blot using HRP-conjugated streptavidin (*see* **Fig. 4B**).
13. Pool the fractions containing a high amount of the biotinylated target protein.
14. Freeze and lyophilize the pooled fractions if necessary.

The chitin resin can be regenerated four to five times. First, wash the column with 9 mL of 0.3 *M* NaOH and allow the resin to soak in NaOH for 30 min. Next, wash the column with another 20 mL of NaOH before rinsing with 60 mL of dH_2O followed by 15 mL of column buffer. The regenerated resin can then be stored in column buffer at 4°C for reuse.

3.5. Spotting of Biotinylated Proteins onto Avidin Slides and Fluorescent Antibody-Based Detection

No further treatment of the protein sample is needed prior to spotting because a trace amount of Cys-biotin in the pooled fractions does not seem to interfere with

binding of biotinylated proteins. The pooled fractions of the purified and biotinylated protein can be spotted directly onto the slides following the steps described below.

3.5.1. Preparation of Avidin-Functionalized Slides

1. Clean glass slides in piranha solution (H_2SO_4:H_2O_2, 7:3) for at least 2 h.
2. Wash the slides copiously with deionized H_2O, rinse with 95% ethanol, and, finally, dry the slides.
3. Soak the freshly cleaned slides in 1% solution of 3-glycidyloxypropyltrimethoxysilane (95% ethanol, 16 m*M* acetic acid) for 1 h.
4. Place the derivatized slides in a slide holder and wash two to three times with 95% ethanol.
5. Cure slides at 150°C for at least 2 h (overnight curing gives the same result). Rinse the slides with ethanol and dry.
6. Add 40–60 μL of 1 mg/mL avidin in 10 m*M* $NaHCO_3$ to the slides, cover with cover slips, and incubate for 30 min.
7. Subsequently, wash the slides with deionized H_2O in a slide tray and dry the slides.
8. React the remaining epoxides by adding 2 m*M* aspartic acid in a 0.5 *M* $NaHCO_3$ buffer, pH 9, to the slides and covering with a cover slip.
9. Finally, wash the slides with deionized H_2O and dry them for spotting.

*3.5.2. Immobilization of Biotinylated Protein (*see ***Note 7****)*

1. Prepare source plates by dissolving the lyophilized eluent in PBS buffer (pH 7.4).
2. Spot biotinylated proteins onto the avidin-functionalized slides using an ESI SMA™ arrayer, and incubate for about 10 min.
3. Wash spotted slides with PBS for a few minutes before drying in air.

3.5.3. Antibody Detection of the Immobilized Proteins

1. If necessary, label the antibody with fluorescent dye in 0.1 *M* $NaHCO_3$, pH 9, for 1 h according to the manufacturer's protocol.
2. Purify the labeled antibody with a NAP-5 column and elute the labeled antibody in PBS containing 1% BSA.
3. Incubate the spotted slide with approx 50 μL of the fluorescently labeled, monoclonal antibody for 1 h in the dark.
4. Wash four times, each time for 15 min with 1X PBST, and rinse with dH_2O.
5. Dry slide and visualize spots with an ArrayWoRx™ microarray scanner (*see* **Fig. 5**).

4. Notes

1. In addition to pTVB1, there are other pTVB expression vectors (from New England Biolabs, Beverly, MA) that also allow fusion of the intein tag to either the C-terminus (pTVB2) or N-terminus (pTVB11, pTVB12) of a target protein. Studies show that the choice of either C-terminal or N-terminal fusion vector would greatly affect the expression level of the target protein in a host cell. Thus, it is best to try out the different expression vectors for optimal expression of the fusion protein. Amino acid residues adjacent to the intein cleavage site also have adverse effects on the cleavage of the fusion protein. The authors' experimental results show that some amino acids (e.g., Asp) would cause premature cleavage of the fusion protein (*see* **Fig. 6A**), whereas others (e.g., Cys) greatly reduce cleavage/biotinylation efficiency of the target protein (*see* **Fig. 6B**). Cloning of a target gene into pTVB2 and pTVB12 results in extra vector-derived amino acid residues

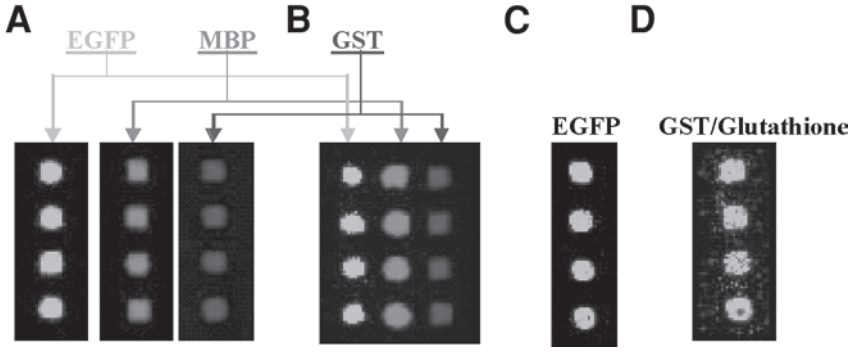

Fig. 5. Site-specific immobilization of biotinylated, functionally active proteins onto avidin slide. (**A**) EGFP, MBP, and GST were individually detected with Cy3-anti-EGFP, Cy5-anti-MBP, and FITC-anti-GST, respectively; (**B**) specific detection of all three proteins with a mixture containing all three antibodies; (**C**) fluorescence from the native EGFP; and (**D**) specific binding between GST and its Cy3-labeled natural ligand, glutathione.

added next to the intein cleavage site. These extra residues are necessary to either prevent in vivo cleavage or improve cleavage/biotinylation efficiency of target proteins for cases whereby unfavorable amino acid residues are present adjacent to the cleavage site (*see* **Fig. 6C**). Alternatively, one can also ensure the presence of a suitable amino acid residue next to the intein cleavage site by the design of primers and the choice of restriction sites used for cloning.

2. Restriction enzyme digestion of a PCR product is usually less efficient compared to plasmid digestion, because of the short flanking nucleotide sequences. This would greatly affect the subsequent cloning efficiency into the pTVB1 expression vector. Alternatively, the PCR fragment can be cloned into a T-vector to enhance the effectiveness of the restriction digest.
3. Expression level of fusion protein from the pTVB vector is greatly influenced by the bacterial host, nature of the fusion protein, and induction conditions (temperature, duration, and IPTG concentration). ER2566 is the *E. coli* strain supplied by New England Biolabs for expression of fusion proteins from pTVB vectors, but other commercially available strain (e.g., BL21) may be tested for optimal expression level of the fusion protein. Different induction conditions (e.g., 30°C for 3 h, 20–25°C for 6–8 h, or 12–16°C for overnight) should be tested for each type of fusion protein to maximize fusion protein expression and to minimize proteolysis.
4. Nonionic detergents (e.g., Trition X-100 or Tween-20), protease inhibitors (e.g., phenylmethylsulfonyl fluoride, pepstatin, leupeptin), and reducing agents such as tris-(2-carobxyethyl) phosphine or tris-(2-cyanoethyl) phosphine may be added to the lysis buffer to ensure stability of the fusion protein during extraction. Thiol reagents (β-mercaptoethanol, 1,4-dithiothreitol) would cause premature cleavage of the fusion protein, resulting in the lost of target protein prior to affinity purification. Therefore, no thiol compound should be present in the lysis or column buffer to ensure maximum recovery of the target protein.

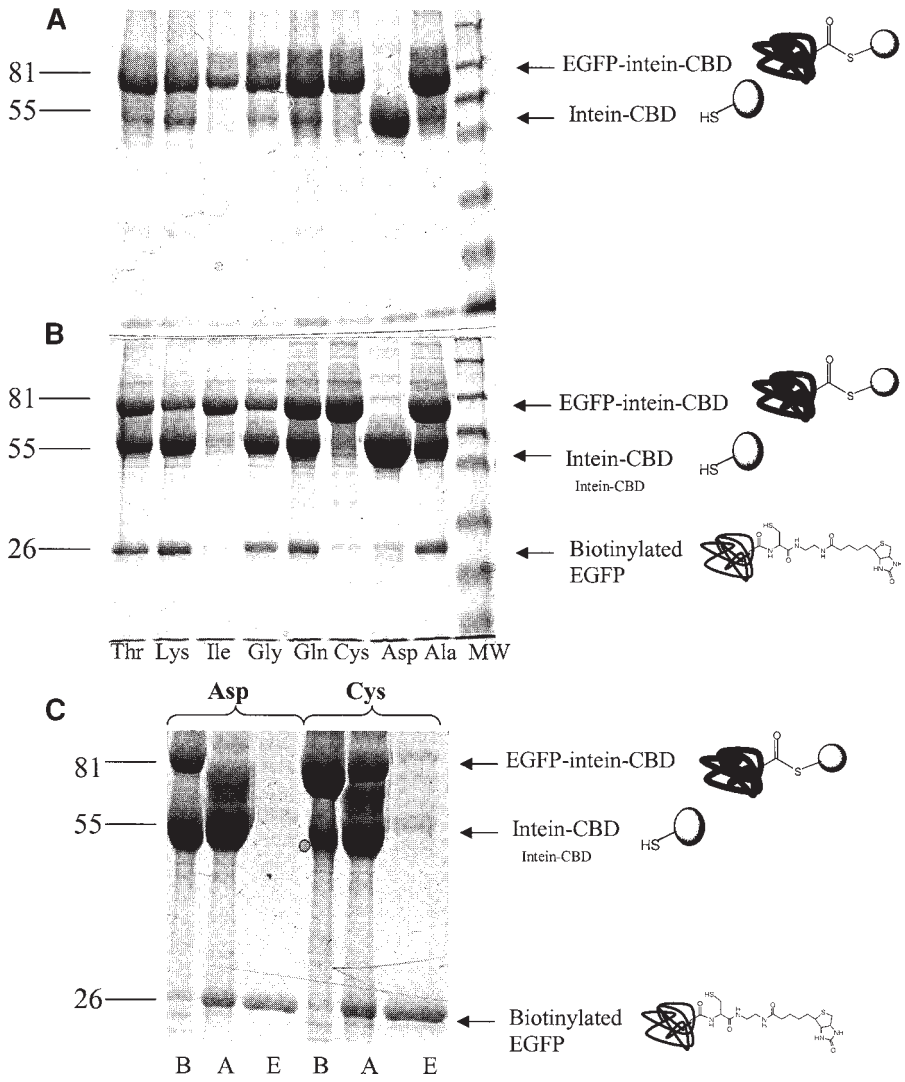

Fig. 6. (**A**) Proteins bound to chitin beads before cleavage. This shows the amount of fusion protein cleaved in vivo prior to Cys-biotin cleavage for the different amino acids adjacent to the intein cleavage site. (**B**) Proteins remaining on the chitin beads after Cys-biotin cleavage. This shows the amount of fusion protein cleaved by Cys-biotin, thus indicating the cleavage/biotinylation efficiency for the respective amino acid residues. (**C**) EGFP is cloned into pTVB2 vector to add an extra glycine residue adjacent to the intein cleavage site. B: Proteins bound to chitin beads before Cys-biotin cleavage; A: proteins remaining on chitin beads after Cys-biotin cleavage; E: biotinylated EGFB. It shows a reduction of in vivo cleavage and improved cleavage/biotinylation efficiency with the extra glycine residue added.

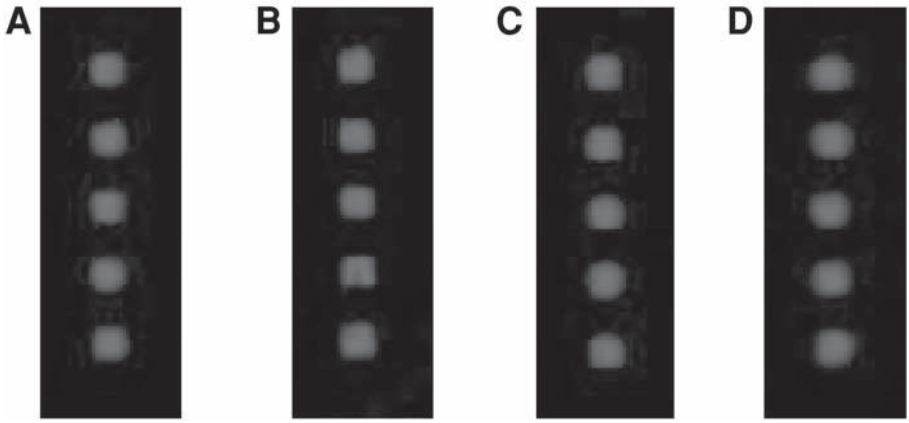

Fig. 7. Biotinylated GST on an avidin slide treated with different washing conditions: (**A**) 1 *M* acetic acid solution pH 3.3; (**B**) 60°C H_2O; (**C**) 4 *M* GuHCl, all for 30 min; and (**D**) control slide with no treatment. Slides were probed with FITC-anti-GST.

5. In addition to sonication, the *E. coli* cells can also be disrupted either by French press or freeze-thawing methods. Lysozyme is not a preferred method for cell lysis because it is known to bind and digest chitin beads. However, if no alternative method is available, a low level of lysozyme may be used (incubate at 4°C for 1 h) for cell lysis.
6. Cleavage/biotinylation efficiency of target proteins is affected by the amino acid residue that is directly adjacent to the intein tag (*see* **Note 1**) and by the cleavage conditions (temperature, duration, and pH). Generally, target protein with suitable amino acid residues at the cleavage site can be cleaved efficiently from its intein tag at 4°C overnight. For fusion protein that does not cleave effectively under this condition, prolonged incubation (e.g., 40 h) at higher temperature (e.g., 16–23°C) and different pH conditions (e.g., pH 9.0) may be required to obtain a sufficient yield of the target protein.
7. The authors' experimental results show that biotin–avidin interaction between the biotinylated protein and the avidin-functionalized slide was able to withstand most harsh conditions. The glutathione-*S*-transferase (GST) immobilized slides showed no reduction in the intensity of spots when probed with FITC-anti-GST after treatment with 1 *M* acetic acid solution at pH 3.3, 60°C H_2O, or 4 *M* GuHCl (*see* **Fig. 7**). For comparison, the authors have also prepared Ni-NTA slides according to published protocols. Briefly, epoxy slides were incubated with NTA dissolved in $NaHCO_3$. The slides were washed in H_2O and soaked in 100 m*M* $NiSO_4$ for at least 1 h, then washed with 0.2 *M* acetic acid and 100 m*M* NaCl to produce the Ni-NTA slides. The authors expressed a GFP fusion protein with an His tag and spotted it onto Ni-NTA slides as described by Zhu et al. *(7)*. When this GFP-containing slide was treated with any of the previously mentioned harsh conditions, immobilization of the His tag protein on the Ni-NTA was completely removed.

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9

Processing of Photoaptamer Microarrays

Helen Petach, Rachel Ostroff, Chad Greef, and Gregory M. Husar

Summary

Photoaptamers are single-stranded nucleic acids selected for their high affinity to specific proteins of interest. Photoaptamer microarrays capture and quantify proteins from complex samples using a unique protocol that leverages both high-affinity capture with covalent retention of analytes. The initial capture of proteins from solution is similar to the well-known antibody capture, but the "secondary binding event" affected by photoaptamers is a covalent crosslink between the photoaptamer capture agent and the protein analyte. The nature of this specific covalent reaction allows a unique microarray processing that is described in detail in this chapter.

Key Words:

Photoaptamer; aptamer; photocrosslinking; protein microarray; protein array; SELEX; PhotoSELEX.

1. Introduction

1.1. Relevance of Photoaptamer Microarrays

The key, alluring feature of protein microarrays is the multiplexing capacity that permits massive parallel analysis of complex mixtures of proteins from serum, plasma, cell lysates, and other sample matrices. As protein microarrays become a mainstream diagnostic tool, the single biomarkers frequently used as disease indicators will be replaced with microarray results that generate multianalyte profiles or proteomic patterns. A significant benefit to diagnostics afforded by multiple disease markers is substantial improvement of the positive predictive value that results from combining the diagnostic value of numerous analytes. For example, recent studies of prostate and ovarian cancer patient protein profiles suggest that a suite of markers will provide more precise indication of disease ***(1,2)***. As such, the development of protein microarrays for protein expression profiling has gained heightened interest ***(3)***.

Proteomic microarrays are under development using various capture molecules, the most common being multiplexed enzyme-linked immunosorbent assays (ELISAs)

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on a solid support. These sandwich assays are dependent on the reaction of a secondary detection antibody (which provides the second dimension to an ELISA). In addition to the problems of reagent stability and compatibility, a significant limitation of multiplexed ELISA arrays has proved to be the crossreactivity of the detection antibody with the immobilized capture antibodies and other captured proteins on the array ***(4)***. In fact, recent reports of multiplexed ELISA arrays describe arrays that are limited to fewer than 40 antibody pairs because of the high levels of crossreactivity ***(5)***.

To improve the multiplexing capability of ELISAs, antibody arrays have also been developed for protein quantification without the use of labeled antibodies (relying completely on the specificity of capture antibodies). Mixtures of antigens targeted to the microarray antibodies were labeled with Cy5, then mixed with a reference sample containing known amounts of each antigen labeled with Cy3. Antigen quantification was determined by the ratio of Cy5 to Cy3 fluorescence ***(6)***. In this work, some 20% of the capture antibodies on the spots retained their activities after immobilization. Although this technique does not require a labeled antibody, it is dependent on exquisite specificity of the capture antibody and precise replication of reference mixtures. In general, antibodies do not have adequate specificity to be used in complex mixtures such as serum without a specific labeled secondary antibody, although one might hope that new selection techniques (performed in vitro) for antibodies could yield higher specificities ***(7)***.

1.2. Benefits of Photoaptamer Capture Agents

Photoaptamers are the basis for a different approach to multianalyte analysis in complex biological fluids that may provide superior limits of quantitation and scalability. Because photoaptamers do not require the use of a second specific (detection) agent, they eliminate the challenges associated with detection antibodies: (a) identifying detection antibodies, (b) screening antibody pairs for compatibility, (c) maintaining large numbers of labeled reagents in solution, and (d) dealing with the potential for crosstalk introduced by labeled detection antibodies. If a protein is labeled with a detection antibody, it will generate a signal whether it has bound to its intended capture antibody or interacted nonspecifically with another antibody. In contrast, photoaptamers enable two dimensions of specificity without requiring a secondary binding agent and, thus, reduce these challenges ***(8)***.

Aptamers (first described by Tuerk and Gold, 1990 ***[9]***) are generated via the in vitro high-throughput, automated systematic evolution of ligands by exponential enrichment (SELEX) protocol ***(10)*** and are stable, tenacious capture agents that can be directed to a diverse range of target protein molecules. Photoaptamers are readily generated for a wide range of proteins, including those that are acidic, basic, large, small, glycosylated, chemically modified, or hydrophobic.

Photoaptamers bind proteins with two dimensions of specificity: First, the protein is captured via a high-affinity noncovalent interaction, typically with Kd values of 20–500 picomolar (p*M*), and second, the protein is photocrosslinked to the photoaptamer if the protein is bound tightly and in the proper orientation to provide for a photocrosslinking site.

Crosslinking requires the precise alignment between a bromodeoxyuridine (BrdU) in the photoaptamer and a specific tyrosine or other reactive amino acid (Trp, Tyr, His, Phe, Cys, Cys–Cys, Met, and Thr) in the target protein ***(11)***—clearly some of these amino acids will provide better targets for crosslinking than others, especially in the context of the conformational epitope recognized by the aptamer. The activated BrdU, formed by irradiation with light at 308 nm, preferentially reacts with Tyr and Trp. High specificity is achieved by combining the intrinsic affinity of an aptamer to its target protein and the demand for a photoactivated crosslink between an aptamer's BrdU and a protein's target amino acid residue. The target amino acids must be positioned appropriately to allow this photocrosslink. The specific photocrosslinking reactions and specificities are described elsewhere ***(12,13)***.

The covalent crosslink is stable to stringent wash conditions, allowing the removal of nonspecifically bound proteins prior to labeling and detection. These stringent wash conditions reduce background, leading to improved signal-to-noise ratios. The result of stringent washing should be improved limits of detection and quantification that will make photoaptamer-based protein microarrays truly useful tools by enabling the parallel measurement of even low-abundance proteins.

Another important characteristic of photoaptamers derives from the fact that they are generated in vitro under defined conditions. There are at least two distinct advantages to making multiplexed detection reagents in vitro. The conditions under which the photoaptamers are made can be adjusted to accommodate the requirements of the final application, and all of the photoaptamers used for a given application can be made using the same SELEX conditions. This ensures the compatibility of all components of the incorporated into the multiplexed array.

These characteristics of photoaptamers are used to create an assay for protein quantification on photoaptamer microarrays.

2. Materials

1. Selection buffer: 40 m*M* HEPES, pH 7.5, 111 m*M* NaCl, 5 m*M* KCl, 1 m*M* $MgCl_2$, 1 m*M* $CaCl_2$, and 0.05% Tween-20.
2. Denaturing buffer (*see* **Note 1**):
   (a) 20 m*M* NaOH.
   (b) Salt washes: 20 m*M* NaH_2PO_4 at pH 7.4, 150 m*M* NaCl, 0.1% sodium dodecyl sulfate (SDS), 1 m*M* ethylenediaminetetraacetic acid (EDTA).
   (c) 8 *M* Urea.
   (d) Detergents.
3. Dye buffer: 0.01 mg/mL of NHS-Alexa-555, diluted from concentrated dimethyl sulfoxide (DMSO) stock into carbonate buffer composed of 0.1 *M* sodium carbonate, pH 8.75, 1 m*M* EDTA, and 0.1% Tween-20.

3. Methods

3.1. Photoaptamer Microarray Construction

The photoaptamer microarrays can be constructed on any solid surface, including glass slides, 96-well plates, multiplexing beads, and other solid support systems. Spe-

cific requirements for the surface may include a hydrophilic, organic environment that minimizes nonspecific adsorption of the photoaptamer and allows it to retain its three-dimensional conformation; a robust attachment of the organic layer to the substrate surface, to allow harsh washing; and a surface modification that facilitates the covalent attachment of the photoaptamer to the surface. Photoaptamers are synthesized chemically and, thus, can be made with various of 5' or 3' functional groups permitting covalent attachment to surfaces including amine, thiol, acrydite, and others, so various surface chemistries are available for photoaptamer microarrays.

After deposition of photoaptamers on the surface, the surface is further treated to eliminate residual functional groups that remain active after photoaptamer deposition and to modify the environment around the photoaptamer features. Typical postspotting treatments carried out to inactivate residual surface functionality include alkaline washes to hydrolyze labile groups and acetylation of primary amines or free sulfhydryls. Additional postspotting treatments can include the reaction of surface functional groups with alkyl or polyethylene glycol chains to modify the environment around the photoaptamers themselves.

A specific example of an acceptable surface chemistry is an organic film attached to a glass surface in which *N*-hydroxy-succinimide groups are exposed on the surface of the organic film for attachment of amine-modified photoaptamers. Photoaptamer deposition is followed by hydrolysis with 20 m*M* NaOH to ensure that residual *N*-hydroxy-succinimide groups are completely hydrolyzed.

Typical photoaptamer arrays contain several hundred photoaptamer spots, 100 μm in diameter with 250 μm center-to-center spacing, although the array density and spot size is not constrained to these dimensions. A typical photoaptamer feature contains 10^4 to 10^5 aptamers/μm^2.

3.2. Photoaptamer Microarray Assay

Photoaptamer microarrays are used to quantify a wide range of protein concentrations in complex matrices, such as human serum and cell culture supernatants. The assay protocol is specific to photoaptamer arrays and includes the following steps as shown in **Fig. 1**:

1. Incubation with analytical sample to bind protein to the photoaptamers.
2. Precrosslink wash to remove nonspecifically bound material.
3. Irradiation with ultraviolet (UV) light for photocrosslinking (to impose the second dimension of specificity).
4. Denaturing wash to remove noncrosslinked protein on the array.
5. Staining with a universal protein stain.
6. Reading the fluorescence of the array features.
7. Image capture for documentation and data analysis.

Each step in the protocol is elaborated as follows:

1. The analytical sample, typically 50–100 μL in volume, is incubated over the array. To allow optimal affinity association of photoaptamers with their cognate proteins, the incubation is carried out in the selection buffer in a humid chamber at 30°C. The time for this incubation is determined by the efficiency of the mixing of the sample solution with the

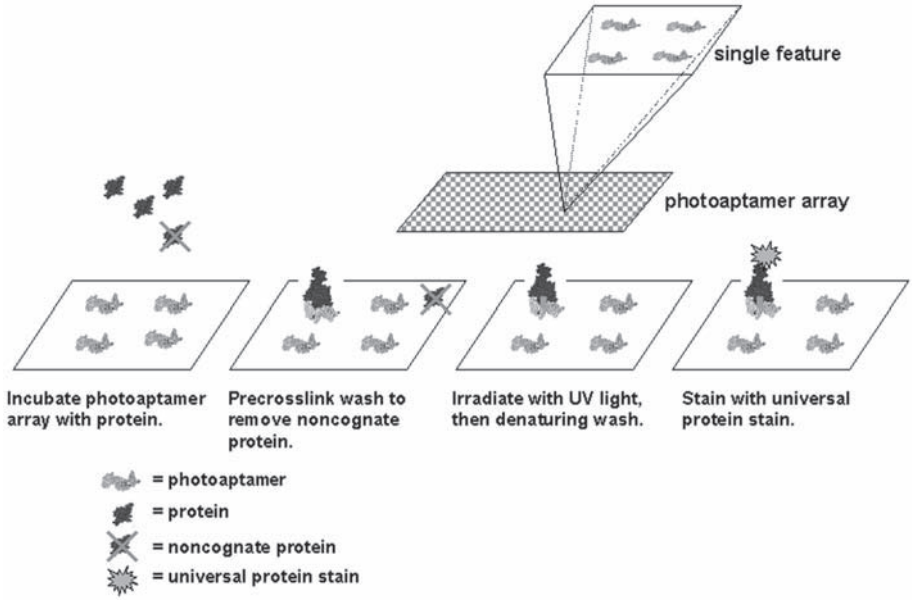

Fig. 1. Schematic representation of the steps required for a photoaptamer array assay of protein.

array components. For example, in the absence of any mixing, the best results are obtained after 14 h of incubation, long enough for the binding between sample analyte and the photoaptamers to approach equilibrium. With good mixing, the incubation time can be reduced. During incubation, immobilized photoaptamers establish noncovalent affinity associations with their cognate proteins through a combination of electrostatic, ionic, and hydrophobic interactions.

2. Following incubation with the analytical sample, but prior to photocrosslinking, the photoaptamer array is washed using the incubation buffer to selectively remove noncognate protein on the array. Although the kinetic properties of individual photoaptamers vary, they typically have affinity constants (Kd) of 20–500 pM and dissociation rates of 20–30 min. The interactions of photoaptamers with noncognate proteins are weaker, and, thus, nonspecific proteins should be distinguished from cognate proteins by their removal during a precrosslink wash.
   The precrosslink wash step can be carried out at increased ionic strength to facilitate the dissociation of noncognate protein from the photoaptamer or the solid surface. As with the incubation step, improved mixing of the wash solution with the array can reduce the time required for this step.
3. Photocrosslinking of cognate protein to photoaptamer features is carried out by irradiation with UV light. The optimal wavelength for this irradiation is 308 nm (a compromise between optimal excitation of the BrdU nucleotides and minimal degradation of the photoaptamers themselves) and is delivered to a "wet" microarray in which a thin layer of aqueous buffer is present over the array surface. For monochromatic irradiation, the light is delivered from a XeCl Excimer laser to achieve a dose of 3 J/cm^2 or less (*see* **Note 2**).

Although monochromatic light sources provide a convenient method to deliver a precise light dose, the UV irradiation can also be carried out using a high-energy mercury lamp source, provided appropriate filters are placed in the light path to remove wavelengths below 312 nm and limit the infrared (IR) wavelengths reaching the array.

Because excessive irradiation or exposure to light of inappropriate wavelength can damage the photoaptamers, the light must be controlled to ensure adequate photocrosslinking without damaging the photoaptamers. The efficiency of photocrosslinking varies between different photoaptamers ***(14)***, but the rate of photocrosslinking is usually at least five times greater than the rate of degradation.

4. Following the photocrosslinking step, the cognate protein is bound covalently to the immobilized photoaptamers. To remove nonspecific protein from the array surface, associated with the aptamers but not covalently bound by photocrosslinking, harsh denaturing washes are used on the array as described in **Subheading 2.** These wash conditions are designed to remove all residual nonspecifically bound protein and to denature the covalently bound protein to expose the functional groups on the amino acid side chains susceptible to staining in the next step (*see* **Note 3**). The harshness of the denaturing wash is only limited by the ability of the organic surface coating to maintain structural integrity.
5. Following the denaturing washes, the only proteins bound to the array are those covalently crosslinked to photoaptamers. As such, a single protein stain permits the quantification of protein bound to each of the addressed photoaptamer features on the array. This universal protein staining agent can react with any of the protein-specific features, such as the peptide backbone, the primary amines on the lysines, the carboxylates on the aspartates and glutamates, the thiols on the cysteines, and so forth (*see* **Note 4**). For example, amine-reactive fluorophores such as *N*-hydroxy-succinimide-fluorescent dyes or thiol-reactive maleimide dyes can be used to label the protein bound to the array.

   The universal protein stain facilitates protein quantification without the complications associated with complex mixtures of specific secondary binding agents for each protein target. Elimination of multiple detection agents expands the multiplexing capacity of photoaptamer arrays.

   To enhance the signal on the array, amplification techniques can be used. For example, an NHS-biotin conjugate can be used to label primary amines on the protein, and streptavidin conjugates can carry an amplifiable signal (e.g., streptavidin coupled to horseradish peroxidase). Numerous amplification schemes are currently available and can be adapted to photoaptamer arrays, including rolling-circle deoxyribonucleic acid ***(15)***, precipitating fluorescent enzyme-labeled fluorescence (ELF) substrates, chemiluminescent substrates, and so forth.

   An alternative to direct fluorescent dye labeling is chemical modification of specific amino acids that are then detected by an antibody that specifically recognizes the modified amino acid. Two examples of this approach are: (a) nitrosylation of tyrosine followed by reaction with antinitrotyrosine antibody, or (b) acetylation of lysine followed by reaction with antiacetyl-lysine. Although both methods incorporate an antibody in the detection step, a single antibody is used to detect all proteins, so these antibodies function as universal proteins stains. The antibody can be directly or indirectly labeled for amplification. These universal antibody stains can increase signal-to-noise ratios by reducing background interference, because nitrotyrosine and acetyl-lysine would be unusual components of a slide surface.

   A protocol for using the direct fluorescent label for primary amine staining is to apply 0.01 mg/mL of NHS-Alexa-555 in dye buffer, as described in **Subheading 2.**, on the

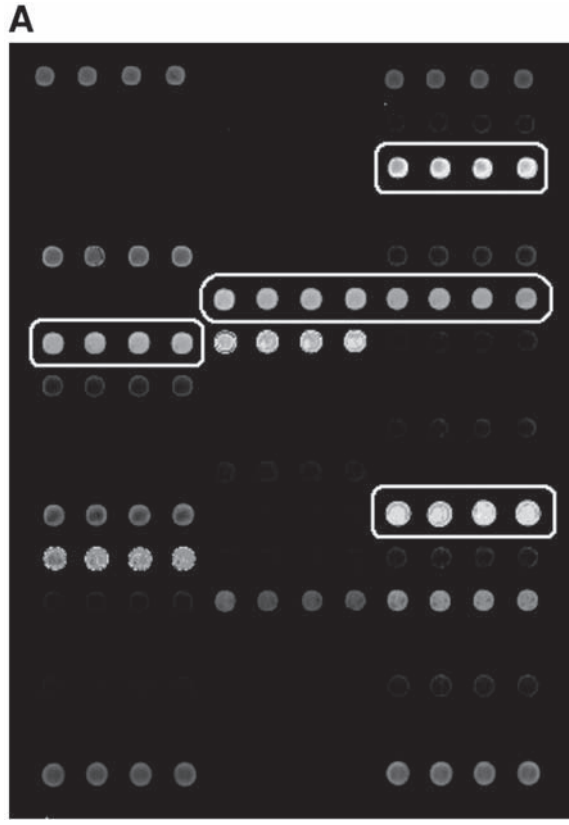

Fig. 2A. Image of a photoaptamer array after incubation with 10% serum. The array contains 40 different photoaptamers, each specific for a different protein. The boxed spots moving from the top of the image to the bottom represent proteins bound to the photoaptamers to von Willebrand Factor, thrombin, C1q, and fibronectin. The shading on the image indicates the amount of protein bound to each spot.

array for a 30-min incubation in a humid chamber at room temperature, followed by harsh washing for 15 min in each of the following wash solutions: (a) 0.1% SDS in H_2O, (b) 20 m*M* NaOH, (c) H_2O. This scheme can be readily modified to include amplification by reacting NHS-biotin with the primary amines on the protein, following with a streptavidin–enzyme conjugate that converts a fluorescent substrate such as ELF.

6. Fluorescent signals on photoaptamer spots can be visualized using standard microarray fluorescent readers that contain the appropriate lasers for the fluorescent stains (*see* **Note 5**).

3.3. Sample Protein Quantification Results

Following analysis of a protein-containing sample by the photoaptamer array protocol, results such as those shown in **Fig. 2** can be obtained. **Figure 2A** shows a photoaptamer array on which 42 protein targets were analyzed simultaneously. The

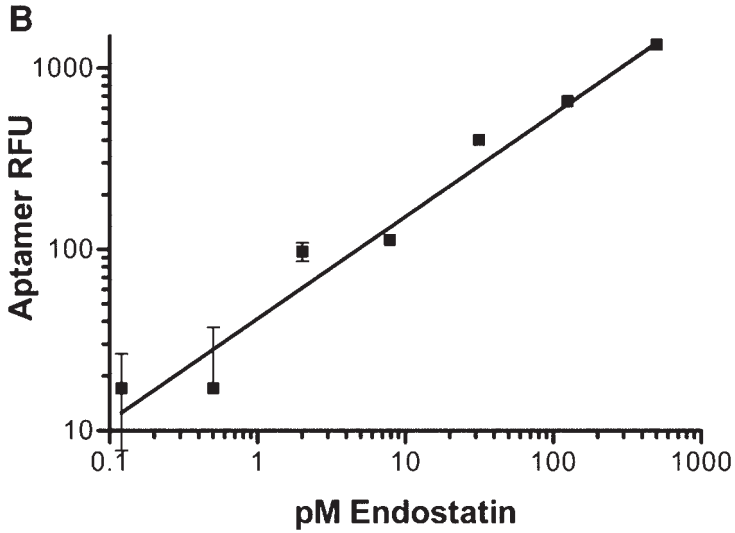

Fig. 2B. Dose response curve for endostatin bound to the endostatin photoaptamer on the array surface. The endostatin is quantified by measuring the fluorescence of Alexa-555 stain bound to the endostatin on the photoaptamer feature. The limit of detection for endostatin in this example is 1 pM. This dose response curve was generated by spiking a known concentration of endostatin into selection buffer. The endostatin concentrations ranged from 100 f*M* to 1 n*M*.

quantification of a dose response to one protein, endostatin, is shown in **Fig. 2B**. This dose response profile was obtained by exposing replicates of the array shown in **Fig. 2A** to solutions containing different concentrations of endostatin. The lower limits of detection are constrained by the background resulting from the staining system and are currently approx 1–10 pM (20–200 pg/mL) using standard microarray surfaces. Improvements are expected as surfaces designed and optimized for photoaptamer arrays are developed.

As multiparameter proteomic analyses become more relevant for both research and diagnostic applications, large protein microarrays will be essential. Photoaptamer microarrays provide a means to increase protein array size without compromising performance because of secondary binding-agent limitations ***(16,17)***.

4. Notes

1. Denaturing wash solutions vary depending on the specific slide surface and assay, and representative solutions are listed under **Subheading 2.** Washing is carried out at elevated temperature (e.g., 40°C), and combinations of these reagents have been used to improve array performance.
2. Low signals can be observed if the UV light source has degraded and the dose for wavelengths near 308 nm is too low. Emission from UV lamps and laser sources is known to vary over time. If the fluorescence intensity on the array drops off over time, the light

source should be recalibrated. The simplest way to recalibrate the light source is to run a titration of light irradiation over the array surface and determine the dose providing the maximum fluorescent signal while maintaining the minimum nonspecific noncognate protein binding. (Note: Overirradiation damages the photoaptamers and can lead to more nonspecific binding.)

3. Blotches on the array surface are occasionally observed. Often the variability in the fluorescence can be minimized by adequate rinsing following the use of detergent washes such as SDS-containing solutions. Variable results can also be observed when the array surface dries out during processing. The array processing is carried out on wetted surfaces until the final rinsing is complete, and the slide is dried with a stream of nitrogen gas.
4. Fluorescent stains such as *N*-hydroxy-succinimide dyes are known to hydrolyze in aqueous solutions and when exposed to atmospheric conditions. To minimize the competing hydrolytic reactions of these dyes, the concentrated NHS-activated dyes are stored in dry DMSO prior to their dilution into aqueous buffer, and the diluted reagent is used immediately after dilution. When the surface chemistry can tolerate organic solvents, the activated dyes can be used in dry solvents instead of aqueous buffers.
5. Fluorescent quenching is known to occur on some substrates (e.g., gold, silicon) when the fluorophore is in close enough proximity to the surface. For substrates that are able to quench fluorescence, chemiluminescent detection may provide a more accurate result.

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10

Use of a Small Molecule-Based Affinity System for the Preparation of Protein Microarrays

Karin A. Hughes

Summary

This chapter describes a method for preparing protein microarrays, using a small-molecule, chemical affinity system.

Key Words:

Protein microarrays; protein conjugation; protein chips; immunoassay; proteomics.

1. Introduction

Proteomics, the systematic study of protein structure and function, is a natural extension of genomics. As with genomics, proteomics requires high-throughput platforms that enable screening of multiple proteins in a single assay. The protein microarray holds promise as such a platform.

Originally, microarrays were developed for the high-throughput analysis of nucleic acids. However, tools and technologies designed for DNA microarrays have found limited success when applied to protein microarrays. Whereas the synthesis of the correct DNA sequence is important to nucleic acid microarrays, reliable maintenance of three-dimensional (3D) structure and protein activity postspotting as well as optimal presentation of active regions are critical to successful protein microarray generation. Issues inherent in the fundamental nature of proteins—their fragility and diversity—present additional challenges in the adaptation of DNA microarray tools for protein analysis ***(1–5)***. Key to the successful implementation of protein microarrays is the approach used to attach proteins to a substrate surface as well as the characteristics of the substrate surface itself.

Several methods exist for the immobilization of proteins on surfaces ***(6)***, including passive adsorption, covalent coupling, and biological affinity-based binding. Passive adsorption relies on noncovalent interactions between a protein and a hydrophobic or

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charged surface ***(1,3,7–9)***. Although this strategy requires no specialized chemistry and is easy to use, the main drawbacks include partial protein denaturation and the inability to control protein orientation on the surface. In addition, this type of linkage can be unstable, and protein may be lost during wash steps. Covalent coupling uses various chemically activated surfaces (e.g., aldehyde, epoxy, activated esters) to react with specific sites on proteins or peptides ***(10–16)***. Such chemistries can allow for oriented immobilization of proteins on surfaces, are moderately reproducible, and result in a stable linkage of proteins to solid supports. However, not all activated substrates are stable to the conditions required for protein array printing. Furthermore, reduced protein activity is often observed and multiple surfaces are required to specifically orient proteins using different protein functional groups. Use of biological affinity-based binding [e.g., (strept)avidin/biotin, recombinant-tagged proteins, protein A] ***(10,15,17–18)*** is specific by virtue of the affinity interaction used. These methods also allow for proteins to be attached to surfaces in a defined orientation, but linkages resulting from the use of recombinant tags are often weak and may not be stable over the course of the assay ***(9,19)***. Use of (strept)avidin/biotin results in a highly stable protein–surface linkage but requires the use of a large protein, limiting capacity, and special handling ***(19)***.

To address the above issues, the authors report here an alternative strategy for protein microarray preparation, using a nonbiological chemical affinity system based on phenyldiboronic acid (PDBA) and salicylhydroxamic acid (SHA) ***(20–24)*** (*see* **Fig. 1**). To prepare a protein microarray, a PDBA derivative is selected (*see* **Table 1**) to modify solvent-accessible functional groups present on a capture protein but avoiding the active site. Conjugation of proteins with PDBA occurs in solution under mild reaction conditions separate from immobilization, resulting in a more uniform distribution of affinity moieties but insuring a high retention of protein activity relative to other methods. After conjugation, and without the need for purification, the PDBA-modified protein is immobilized on a 3 × 1 inch glass slide coated with a 3D polymer containing SHA. SHA is incorporated into a polymer grown from the surface under living polymerization conditions, resulting in a brush polymer with high graft density and no crosslinking. The 3D-SHA coated slides are easy to work with, require no preparation before use, and can be employed under conditions normally used for protein handling. The slides exhibit excellent stability before, during, and after spotting and afford arrayed proteins with predictable and regular morphology (i.e., shape and signal uniformity), thus facilitating data analysis. After the PDBA-capture proteins are arrayed on an SHA slide, a crude mixture containing the protein target is applied, and the immobilized proteins capture and retain any "targets." The slide is washed to remove any impurities, and the bound targets are detected.

2. Materials

2.1. Conjugation of Protein-Capture Ligands

2.1.1. Conjugation of Lysine Residues

1. Protein-capture ligand containing solvent-accessible lysines.
2. Amine-modifying reagent, proteomics grade PDBA-X-*N*-hydroxysuccinimide (NHS) (FW = 500.11) (Prolinx, Bothell, WA, cat no. VMT5000-5); store desiccated at –20°C.

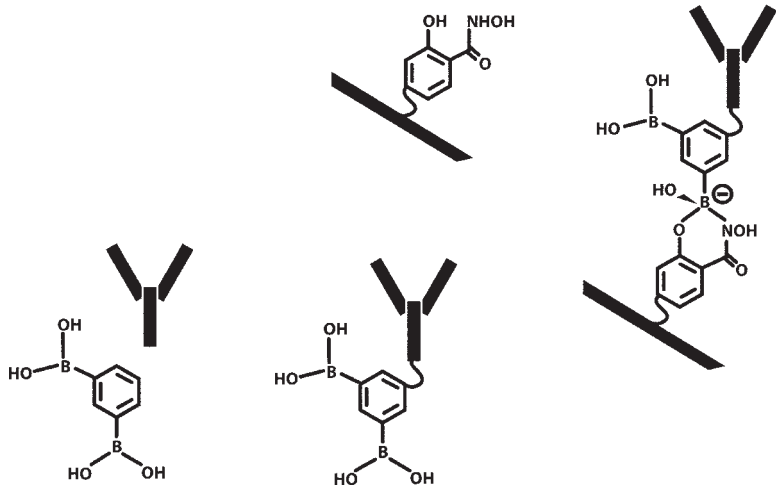

Fig. 1. Strategy for use of PDBA and SHA for protein microarray generation. PDBA is covalently bound to proteins using known chemistries. SHA is incorporated into a three-dimensional matrix bound to a 3 × 1 in glass microarray slide. Proteins are immobilized on the surface by the specific complex formation of PDBA with SHA.

Table 1
Reagents Used for Protein Conjugation With Phenyldiboronic Acid

Reagent	Reagent reactive group	Targeted protein site
Amine-modifying reagent	NHS ester	ε-amino group of lysine and N-terminal amino group
Sulfhydryl alkylating reagent	maleimide	sulfhydryl of cysteine
Carbohydrate-modifying reagent	hydrazide	oxidized carbohydrates

3. Sodium bicarbonate (0.1 *M*), pH 8.
4. *N*,*N*-dimethyl formamide (DMF), anhydrous.
5. Optional: size-exclusion column (e.g., protein desalting columns, Pierce Chemical, Rockford, IL, cat no. 98949).

2.1.2. Conjugation of Glycoproteins

1. Protein-capture ligand containing sites of glycosylation.
2. Carbohydrate-modifying reagent, proteomics grade PDBA-X-hydrazide (FW = 373.41) (Prolinx, Bothell, WA, cat no. VMT5100-5); store desiccated at –20°C.
3. Sodium periodate (350 m*M*): prepare fresh, light sensitive.
4. Hydrazide reaction buffer: 0.1 *M* sodium acetate, 0.1 *M* sodium chloride, adjusted to pH 5.5 with 6 *M* HCl.
5. 0.1 *M* sodium bicarbonate, pH 8.0.
6. 400 m*M* sodium sulfite.
7. Methyl sulfoxide.

8. Optional: size-exclusion column (e.g., protein desalting columns, Pierce Chemical, Rockford, IL, cat no. 98949).

2.1.3. Conjugation of Sulfhydryl Residues

1. Protein-capture ligand containing sulfhydryl residues.
2. Sulfhydryl alkylating reagent, proteomics grade PDBA–X–maleimide (molecular weight [MW] = 459.07) (Prolinx, Bothell, WA, cat. no. VMT5050-5); store desiccated at –20°C.
3. Dithiothreitol (DTT), molecular biology grade.
4. DMF, anhydrous.
5. 0.1 *M* sodium phosphate buffer, pH 7.0.
6. Size-exclusion column (e.g., protein desalting columns, Pierce Chemical, Rockford, IL, cat no. 98949).

2.2. Printing the Array

1. Spotting solution II (Prolinx, Bothell, WA, cat. no. VMT2150-1).
2. Versalinx protein microarray technology SHA slides (Prolinx, Bothell, WA, cat. no. VMT1000-4).

2.3. Washing and Developing the Array

1. Slide wash jars (Prolinx, Bothell, WA, cat. no. VMT2200-10).
2. Wash buffer no. 1 (Prolinx, Bothell, WA, cat. no. VMT2101-1).
3. Wash buffer no. 2 (Prolinx, Bothell, WA, cat. no. VMT2102-1).
4. Wash buffer no. 3 (Prolinx, Bothell, WA, cat. no. VMT2103-1).
5. Crude protein mixture containing target protein(s).
6. Detection protein(s).
7. Distilled, deionized H_2O (ddH_2O).
8. N_2 (g).

3. Methods

The methods described below outline (a) the conjugation of the protein-capture ligand(s) with PDBA, (b) the immobilization of PDBA–protein conjugates on SHA glass slides (printing the array), (c) the binding of target protein(s) to the arrayed elements, and (d) the subsequent detection and imaging of specifically bound targets.

3.1. Conjugation of Protein-Capture Ligands

To immobilize a capture ligand on an SHA slide, it is necessary to first conjugate the protein ligand with PDBA. To choose which PDBA derivative to use, it is useful to know the active site of the protein ligand responsible for binding the target protein and to avoid modifying that active site during the conjugation reaction. If the active site is unknown, a small amount of the capture ligand may be conjugated using each of three PDBA derivatives at varying molar-input ratios, and the resulting conjugates may be tested to determine which gives optimal capture of the protein target.

3.1.1. Conjugation of Lysine Residues

1. Prepare a 1–10 mg/mL protein ligand solution (*see* **Note 1**) in 0.1 *M* sodium bicarbonate buffer, pH 8.0 (*see* **Note 2**). If the protein is already in solution, perform a buffer exchange, either by dialysis or using a size-exclusion column.

2. Measure the ultraviolet (UV) absorbance of the protein ligand solution. Using the literature value for the absorptivity and MW of the protein ligand, calculate the concentration of the stock solution (in micromolar units) and the micromoles of protein ligand to be conjugated.
3. Prepare a 100 m*M* solution of PDBA-X-NHS in anhydrous DMF. Vortex to dissolve.
4. Add 10-mol equivalents of 100 m*M* PDBA-X-NHS solution to the protein ligand solution (*see* **Note 3**), keeping the final concentration of DMF below 10% (v/v) (*see* **Note 4**).
5. Incubate the reaction on wet ice for 1 h (*see* **Note 5**).
6. Optional, *see* **Note 6**: Purify the protein conjugate of unwanted byproducts and reactants using a size-exclusion column.
7. If desired, the concentration of the protein conjugate may be estimated as described in **Note 7.**

3.1.2. Conjugation of Carbohydrate Moieties of Glycoproteins

1. Dissolve the glycosylated protein in hydrazide reaction buffer to a final concentration of 1–10 mg/mL protein solution (*see* **Note 8**). If the protein is already in solution, perform a buffer exchange, either by dialysis or using a size-exclusion column.
2. Measure the UV absorbance of the protein ligand solution. Using the literature value for the absorptivity and MW of the protein ligand, calculate the concentration of the stock solution (in micromolar units) and the micromoles of protein ligand to be conjugated.
3. Freshly prepare a 350-m*M* solution of sodium periodate in H_2O. Protect this solution from the light.
4. Add the sodium periodate solution to the protein ligand solution a final concentration of 10 m*M* sodium periodate.
5. React on wet ice, in the dark, for 30 min.
6. Quench the reaction by adding freshly prepared 0.4 *M* sodium sulfite solution to a final concentration of 20 m*M* sodium sulfite. Vortex to mix. The quenching reaction occurs instantaneously.
7. Prepare a 100-m*M* solution of PDBA–X–hydrazide in 50% (v/v) dimethyl sulfoxide and hydrazide reaction buffer.
8. Add 10-mol equivalents of 100 m*M* PDBA–X–hydrazide solution to the protein ligand solution (*see* **Note 3**), keeping the final concentration of DMF below 10% (v/v) (*see* **Note 4**).
9. Incubate on wet ice for 4 h.
10. Optional (*see* **Note 6**) Purify the protein conjugate of unwanted byproducts and reactants using a size-exclusion column.
11. If desired, the concentration of the protein conjugate may be estimated as described in **Note 7**.

3.1.3. Conjugation of Sulfhydryl Residues

1. Dissolve the sulfhydryl-containing protein ligand in 0.1 *M* sodium phosphate buffer, pH 7.0 (*see* **Note 9**) at a concentration of at least 0.5 mg/mL. If the protein is already in solution, perform a buffer exchange, either by dialysis or using a size-exclusion column.
2. Measure the UV absorbance of the protein ligand solution. Using the literature value for the absorptivity and MW of the protein, calculate the concentration of the stock solution (in micromolar units) and the micromoles of protein ligand to be conjugated.
3. Warm the protein solution to 37°C in a water bath.
4. Add 0.5 *M* DTT solution to the warmed protein solution to afford a final concentration of

Table 2
Spotting Methods and Corresponding Parameters for Printing Protein Microarrays

Spotting method	Spotting parameters		
Manual micropipet	0.3–0.5 µL vol.		
Automated	Volume	Pitch	Humidity (room temperature)
Contact spotter	30–35 nL	500 µm	60–70%
Noncontact spotter	350 pL	300–500 µm	60–70%

0.5 m*M* DTT (*see* **Note 10**). Incubate the solution for an additional 10 min at 37°C.
5. Remove the reducing agent by passing the protein solution through a size-exclusion column (e.g., Pierce Chemical protein desalting columns).
6. Prepare a solution of 100 m*M* PDBA–X–maleimide in anhydrous DMF. Vortex to mix.
7. Add 2-mol equivalents of the 100 m*M* PDBA–X–maleimide solution to the protein solution (*see* **Note 3**), keeping the final concentration of DMF below 10% (v/v) (*see* **Note 4**). Vortex to mix.
8. Warm the reaction mixture at 37°C for 30 min.
9. Optional (*see* **Note 6**) Purify the protein conjugate of unwanted byproducts and reactants using a size-exclusion column.
10. If desired, the concentration of the protein conjugate may be estimated as described in **Note 7**.

3.2. Printing the Array (Immobilization of PDBA–Protein-Capture Ligands on SHA Slides)

1. Dilute the PDBA–protein-capture ligand(s) into spotting solution II to a final concentration of 50–100 µg/mL for spotting (*see* **Notes 11** and **12**).
2. Array PDBA conjugated proteins onto SHA slides (*see* **Note 13**) using a micropipet (*see* **Note 14**) or a commercial spotter according to the parameters shown in **Table 2**. Do not let spots dry out (*see* **Note 15**).
3. Incubate spotted slides for 1 h in a covered, humid environment. Do not let spots dry out (*see* **Notes 15** and **16**).

3.3. Binding of Target Protein(s)

1. Wash slides by slowly dipping them in slide wash jars containing 25–30 mL of wash buffer 1 (*see* **Note 17**).
2. Incubate slides at room temperature in wash buffer 1 for 2 min.
3. Optional: If necessary, block the slides (*see* **Note 18**).
4. Without allowing the slides to dry, decant the wash buffer, and add the sample containing the target proteins in wash buffer 2 (*see* **Notes 19** and **20**).

3.4. Washing and Developing the Array

1. Decant wash buffer 2 containing the target protein(s) and add approx 35 mL of wash buffer 2 (*see* **Note 19**) containing the fluorescent dye-labeled detection protein(s) at a final concentration of 0.1 µg/mL to 10 µg/mL (*see* **Notes 21** and **22**).
2. Incubate slide for 30 min with gentle agitation.
3. Decant the wash buffer 2 containing the fluorescent dye-labeled detection protein and

add approx 35 mL of wash buffer 3.
4. Gently agitate for 5 min.
5. Decant wash buffer 3 and add approx 35 mL of ddH_2O.
6. Gently agitate for 5 min.
7. Decant ddH_2O (*see* **Note 23**).
8. Dry slide under a slow stream of N_2 gas or in a centrifuge equipped with slide racks for 1 min.

3.5. Imaging the Array

1. Read slide using a commercial fluorescence scanner designed for microarray detection (*see* **Note 24**). Sample results are shown in **Fig. 2**.

4. Notes

1. To maximize the modification of lysines and minimize the effects of hydrolysis of the NHS ester, it is desirable to maintain a high concentration (1–10 mg/mL) of protein in the reaction. However, successful conjugations can be performed with protein concentrations as low as 0.1 mg/mL by increasing the input ratio of PDBA:protein.
2. Although sodium bicarbonate is suggested as the buffer of choice, other buffers may be used in the pH range of 7–9. Exceptions include those buffers that contain free amine or sulfhydryl groups such as Tris-HCl and glycine, β-mercaptoethanol and DTT or dithioerythritol (DTE). The mechanism of this reaction is based on nucleophilic attack of the deprotonated amine groups (i.e., the ε-amine groups of lysine in proteins) on the NHS ester. The pH is best kept below 9.0 to minimize the competing hydrolysis reaction of the NHS ester.
3. The input ratio of PDBA-conjugation reagent to protein may need to be optimized for a particular ligand. By adjusting the molar ratio of PDBA-conjugation reagent to the target protein, the level of modification may be controlled to create a PDBA ligand with optimal activity.
4. Above concentrations of 10% (v/v) DMF, protein ligands may begin to denature.
5. If using a buffer with a pH <8.0, the reaction time may need to be increased for the reaction to go to completion. Alternatively, a higher input ratio of PDBA:protein (e.g., 20:1) may be used.
6. Purification of PDBA-conjugates is not necessary for most applications and is efficiently removed by washing. However, if you wish to estimate the moles of PDBA per mole of protein (*see* **Note 7**), you will need to purify the conjugate to remove free PDBA.
7. The actual moles of conjugated PDBA per mole of protein can be estimated by quantitatively comparing the absorbance at 260 nm of unmodified protein (P) vs modified protein (PDBA-P). An increase in absorbance at this wavelength results from PDBA addition. The molar absorptivity of PDBA at 260 nm is 4000. For a protein that absorbs at 280 nm, dilute (as necessary) an aliquot of the PDBA-modified protein with an appropriate buffer. Dilute (as necessary) an aliquot of the unmodified protein with the same buffer. Measure A_{280} and A_{260} of both solutions. Use the following equations to estimate the degree of modification. *DF* refers to the dilution factor used, whereas a_{280}^{P} is the molar absorptivity of the unmodified protein at 280 nm, and *MW* is the molecular weight of the protein.

$$[\text{PDBA-P, mg/mL}] = A_{280}\text{PDBA-P}/a_{280}^{\text{P}} \quad (1)$$

$$[\text{PDBA-P, M}] = [\text{PDBA-P, mg/mL}]/\text{MW}^{\text{P}} \quad (2)$$

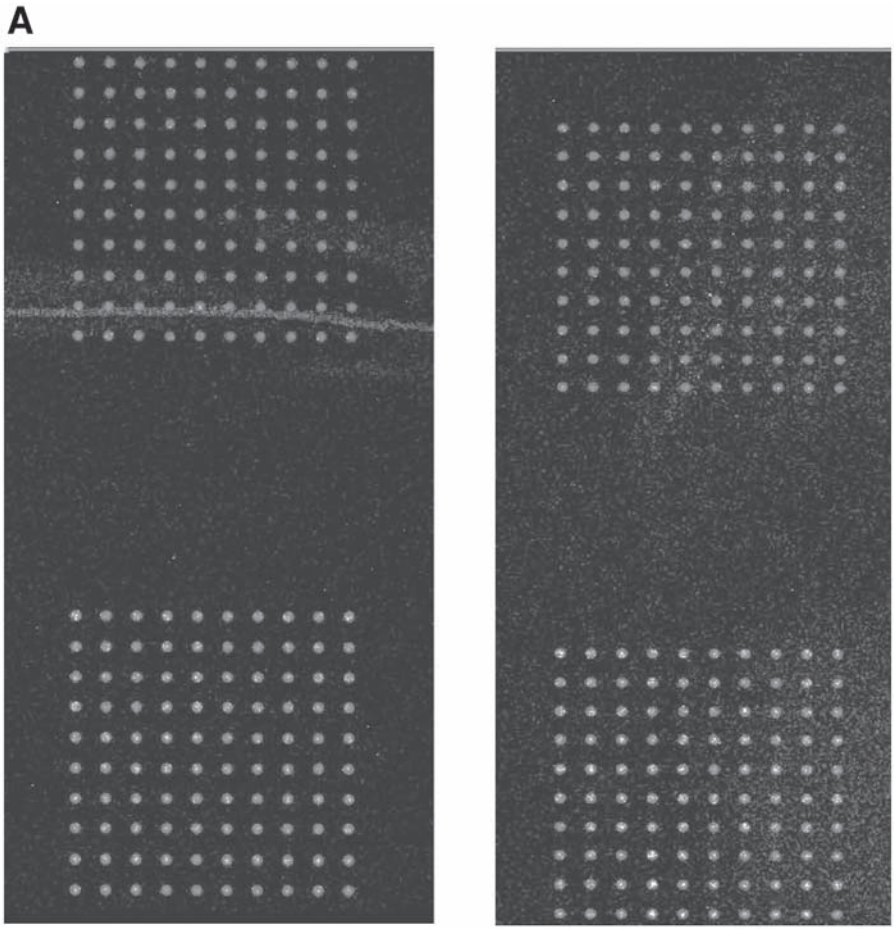

Fig. 2. Image of four 10 × 10 subarrays of dual-modified PDBA-BSA-Cy3 conjugate on two SHA slides. Each subarray contains BSA modified through the N-terminus and lysine residues with PDBA-X-NHS and Cy3-X-NHS. BSA was conjugated with PDBA-X-NHS (45 min, room temperature, 15:1 input ratio NHS:BSA in 0.1 *M* sodium bicarbonate, pH 8) followed by reaction with Cy3-NHS (45 min, room temperature, 15:1 input ratio NHS:BSA in 0.1 *M* sodium bicarbonate, pH 8.0). The resulting conjugate was purified by size-exclusion chromatography (NAP-25) to remove excess dye. The conjugate was spotted in two 10 × 10 arrays on two SHA slides at a protein concentration of 100 μ*M* using a noncontact Packard Bioscience BioChip Arrayer (350 pL/spot, 180 μm diameter, 500 μm pitch). The slide was incubated in a humid chamber for 1 h, then washed, rinsed with H_2O, dried, and scanned using a Packard Bioscience ScanArray Lite (100% laser power, 85% PMT gain).

$$A_{260}^{PDBA} = (DF)(A_{260}^{PDBA\text{-}P}) - [(DF)(A_{280}^{PDBA\text{-}P})*(A_{260}P/A_{280}^{P})] \quad (3)$$

$$[PDBA, M] = A_{260}^{PDBA}/4000 \quad (4)$$

$$PDBA{:}P = [PDBA, M]/[P, M] \quad (5)$$

8. Successful conjugations can be performed with protein concentrations as low as 0.1 mg/mL by increasing the input ratio of PDBA:protein (e.g., 20:1).
9. The pH of this reaction needs to be controlled to minimize reaction with free amines on the protein ligand. Maleimides react specifically with sulfhydryl groups in the pH range of 6.5–7.5. At more basic pH, however, maleimides show crossreactivity with amines such as those found on lysine residues. The input concentration of PDBA-X-maleimide may need to be optimized to minimize reaction with free amines on the protein ligand.
10. DTT serves to reduce disulfide bonds generating free sulfhydryl groups that can react with the maleimide. Alternatives to DTT include tris-(2-carboxyethyl)-phosphine hydrochloride, and mercaptoethylamine hydrochloride. If the protein ligand already has free sulfhydryl groups, this step may be eliminated.
11. Optimal spotting concentration is dependent on the protein MW and spotting method and may need to be optimized. For crude conjugation solutions (those that contain excess PDBA), use the input protein concentration for this calculation.
12. Proteins diluted in spotting solution II must be used within 5 h or they will not bind to the slide surface.
13. Handle slides on the edges or by the frosted edge with powder-free gloves. Spot the slides on the side with the frosted surface.
14. If spotting by hand using a pipet, apply a spot without allowing the pipet tip to touch the slide's surface.
15. Although stability is somewhat protein dependent, many proteins undergo irreversible denaturation on drying.
16. Humidity is important to maintain low background. If spots dry before development, background is significantly higher. During incubation, a temperature of about 1°C above the dew point is sufficient to keep the slides from drying out without developing excess condensation, either of which can negatively impact spot morphology and affect array quantification. The spotted arrays can also be incubated at room temperature with a humidity of around 70%.
17. It is best to hold the jar at a 45° angle and slowly dip the slide into the wash buffer 1 with the spots on the bottom face of the slide.
18. Although the 3D-SHA-coated slides inherently have low nonspecific protein binding, for certain assays blocking has been shown to improve the resolution of low-abundance targets. Blocking conditions will be assay dependent. In general, good performance has been achieved blocking slides with a solution of 1.5% (w/v) bovine serum albumin (BSA), 5% (w/v) nonfat milk, 0.01 *M* sodium phosphate, 0.14 *M* sodium chloride, 0.003 *M* potassium chloride, pH 7.4 for 1 h at room temperature. After blocking, wash slides in 25–30 mL of wash buffer 2 for 2 min at room temperature.
19. To reduce volumes, a slide well or hybridization chamber (e.g., Grace Biolabs' perfusion chamber, part no. PC1L-1.0, with adhesive seal tabs, part no. ST200) can be fixed onto the slide allowing incubation with reduced volumes of wash buffers and detection solutions. Sections of the slide surface can also be separated for differential incubations by using Jacquard Gutta resist clear polymer (Rupert Gibbon and Spider, Inc., Headsburg,

CA), available from most art supply stores. Use of Pap pens to create sections on the slide surface is *not* recommended.

20. The concentration of the sample, the sample to diluent ratio, and the incubation time is assay dependent. In general, good performance has been achieved using an incubation time of 30 min.
21. If using a primary and secondary antibody pair for detection, add the antibodies serially in wash buffer 2 and wash between additions with wash buffer 2.
22. Optimal concentration of dye-labeled protein is dependent on protein titer, MW, and spotting method and may need to be optimized.
23. You may need to rinse several times with ddH_2O to remove residual wash solution streaks.
24. Scanning parameters will be instrument dependent. Taking a preliminary low-resolution scan using the highest photomultiplier tube (PMT) gain and laser power is useful in determining the array coordinates and estimating suitable laser power and PMT-gain settings to be used for a high-resolution scan.

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11

Evaluation of Antibodies and Microarray Coatings As a Prerequisite for the Generation of Optimized Antibody Microarrays

Philipp Angenendt and Jörn Glökler

Summary

Antibody microarrays are becoming a major tool for the parallel analysis of complex samples. So far, many efforts have been made to increase the complexity and sensitivity of antibody microarrays. In contrast to enzyme-linked immunosorbent assay (ELISA) experiments, not all antibodies remain functional in the microarray format. Sensitivity is very much dependent on the type of coating and its application. The method described in this chapter is a quick and reliable method that has been very useful in determining the functionality of antibodies and the suitability of coatings for antibody microarrays. At the same time, a detailed description of how to prepare an inexpensive and highly efficient antibody microarray surface is given.

Key Words:

Antibody microarray; functionality; poly-L-lysine; surface coating; ELISA.

1. Introduction

With the advent of proteome research, new tools for the handling and investigation of thousands of different proteins are being developed. One of the key technologies in this field is antibody microarray technology, in which an array of different antibodies is immobilized on a standard microscope slide ***(1–4,15)***. Because provision of an optimal slide coating is crucial for immobilization, many efforts have been undertaken to develop slide coatings that offer high capacity, low autofluorescence, and a nondenaturing environment.

In previous years, polyvinylidene difluoride membranes were the support of choice for high-density protein macroarrays ***(5)*** and microarrays ***(6)***. Currently, two major groups of microarray slides can be distinguished. The first comprises modified plain glass or plastic surfaces displaying chemical groups, such as aldehyde residues ***(7)***, amino terminus of L-lysine ***(8)***, or avidin-coated slides ***(9)***. The other group consists of

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gel-coated surfaces, such as polyacrylamide ***(10)*** or agarose ***(11)***. Beside the evaluation of slide coatings ***(14)***, the assessment of the antibodies is crucial because not all remain functional on immobilization ***(12)***. The characterization of two antibodies and two coatings will be used to illustrate the methodology of evaluation. The authors suggest that these methods can be extended for the screening of complex solutions as described in further detail in Chapter 4 of this volume.

2. Material

2.1. Derivatization of Glass Slides With Poly-L-Lysine

1. Glass microscope slides.
2. Cleaning solution: 0.25 *M* NaOH, 50% (v/v) ethanol absolute.
3. Plastic slide holder and incubation chamber (Carl Roth; Karlsruhe, Germany).
4. Microscope slide shipping container (Neolab, Heidelberg, Germany).
5. Phosphate-buffered saline (PBS) (pH 7.5): 0.137 *M* NaCl, 0.0027 *M* KCl, 0.012 *M* Na_2HPO_4, 1.76 m*M* KH_2PO_4.
6. Poly-L-lysine solution (cat. no. P8920, Sigma-Aldrich GmbH, Munich. Germany).
7. Poly-L-lysine coating solution: 10% (v/v) poly-L-lysine solution, 10% (v/v) PBS.
8. Kimwipes (Kimberly-Clark, Irving, TX).

2.2. Derivatization of Glass Slides With a Polyacrylamide Gel

1. Plastic slide holder and incubation chamber (Carl Roth; Karlsruhe, Germany).
2. Glass slide holder and incubation chamber (Neolab, Heidelberg, Germany).
3. Technical ethanol.
4. 55°C oven.
5. Bind-silane (Amersham Biosciences, Uppsala, Sweden).
6. Bind-silane solution: 0.1% (v/v) acetic acid (glacial), 0.3% (v/v) bind-silane, 99.6% (v/v) ethanol absolute.
7. Repel-silane ES (Amersham Biosciences, Uppsala, Sweden).
8. Metal stripe (d = 0.03 mm) (Perschmann, Braunschweig, Germany).
9. Mauly letter clips (Jakob Maul, Bad König, Germany).
10. 8% polyacrylamide gel solution: 26.6% (v/v) 30%–0.8% (w/v) acrylamide/*bis*-acrylamide solution, 0.5% (v/v) 10% (w/v) adenosine 5'-phosphosulfate (APS), 0.2% (v/v) TEMED.
11. 1X PBS.

2.3. Fluorescent Labeling of Antigen and Preparation for Spotting

1. Cy3-*N*-hydroxysuccinimide (NHS)-dye (Amersham Biosciences, Uppsala, Sweden).
2. Cy5-NHS-dye (Amersham Biosciences, Uppsala, Sweden).
3. Dimethyl formamide (DMF).
4. 1X PBS/0.1% NaN_3:99.9% (v/v) PBS, 0.1% (w/v) NaN_3.
5. Sodium carbonate buffer (pH 9.6): 1M Na_2CO_3, pH 9.6.
6. Centrifugal filter (Millipore Corp. Bedford, MA).
7. Glycerol.

2.4. Preparation of Antibodies for Spotting

1. Lyophilized antibody.
2. 1X PBS/0.1% NaN_3.
3. 384 v-well polypropylene plates (Genetix, Hampshire, UK).

2.5. Spotting

1. QArray spotting robot equipped with 16 solid spotting pins (Genetix, Hampshire, UK).
2. Dye (e.g., Orange G).
3. 80% ethanol: 80% (v/v) Ethanol technical.

2.6. Blocking and Incubation of Microarrays

1. Tris-buffered saline (TBS): 0.15 *M* NaCl, 0.05 m*M* Tris-HCl, pH 7.5.
2. TBST: 0.1% (v/v) Tween®-20, 99.9% (v/v) TBS.
3. Nonfat dry milk powder (Bio-Rad, Hercules, CA).
4. 3% nonfat dry milk powder/TBST: 3% (w/v) nonfat dry milk powder, 97% (v/v) TBST.

2.7. Fluorescent Scanning

1. ScanArray 4000.

2.8. Data Analysis

1. GenePix Pro 4.0 (Axon Instruments, Union City, CA).

2.9. Enzyme-Linked Immunosorbent Assay

1. 96-well Cliniplate® (Thermo Labsystems, Helsinki, Finland).
2. Nonfat dry milk powder (Bio-Rad, Hercules, CA).
3. PBS.
4. 3% nonfat dry milk powder/PBS: 3% (w/v) nonfat dry milk powder, 97% (v/v) PBS.
5. PBST: 0.1% (v/v) Tween®-20, 99.9% (v/v) PBS.
6. 2,2'-Azino-*bis*-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS).
7. ABTS solution: 5×10^{-5}% (w/v) ABTS, 25 m*M* trisodiumcitrate, 25 m*M* citric acid, 2×10^{-4}% 30% (w/w) H_2O_2.
8. Photometer.

3. Methods

The methods described in this section outline (a) the coating of standard microscope slides with chemical groups as well as with a polyacrylamide gel, (b) the spotting of a microarray, (c) the subsequent blocking and incubation, (d) the fluorescent scanning, (e) the data analysis, and (f) the use of ELISA to validate antibodies that were not functional on microarrays.

3.1. Preparation of Microarray Coating, Antibody, and Antigen

The preparation of microarray substrates, antibody, and fluorescently labeled sample is described in **Subheadings 3.1.1.–3.1.3.** This includes (a) coating of a standard microscope slide with a poly-L-lysine, (b) coating of a standard microscope slide with a polyacrylamide gel, (c) preparation of antibodies, and (d) labeling of sample and preparation for spotting.

3.1.1. Derivatization of Glass Slides With Poly-L-Lysine

Within the derivation of the slides, glass slides are first cleaned and abraded with a mixture of NaOH and ethanol. Onto this surface, polymers of L-lysine attach electrostatically.

1. Incubate slides in cleaning solution for 1 h on an orbital shaker at room temperature.
2. Wash slides vigorously two to three times by plunging racks up and down in plenty of ddH_2O to remove all traces of ethanol and NaOH.
3. Check pH to assure the removal of NaOH and incubate slides in poly-L-lysine coating solution on an orbital shaker for 45 min at 4°C (*see* **Notes 1** and **2**).
4. Rinse slides with ddH_2O.
5. Place a Kimwipe at the bottom of the incubation chamber and place the rack on the Kimwipe. Spin dry in a centrifuge at 160*g* for 10 min.
6. Transfer coated substrates into a microscope slide shipping container capable of five slides each and seal with Parafilm.
7. Store slides at 4°C, and use slide within 6 wk after production (*see* **Note 3**).

3.1.2. Derivatization of Glass Slides With a Polyacrylamide Gel

After cleaning the slides with technical ethanol, one batch of slides is treated to present a repellent surface, whereas the other is treated to bind to the acrylamide gel. Two slides, one of each batch, are assembled, so that a space of 30 μ*M* is created between both, in which a polyacrylamide gel is cast.

1. Prepare polyacrylamide slides by cleaning two batches of glass microscope slides for 10 min in technical ethanol under agitation.
2. Dry slides in an oven at 55°C for 2 h.
3. Take batch 1 and dispense 200 μL bind-silane solution on one side of each slide, and incubate for 30 min under an extraction hood.
4. Incubate the other batch of cleaned slides in repel-silane using a slide rack and chamber made of glass for 30 min under an extraction hood (*see* **Note 4**).
5. Cut metal spacers with a defined thickness of 30 μ*M* into stripes of 22 × 5 mm.
6. Clean both sets of slides with technical ethanol and wipe dry using a piece of leather (*see* **Note 5**).
7. Place bind-silane-treated slide bind-silane side up, and put a spacer at both ends.
8. Place a repel-silane-treated slide on the bind-silane slide leaving a protruding end of about 1 mm and clip on Mauly letter clips at both ends (*see* **Fig. 1**).
9. Cast 8% (v/v) polyacrylamide gel solution into the spacing between the two slides.
10. Remove clips and slides after polymerization and store gel-coated slides at 4°C.
11. Prior to spotting incubate slides in PBS and remove excess liquid by centrifugation at 1000*g* for 1 min.

3.1.3. Fluorescent Labeling of Antigen and Preparation for Spotting

Several fluorophores are available for the detection of labeled proteins. The most commonly used fluorophores in microarray technology are Cy3 and Cy5 because they provide a high quantum yield, are more photostable, and give less background than most other fluorophores. Moreover, they are readily available as NHS conjugates.

1. Reconstitute Cy3/Cy5-NHS-dye in 100 μL DMF, prepare aliquots of 10 μL each, and store them at –20°C.
2. Dilute protein solution with PBS/0.1% NaN_3 to a final concentration of 1 mg/mL (*see* **Note 6**).
3. Add 5 μL of sodium carbonate buffer (pH 9.6) to 100 μL of protein and mix by pipetting up and down.

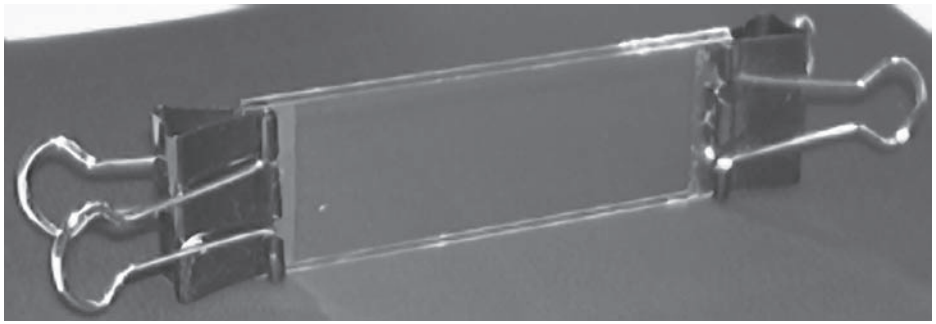

Fig. 1. Photograph of assembled slides with a 30-m*M* gap by metal spacers.

4. Transfer protein solution to one aliquot of Cy3/Cy5-NHS-dye, and incubate in the dark at room temperature for 30 min.
5. Vortex mixture every 10 min, avoiding the generation of foam.
6. Transfer labeled protein/unreacted dye mixture to a centrifugal filter of the appropriate molecular weight cutoff, and centrifuge at 16,000*g* and 4°C until the whole solution is filtered.
7. Discard filtrate, resuspend retentate in 500 µL PBS/0.1% NaN_3, and centrifuge again.
8. Repeat **step 7** until flowthrough is colorless.
9. Transfer remaining labeled protein into a 1.5-mL Eppendorf tube, and resuspend to 100 µL with PBS/0.1% NaN_3 and 20% (v/v) glycerol.
10. Store at –20°C.

3.1.4. Preparation of Antibodies for Spotting

1. Dissolve lyophilized antibodies as recommended by the manufacturer.
2. Prepare dilutions in PBS containing 0.1% NaN_3.
3. Transfer 30 µL of each dilution into 384 v-well polypropylene plates (*see* **Note 7**).

3.2. Spotting

The following procedure is optimized onto the QArray spotting robot. In case other robots are used, adjust procedure accordingly.

1. Design a microarray pattern to yield at least 16 spots per dilution in case only one dilution is spotted. In case dilution rows are spotted, design pattern to yield at least quadruplicates for each concentration (*see* **Note 8**).
2. Clean solid spotting pins by sonication and place pins into the spotting head (*see* **Note 9**).
3. Check datum points of the robot and adjust printing depth of pins. Check printing depth by spotting a dye (e.g., Orange G). In case not all spots are clearly visible, adjust printing depth (*see* **Fig. 2**).
4. Set humidity of the robot to 65%.
5. Program wash routine to wash three times. First, wash 5 s with ddH_2O followed by 3 s airflow to remove the H_2O; the second time, wash 5 s with ddH_2O followed by 3 s airflow. Finally, wash 7 s with 80% ethanol followed by a 5-s airflow.
6. After spotting, place microarrays in a plastic box and incubate at 4°C overnight before use.

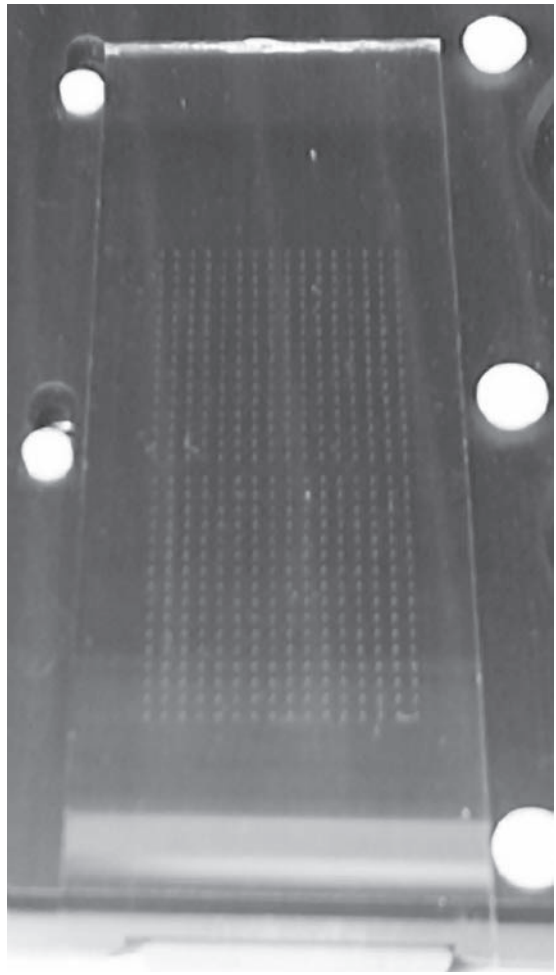

Fig. 2. Photograph of test slide spotted with Orange G to adjust the printing depth. Because all spots are clearly visible, these settings can be used for further spotting.

3.3. Blocking and Incubation of Microarrays

The most critical step in postprocessing is the blocking step, where the remaining adhesive surface is modified to minimize its ability to bind labeled sample. A comparison of four different blocking solutions containing milk powder, bovine serum albumin (BSA), fish gelatin, and fetal calf serum revealed no difference in the quality of background fluorescence.

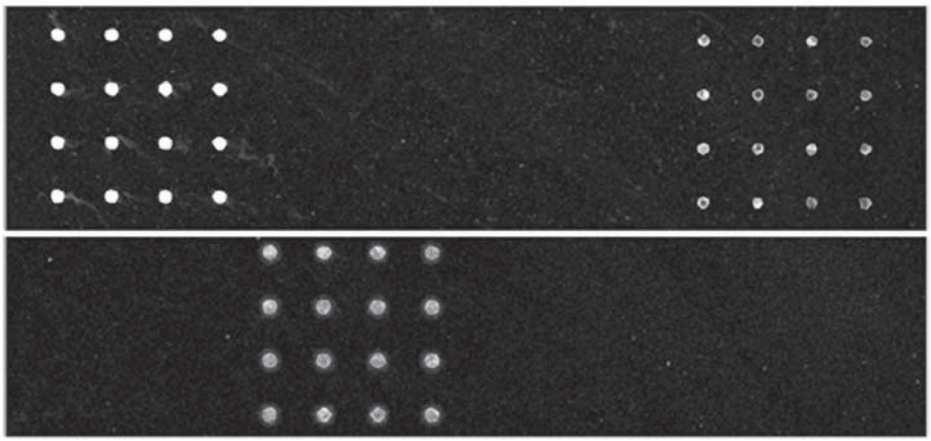

Fig. 3. *Upper part*: Scan with an excitation at 649 nm and emission at 670 nm. *Lower part*: Scan with an excitation at 550 nm and emission values at 570 nm. Samples immobilized in groups of 16 spots (from the right to the left): monoclonal anti-HSA Ab (c = 348 amol/spot), monoclonal antivimentin Ab (c = 333 amol/spot), Cy3-vimentin (c = 266 amol/spot), Cy5-HSA (294 amol/spot). The slide was incubated with 1.6 μg/mLCy3-vimentin and 2 μg/mLCy5-HSA. Both scans show that the antivimentin antibody is nonfunctional on slides.

1. Rinse slides with TBS.
2. Incubate slides in 3% nonfat dry milk powder/TBST for 30 min at 4°C (*see* **Note 10**).
3. Dilute labeled antigen in 3% nonfat dry milk powder/TBST to a concentration of 16 μg/mL.
4. Incubate microarrays in antigen solution at 4°C for 1 h.
5. Rinse microarrays with TBS and wash slides two times by incubation in TBST for 15 min each at 4°C in the dark.
6. Dry slides by centrifugation at 1000*g* for 2 min (*see* **Note 11**).

3.4. Fluorescent Scanning

After binding of the secondary antibody/protein, a fluorescent image of the array is acquired by using a confocal laser scanner (e.g., ScanArray® 4000 scanner) bearing a laser with a wavelength appropriate for the excitation spectra of the dyes being used and a filter for the measurement of the emission at the corresponding wavelengths.

1. Scan slides at a resolution of 50 μ*M* and adjust laser power and photomultiplier gain to maximize the dynamic range without getting saturation of the signal.
2. Scan slide with a resolution of 10 μ*M* (*see* **Figs. 3** and **4**).

3.5. Data Analysis

The intensity of the light emission is quantified using the program GenePix Pro 4.0. For analysis, the median value of signal intensity–background value are applied.

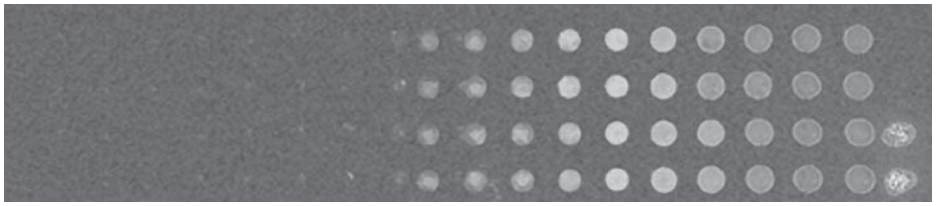

Fig. 4. Scan with an excitation at 550 nm and emission values at 570 nm. A dilution row of polyclonal antifibrinogen Ab was immobilized in quadruplicates from the right to the left, and the slide was incubated with Cy3-fibrinogen. In addition, two spots of Cy3-fibrinogen were immobilized on the right as a positive control.

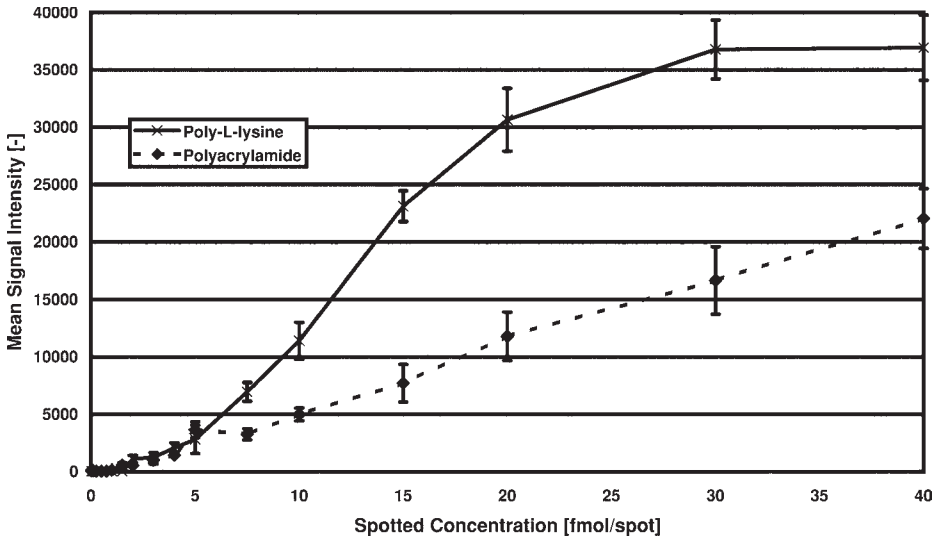

Fig. 5. Comparison of antifibrinogen antibody performance on different slide coatings.

1. Calculate mean values and standard deviation of all signal intensities corresponding to the same concentration of each chip.
2. Calculate coefficient of variation in comparing two chips of the coating.
3. Produce mean signal intensity vs spotted concentration graphs with standard deviation error bars (*see* **Fig. 5**).

3.6. Enzyme-Linked Immunosorbent Assay

All antibodies that do not demonstrate functionality in the array format are tested in ELISA. The antigen is immobilized and detected with a primary antibody specific for the antigen. The primary antigen is detected with an antimouse IgG-horseradish peroxidase (HRP) for the monoclonal primary antibodies and with an antirabbit IgG-HRP for the polyclonal antirabbit.

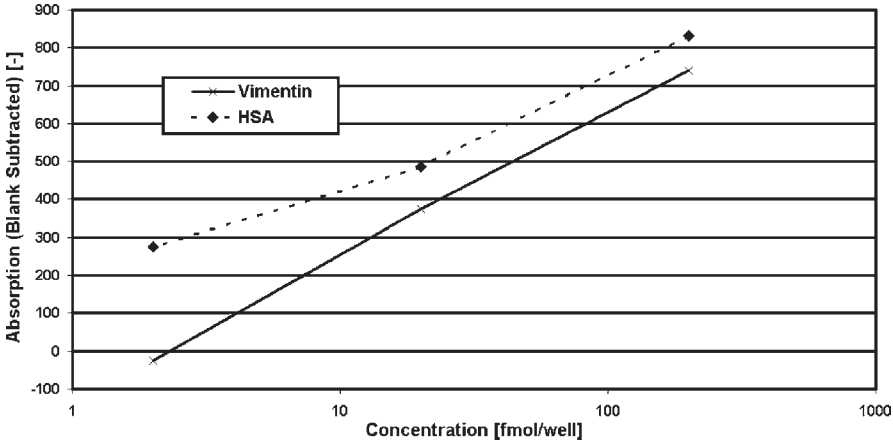

Fig. 6. ELISA of anti-HSA Ab and antivimentin Ab.

1. Transfer 100 µL of antigen into a 96-well Cliniplate and incubated for 1 h at room temperature.
2. Block wells with 400 µL 3% nonfat dry milk powder/PBS for 30 min.
3. Dilute primary antibody 1/2500 and incubate immobilized antigens with 100 µL for 10 min.
4. Wash wells twice with 400 µL PBST and once with 400µL PBS for 10 min each.
5. Dilute secondary antibody 1/5000 and transfer 100 µL into each well.
6. Incubate for 1 h and wash wells twice with 400 µL PBST and once with 400 µL PBS for 10 min each.
7. Add 100 µL of ABTS solution and incubate for 10 min at room temperature.
8. Measure absorption at 405 nm and prepare adsorption vs concentration graphs (*see* **Fig. 6**).

4. Notes

1. Be sure to use plastic slide racks, incubation chambers, and graduated cylinders to avoid coating the whole rack and chamber. Racks and chambers made of polyoxymethylene are a good choice because they can be used in all steps except the treatment of the slides with repel-silane. Moreover, they are more robust than the ones made of glass, which are very sensitive to temperature. Another advantage is that they are black and, therefore, are ideal for the incubation of your fluorescently labeled antigens because they avoid bleaching of the fluorophores by light. Negative effects on using the same rack and chamber for both coating and incubation have not been observed.
2. The reuse of the poly-L-lysine coating solution resulted in slides with increased hydrophobicity, which does not allow the transfer of liquid during spotting. Similar observations were made with slides that were already stored for an extended period.
3. Plain glass slides that have been coated with chemical groups are best stored in dry conditions at 4°C in an exsiccator. This is especially important on using very reactive surface chemistry such as epoxy groups because they react rapidly with the humidity in the air. If possible, an N_2 atmosphere within the exsiccator is ideal because amino groups, for example, react with CO_2 in the air. However, the use of poly-L-lysine-coated slides, stored in Parafilm-sealed plastic containers at 4°C for 6 wk gave good results.

4. Repel-silane can be used repeatedly. Remove brown bubbles that appear on the solution and avoid particles within the solution.
5. The use of a piece of leather before casting of polyacrylamide slides avoids lint inside the gel, which will cause background fluorescence on scanning. The best way to avoid such effects is to briefly wipe away all lint from both slides shortly before assembly. An additional possibility is to remove lint by applying compressed air to the space between both slides.
6. The concentration of the protein solution to be labeled can vary. Protein solutions of up to 10 mg/mL have been labeled successfully.
7. Use 384-well plates made of polypropylene as source plates for spotting because they will not bind proteins as extensively as plates made of polystyrene.
8. The number of spots per concentration is a guideline and is taken from the analysis of previous experiments ***(13)***. Remember a rule from statistics: The more events you have, the more accurate the results.
9. The spotting of antibodies dissolved in PBS does not require extensive cleaning of the pins and head of the robot. However, the spotting of proteins dissolved in buffers containing urea requires caution. Try to dilute them as much as possible because the urea will leave crystals on your chip and on your pins. The crystals on the chip will cause excessive fluorescence on scanning. Crystals on the pins and in the head will cause the pins to stick, which will lead to irregular patterns on the chip. As a rule of caution, sonicate head and pins in large amounts of H_2O every time buffers containing urea were used for spotting.
10. Not all incubation times in the postprocessing of the slides have to be followed precisely. As a general guideline incubations of nongel coated slides may vary between 30 min and overnight. Incubations of gel-coated slides may vary between 1 h and overnight. However, be sure to have the right environment during extended incubation periods (e.g., 4°C for sensitive proteins).
11. Drying of slides can be done in three ways. The first possibility is to place the slides in a 55°C oven. The second way is to blot the slide on a Kimwipe to remove excess liquid. Afterward, the slide is placed vertically in a box, with a Kimwipe on the bottom, and the box is set at room temperature or at 4°C. The third way is to spin the slides dry in a centrifuge. For best results, place slide in a Falcon tube (*see* **Fig. 7**) to have the slide surface in parallel to the centrifugal force. Although all three methods work, the last one is the least time-consuming one and is preferable, particularly compared to the 55°C oven, especially when proteins or antibodies are captured on the surface.

Acknowledgments

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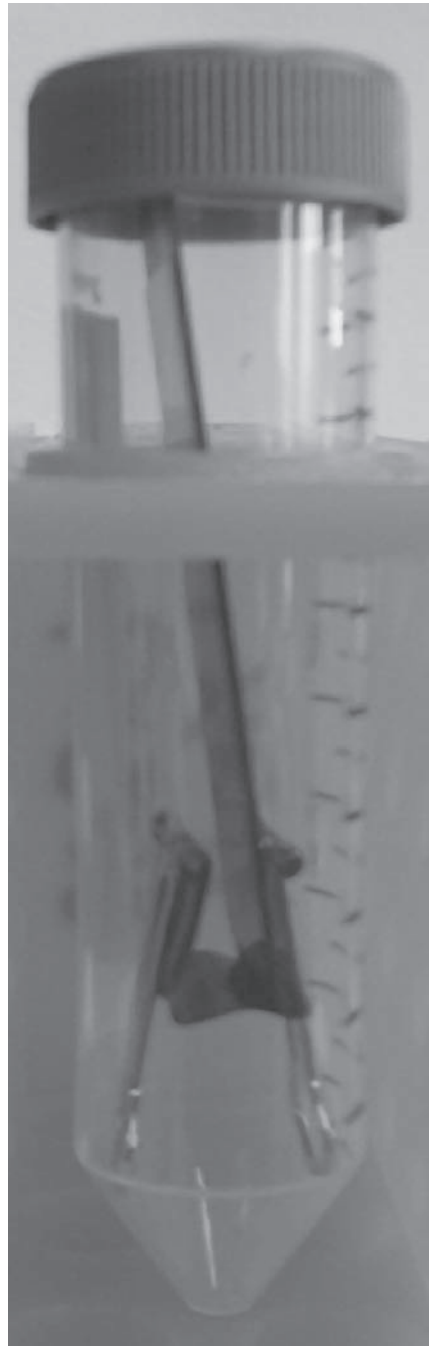

Fig. 7. Photography of Falcon tube with Mauly clip.

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12

Printing Functional Protein Microarrays Using Piezoelectric Capillaries

James B. Delehanty

Summary

The use of piezoelectric dispensers for the fabrication of protein biochips has become a popular approach owing to its inherent advantages of noncontact with the printing surface and the opportunity for recovery of unused sample. Despite the inherent advantages of this printing method, little attention has been devoted to the quantitative aspects of dispensing small volumes of dilute protein solutions from the borosilicate glass capillaries employed by piezoelectric dispensers. Here, a method is detailed that demonstrates the benefits of using a low-ionic-strength phosphate printing buffer containing the carrier protein bovine serum albumin (BSA) to deposit capture antibody microspots. When this buffer is used to deposit biotinylated capture antibodies, 3.6- to 44-fold more protein is delivered to an avidin-coated surface compared to when the antibodies are deposited in standard phosphate-buffered saline (PBS) in the absence of BSA. Further, this method results in the deposition of capture antibodies that adopt a more uniform spot morphology.

Key Words:

Antibody; biosensor; fluorescence; immunoassay; microarray; noncontact printing; piezoelectric dispenser.

1. Introduction

With the advent of protein microarrays, a number of printing platforms have been developed to achieve the deposition of the protein sample to the microarray substrate ***(1–7)***. Among these, piezoelectric dispensers have emerged as a popular platform because they do not contact the printing substrate, and they allow for the recovery of the portion of the sample that is not dispensed. These printers are equipped with borosilicate glass capillaries surrounded by a piezoelectric element collar. The sample is dispensed by the application of a voltage to the piezoelectric collar, typically resulting in the release of a droplet of less than 1 nL in volume. Despite the benefits of the noncontact method employed by the piezoelectric dispenser, only cursory regard has

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been given to the quantitative aspects of dispensing minute volumes of protein solutions (1 nL or less) at the low concentrations (20 μg/mL or less) that are often used in microarray printing. Here, a printing methodology compatible with piezoelectric dispensers is described that employs a low-ionic-strength phosphate buffer containing BSA. This method effectively minimizes the ionic-strength-dependent phenomenon of nonspecific protein adsorption to borosilicate glass ***(8–10)*** and results in the deposition of microspots that exhibit a more uniform spot morphology compared to when printing is performed using a standard PBS printing buffer without BSA ***(11)***. The dispensing of a reference immunoglobulin G (IgG) and a direct immunoassay for cholera toxin are used to illustrate the benefits of the printing method.

2. Materials

1. Phosphate-buffered saline (PBS): 137 m*M* NaCl, 3 m*M* KCl, 10 m*M* phosphate, pH 7.4.
2. Printing buffer 1: 10 m*M* PBS containing 0.1% (w/v) BSA (pH 7.4). Prepare by dilution of PBS with dH_2O and amend with BSA to a final concentration of 0.1% (w/v).
3. Printing buffer 2: PBS (pH 7.4) containing no BSA.
4. Purified polyclonal mouse immunoglobulin G (IgG; Sigma, St. Louis, MO).
5. Goat anticholera toxin antibody (Biogenesis, Brentwood, NH).
6. Biotin-LC-*N*-hydroxysuccinimide (NHS) ester (Pierce Chemical, Rockford, IL).
7. Biogel P-10 (Bio-Rad, Hercules, CA).
8. Cy5 dye (bis-functional derivative, Amersham Life Sciences, Arlington Heights, IL).
9. Glass microscope slides (Daiggerbrand, Wheeling, IL).
10. NeutrAvidin biotin-binding protein (Pierce, Rockford, IL).
11. Biotin-LC-NHS ester (Pierce, Rockford, IL).
12. BSA, Fraction V, (Sigma, St. Louis, MO).
13. Blocking buffer (10 m*M* phosphate, 10 m*M* NaCl, 1% w/v BSA, 0.01% (w/v) NaN_3). Store at 4°C.
14. Tween-20 (Sigma, St. Louis, MO).
15. Poly-(dimethylsiloxane [PDMS]) (NuSil Technology, Carpenteria, CA).
16. (3-mercaptopropyl) trimethoxysilane (Fluka, Ronkonoma, NY).
17. *N*-(γ-maleimidobutyryloxy) succinimide ester (Fluka, Ronkonoma, NY).

3. Methods

The methods described in this section outline (a) the preparation of the microarray substrate, (b) the modification of proteins for printing and assays, (c) the printing of microarrays and the performance of assays, and (d) the imaging and analysis of the microarrays.

3.1. Preparation of the Microarray Substrate

The steps described in **Subheadings 3.1.1.** and **3.1.2.** outline the cleaning and silanization of conventional glass microscope slides and the covalent immobilization of NeutrAvidin to the slide surface to produce a microarray substrate that is suitable for the deposition of biotinylated proteins.

3.1.1. Cleaning of Microscope Slides

Clean conventional glass microscope slides are prepared using the KOH/isopropanol method described in ***(12)***.

1. Clean the slides by immersion in 10% KOH/isopropanol for 30 min at room temperature followed by exhaustive rinsing with deionized H_2O until no schlieren lines are observed.
2. Dry the slides under a stream of nitrogen and use immediately for the immobilization of NeutrAvidin to the slide surface (*see* **Subheading 3.1.2.**).

3.1.2. Covalent Immobilization of NeutrAvidin to Microscope Slides

Cleaned slides are silanized and NeutrAvidin biotin-binding protein is covalently immobilized to the glass surface according to the procedure described in ***(13)***.

1. Treat the cleaned slides for 2 h (under nitrogen) with a 2% solution of (3-mercaptopropyl) trimethoxysilane in toluene.
2. Rinse the silanized slides three times in fresh toluene and briefly allow them to air-dry.
3. Incubate the slides for 30 min in 2.1 m*M* *N*-(γ-maleimidobutyryloxy) succinimide ester in anhydrous ethanol.
4. Rinse the slides three times in deionized H_2O and incubate in 33 µg/mL ImmunoPure NeutrAvidin in PBS overnight at 4°C.
5. The next day, liberally rinse the NeutrAvidin-coated slides in PBS, transfer to blocking buffer, and store at 4°C. The slides can be stored in blocking buffer for up to 4 wk prior to use.
6. Immediately prior to printing, remove the slides from the blocking buffer and rinse three times with PBS. Next, rinse the slides with deionized H_2O and dry under a stream of nitrogen.

3.2. Modification of Proteins

The steps in **Subheadings 3.2.1.** and **3.2.2.** describe the general procedures that are used to prepare biotinylated antibodies and Cy5-labeled proteins for use in assays to demonstrate the effects of different dispensing buffers on the quantitative delivery of proteins from glass capillaries to the microarray substrate.

3.2.1. Biotinylation of Antibodies

1. React the antibody (at a final concentration of 1–1.5 mg/mL) with a fivefold molar excess of biotin-LC-NHS ester in 50 m*M* sodium bicarbonate buffer (pH 8.0) for 1 h at room temperature while stirring.
2. Remove the unincorporated biotin by size-exclusion chromatography using Biogel P-10 equilibrated with PBS.

3.2.2. Labeling of Antibodies and Toxins with Cy5

1. React the protein (at a final concentration of 1–1.5 mg/mL) with a fivefold molar excess of Cy5 dye in 50 m*M* sodium borate buffer (pH 8.0) in the dark for 1 h at room temperature while stirring.
2. Remove the unincorporated Cy5 dye by size-exclusion chromatography using Biogel P-10 equilibrated with PBS.
3. Determine the Cy5:protein ratio by spectrophotometry using the appropriate extinction coefficients (*see* **Note 1**).

3.3. Microarray Printing and Assays

The steps described in this section outline the use of a noncontact microarrayer equipped with piezoelectric glass capillaries to deliver biotinylated antibodies to the NeutrAvidin-coated microarray substrate. **Subheading 3.3.1.** provides general guidelines pertaining to the printing of biotinylated antibodies using piezoelectric glass cap-

illaries. **Subheading 3.3.2.** describes the method used to deposit biotinylated Cy5-labeled mouse IgG to demonstrate the effects of the printing buffer ionic strength and the presence of carrier protein on the quantitative delivery of the IgG from the glass capillary. **Subheading 3.3.3.** describes the method used to deposit biotinylated goat anticholera toxin antibody to demonstrate the functional implications of dispensing biotinylated capture antibodies in either printing buffer 1 (10 m*M* PBS containing 0.1% (w/v) BSA) or printing buffer 2 (150 m*M* PBS containing no BSA) by performing a direct immunoassay for cholera toxin. In both instances, antibodies were deposited onto the NeutrAvidin-coated microarray substrate using a BioChip Arrayer I™ noncontact microarrayer (PerkinElmer Life Sciences, Boston, MA) housed in an atmospherically isolated chamber with a relative humidity of 45% at room temperature.

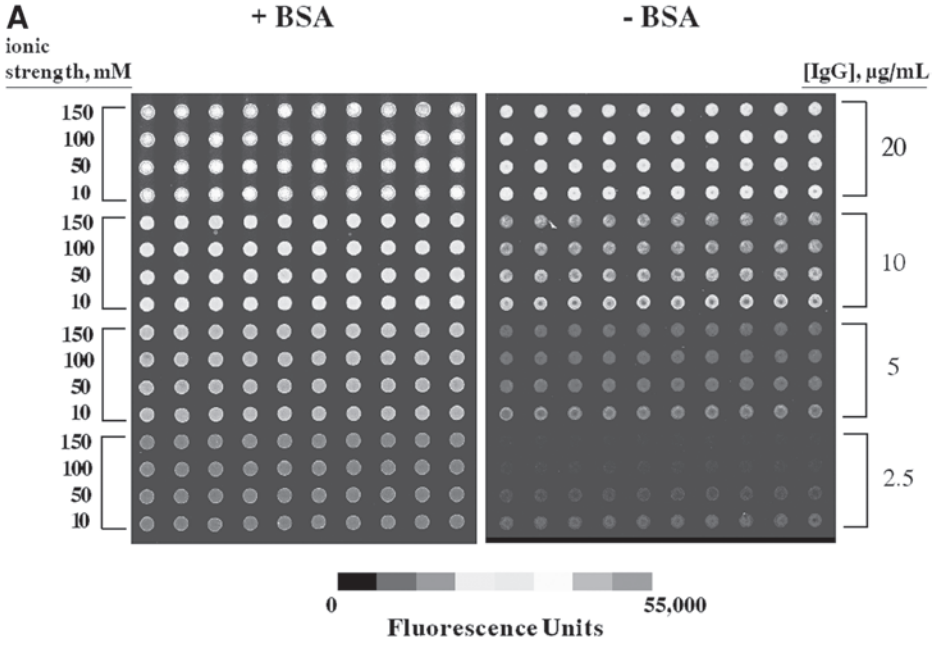
3.3.1. General Guidelines for Printing Capture Antibodies With Glass Capillaries

1. Prepare biotinylated antibodies in the respective printing buffer under investigation and transfer to the wells of a 96-well medium-binding microtiter plate (Costar, Corning, NY).
2. Program the instrument to dispense antibody solutions at 1 nL/spot. When printed in this manner, the resulting capture antibody spots are approx 230 μ*M* in diameter. To control for intercapillary variability, perform all dispensing operations using the same glass capillary tip.
3. Between each sample dispense, perform an aspirating rinse step in which the tip is rinsed liberally with 0.1% Tween-20 (Sigma) in dH_2O followed by a final rinse with dH_2O to remove any residual protein (*see* **Note 2**).

3.3.2. Deposition of Biotinylated Cy5-Labeled Mouse Immunoglobulin G (IgG)

1. Dispense the IgG (10 replicate spots) at concentrations of 2.5, 5, 10, and 20 μg/mL in PBS buffer (pH 7.4) whose ionic strength has been adjusted to final values ranging from 10 m*M* to 150 m*M* by dilution with dH_2O (pH 7.4). Check all solutions prior to use to ensure the maintenance of pH at 7.4. Prepare the samples in duplicate such that one set contains BSA at a final concentration of 0.1% (w/v), whereas the other set contains no BSA.
2. Remove the nonspecifically bound protein from the slide with two washes with 150 m*M* PBS containing 0.05% Tween-20 (PBST).
3. Wash the slide twice with deionized H_2O, and dry the slide under a stream of nitrogen.
4. Image and quantify the fluorescence intensity of the spots as described in **Subheading 3.4.** (*see* **Fig. 1**).

Fig. 1. (*Right*) The effect of the printing buffer on the amount of IgG dispensed from piezoelectric glass capillaries. (**A**) Comparison of arrays printed using different printing buffers. For each IgG concentration (*indicated at the right*), 10 replicate spots of biotinylated Cy5-labeled mouse IgG were dispensed in PBS buffer at four different ionic strengths (*indicated at the left*). The presence (+BSA) or absence (–BSA) of 0.1% (w/v) BSA in the printing buffer is noted above each respective array. All spots were deposited to the surface of the same Neutravidin-coated glass microscope slide. The scale denotes the range of fluorescence intensities registered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ionic strength, mM
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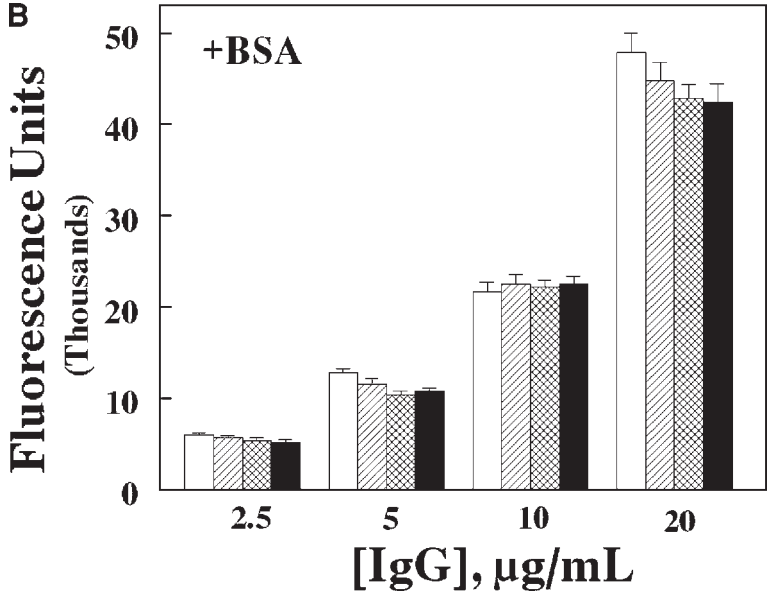
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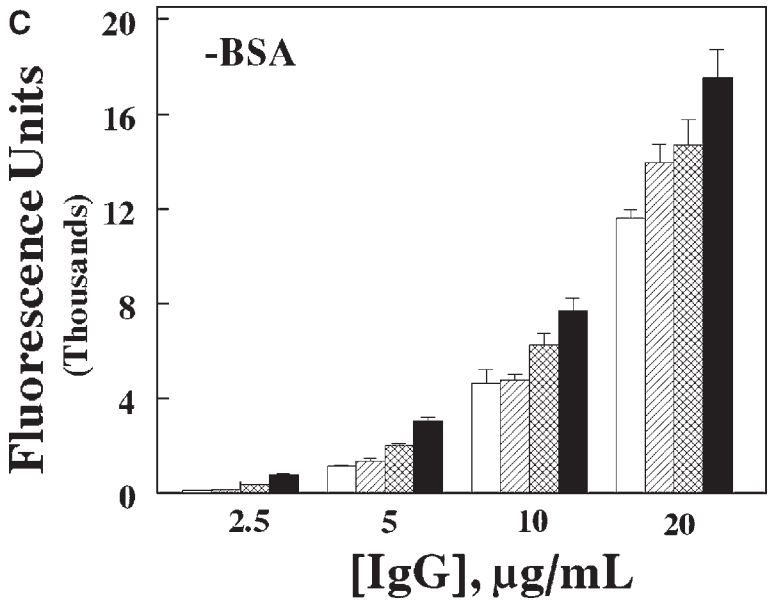
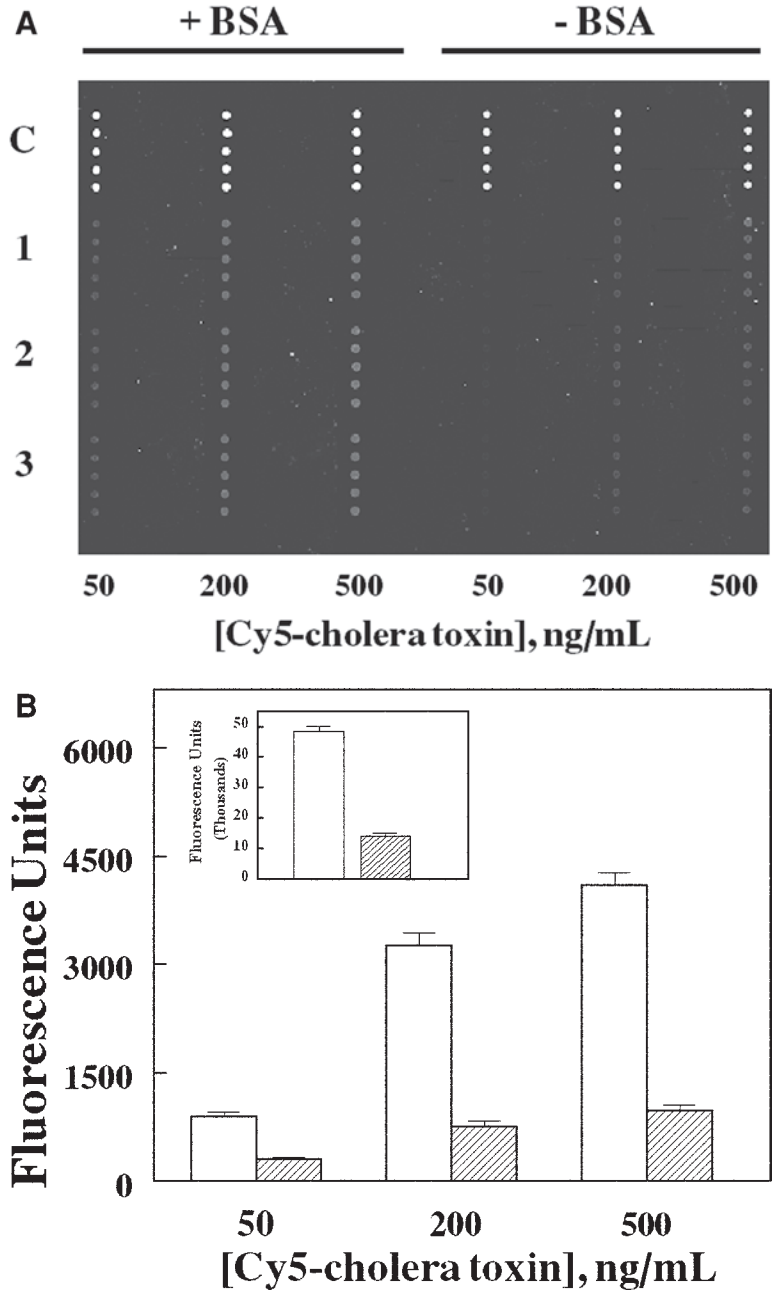


Fig. 1. (*continued from pp. 138–139*) by the microarray scanner. (**B**) and (**C**) The fluorescence intensities of the arrays in (**A**) are depicted graphically. The arrays were printed in the presence (**B**) or absence (**C**) of BSA. For each IgG concentration, the bars correspond to the following ionic strengths of PBS: 150 m*M* (*open bar*), 100 m*M* (*single-hatched bar*), 50 m*M* (*cross-hatched bar*), and 10 m*M* (*solid bar*).

3.3.3. Deposition of Biotinylated Goat Anticholera Toxin Antibody and Direct Detection of Cy5-Labeled Cholera Toxin

1. Dispense the biotinylated goat anticholera toxin capture antibody (15 replicate spots) in a linear orientation for later alignment within one of the six sample channels of a PDMS, (NuSil Technology, Carpenteria, CA) flow module. As an internal printing control, print five replicate spots of biotinylated Cy5-labeled mouse IgG within each sample lane. Dispense all antibodies at a concentration of 5 µg/mL in either printing buffer 1 or printing buffer 2 containing 0.1% BSA.

Fig. 2. (*Right*) The deposition of anticholera toxin capture antibody in a low-salt buffer containing BSA results in the capture of a larger proportion of labeled analyte. (**A**) A direct detection assay was performed for Cy5-labeled cholera toxin. The antibody spots printed in the three sample channels to the left were dispensed in printing buffer 1 (denoted by "+BSA"). The antibody spots printed in the three sample channels to the right were dispensed in printing buffer 2 (denoted by "–BSA"). All antibody spots were printed at 5 µg/mL. Rows marked **1**, **2**, **3** denote the position of replicate spots of biotinylated goat anticholera toxin antibody, whereas the rows marked **C** indicate the position of replicate spots of biotinylated Cy5-labeled mouse IgG used as an internal printing control. Each sample channel column was exposed to 50, 200, or 500 ng/mL of Cy5-labeled cholera toxin as indicated. (**B**) The fluorescence intensities of the



arrays in (**A**) are represented graphically. The bars correspond to antibodies printed in either printing buffer 1 (*open bar*) or printing buffer 2 (*single-hatched bar*). The inset shows the fluorescent intensities of the control biotinylated Cy5-labeled IgG printed in the two buffers.

2. Immediately perform a direct detection assay for Cy5-labeled cholera toxin by using a modification of the flow-based microarray immunoassay system described previously ***(14)***. The details of this assay system are not described here because of space limitations.
3. Flow the Cy5-labeled cholera toxin, at concentrations of 50, 200, and 500 ng/mL in PBST containing 0.1% (w/v) BSA (PBSTB), through the individual channels of the flow module at a rate of 0.3 mL/min.
4. Remove the nonspecifically bound cholera toxin by rinsing the channels with 1 mL of PBSTB at a rate of 1 mL/min.
5. Wash and dry the slide as described in **Subheading 3.3.2.**, and image the slide as described in **Subheading 3.4.** (*see* **Fig. 2**).

3.4. Imaging and Analysis of Microarrays

Microarray images (at a resolution of 10 μ*M* per pixel) were acquired using a ScanArray Lite™ microarray scanner (PerkinElmer Life Sciences) equipped with a 635 nm excitation laser. The fluorescence intensity of the spots was quantified using the QuantArray™ analysis package (version 2.1, PerkinElmer Life Sciences). In all analyses, the fixed-circle algorithm was used to locate and quantify the fluorescence intensity of each spot and its surrounding background. Each spot was enclosed by a circular spot mask (230 μm diameter) and a circular background mask (inner diameter 240 μm; outer diameter 300 μm). The spot intensity was calculated as the mean intensity of the pixels located within the spot mask minus the mean intensity of the pixels in the background mask.

4. Notes

1. It has been the author's experience that the optimal Cy5:protein ratio is approx 3:1. Higher ratios result in the self-quenching of the Cy5 dye, which yields diminished assay sensitivities ***(15)***.
2. The aspirating rinse step with 0.1% Tween-20 in H_2O between each sample is absolutely essential to eliminate sample carryover, to maintain cleanliness, and to extend the working life of the glass capillary.

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13

The Use of Precision Glass Syringes and a Noncontact Microsolenoid Dispenser for the Production of High-Throughput Low-Density Arrays

Arezou Azarani

Summary

The advantages of using nondisposable precision glass syringes in automated high-throughput microdispensers and a single-channel noncontact microsolenoid dispenser in creating highly uniform and reproducible protein, nucleic acid, and organic compound array filters, slides, and plates are described. Using a Hydra®Plus-One system, protein solutions of up to 100 mg/mL (in 100 nL) can be spotted onto slides and plates at a speed of 0.6 s per spot. Using the Hydra microdispenser and Tango™ liquid handling system, as little as 100 nL of polymerase chain reaction (PCR)-amplified human cancer-related genes, housekeeping genes, and protein solutions can be spotted onto nylon membranes, coated slides, or plates. Using these microdispensers equipped with syringes, up to 6144 uniform and reproducible spots per membrane and up to 1000 spots per slide, with a precision variation of less than 10%, were printed at a speed of as fast as 2.5 min per membrane.

Key Words:

Arrays; high-throughput liquid microdispensers; precision glass syringes; noncontact microsolenoid dispensers.

1. Introduction

Arrays are powerful molecular biology tools used for the simultaneous analysis of thousands of proteins, nucleic acids, and compounds ***(1–4)***. They have widespread use in functional genomics research, in drug discovery, and in determination of drug efficacy, as well as in clinical prognostics, diagnostics, pathology, and toxicology. However, array technology is novel, as are the methods by which arrays are created.

Numerous printing technologies are available for the creation of arrays ***(5,6)***. The most popular are contact printing with pins and noncontact printing with ink jet. These two technologies have enabled the successful creation of arrays. However, some complexities in implementing printing with these technologies have been reported ***(5)***.

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Some of the encountered difficulties involve variation in spot size, splashing, evaporation of the samples during the process of delivery, clogging of the pins, and difficulties in effectively cleaning the spotting devices. This chapter's aim is to show two new printing technologies by which many of these difficulties could be circumvented. One involves contact printing by using nondisposable precision glass syringes (used in high-throughput liquid handling dispensers such as the Hydra and Tango systems) ***(7)***, whereas the other involves a new ink jet noncontact printing technology based on a microsolenoid dispenser called the NanoFill™ ***(8)***.

The precision glass syringes and the NanoFill dispenser both offer reliable and precise methods of creating highly reproducible and uniform low-density arrays. Their dispensing capabilities easily reach volumes as low as 100 nL (or even as low as 50 nL for an experienced user) with very high precision (on the order of 90%). As a consequence, the size of the spots created by these two methods is very consistent and reproducible from spot to spot and membrane to membrane.

1.1. The Hydra Automated Dispensing System

The Hydra-PP (plate positioner) microdispenser (*see* **Fig. 1**) can be used for all aspiration and/or dispensing of aqueous and organic solutions in the nano- and microliter range. It is equipped with 96 or 384 nondisposable precision glass syringes arrayed in standard Society for Biomolecular Screening microplate spacing, a computer-controlled plate-positioning stage, and an automated syringe washing system ***(9)***. The stage is composed of two nests; one nest can be dedicated to the source plate and the other to a destination plate. For higher throughput applications, this system can be equipped with a robotic plate handler.

The needles of the syringes come in stainless steel (coated with Teflon®) or a flexible material, made of titanium alloy, called DuraFlex™. Although the stainless steel needles provide a higher dispensing precision of down to 100 nL of aqueous solutions, they can be damaged or crashed if the destination surface is presented misaligned or is improperly positioned. The DuraFlex needles, on the other hand, return to their original shape even after a crash. However, their dispensing precision at low nanoliter volumes is not as high as those achieved by stainless steel needles. The dispensing capabilities of the syringes are presented in **Table 1**.

The use of fixed needles instead of disposable tips in this system facilitates the creation of a convenient, cost-efficient ***(10)***, and environmentally friendly (less wasteful) method of liquid dispensing. However, with this fixed needle system, the need for an efficient syringe-cleaning procedure for the inhibition of carryover contamination is evident. A proper syringe-washing procedure also ensures that the syringes are prevented from clogging. Many wash methods have been developed for different applications to address these issues ***(7,8,11,12)***. Wash procedures for arraying purposes are described in **Section 3** of this chapter and can also be found at Apogent Discoveries Web site ***(10,11)***.

The sizes of the spots printed by syringes (inside diameter of needles used in this study was approx 230 μ*M*) vary from 400 to 600 μ*M*. Therefore, printing can be carried out in a typical laboratory instead of a "clean room" because with these relatively

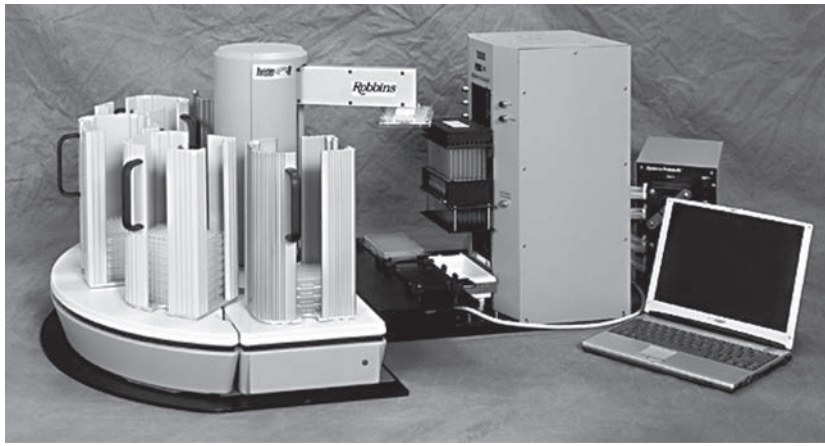


Fig. 1. The Hydra-PP system equipped with a robotic plate handler (Twister, called a Hydra-HTS workstation).

Table 1
Pipetting Performance for 96 and 384 Syringes in the Hydra or Tango Systems

	Minimum dispense volume with CV <10%	
	Wet plates	Dry plates
Teflon-coated, stainless steel needles		
Aqueous	100 nL	250 nL
DMSO	150 nL	250 nL
DuraFlex needles		
Aqueous	250 nL	250 nL
DMSO	250 nL	3 μL

Abbreviations: CV, coefficient of variance; DMSO, dimethyl sulfoxide.

The CV for specific dispensing volumes was calculated. These are typical results obtained across all plate formats (96, 384, and 1536) and may be surpassed under favorable conditions. A wet dispense is a transfer to a surface already containing liquid, whereas a dry dispense is a transfer to a dry surface.

large spot sizes the quantity and the quality of the printed spots are not affected by dust. As a consequence, a significant reduction in operation expenses is achieved. Another advantage of using syringes for arraying purposes is that they can be siliconized to prevent the buildup of air bubbles. With the existing ink jet and pin technologies, bubbles have been reported to create inconsistencies in the volumes printed, causing spot-size variations.

Precision glass syringes are simple to operate, robust, inexpensive, and easily replaceable. Because samples do not evaporate or dry once inside the syringes, there is

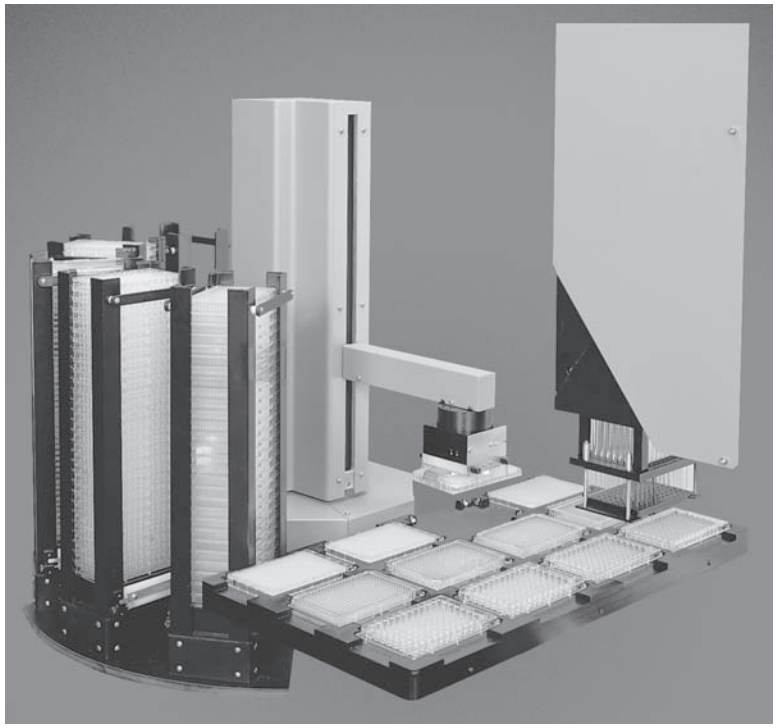


Fig. 2. The Tango system equipped with a plate stacker.

no need for humidification. Consequently, the printed samples dry quickly and sufficiently without spreading or dispersing onto the printing surfaces. In general, many difficulties typically encountered in implementing printing with existing ink jet or pin technologies *(7)*—such as difficulties in thoroughly cleaning the spotting devices, drying samples during the delivery process, shearing samples, and the so-called doughnut-shape effects—are not observed when precision glass syringes are used for spotting.

1.2. The Tango Liquid Handling System

The Tango system (*see* **Fig. 2**) is a higher throughput version of the Hydra system. It has a bigger stage composed of 12 nests and works at a faster speed. This system incorporates 1, 96, or 384 nondisposable precision glass syringes. The syringes are exactly the same as the ones used on the Hydra system. This system can be equipped with a plate stacker. Plates can be removed or placed onto the stage by the plate stacker's rotational gripper. The speeds of creating up to 6144 spots per membrane *(7)* are recorded in **Table 2** for both the Hydra and the Tango systems.

Table 2
Arraying Speed for Nylon Filters (8 × 12 cm) Using the Hydra and Tango Systems Equipped With 96 or 384 Precision Glass Syringes

Number of spots	Arraying speed using Hydra-PP 96 (min) ± SD	Arraying speed using Hydra-PP 384 (min) ± SD	Arraying speed using Tango 384 (min) ± SD
96	3.0 ± 0.5	–	–
384	11.0 ± 0.8	3.0 ± 0.3	0.5 ± 0.1
1536	40.0 ± 2.2	11.0 ± 0.5	3.0 ± 0.4
6144	150.0 ± 4.2	40.0 ± 1.5	13.0 ± 1.0

Abbreviations: SD, standard deviation.

The times indicated include priming, sample spotting, and washing periods for the preparation of a single membrane. Each spot corresponds to a different sample. SDs are shown for each speed. (Reproduced with permission from **ref. *7***.)

1.3. The Hydra-Plus-One Microdispenser

The Hydra-Plus-One microdispenser (*see* **Fig. 3**) is composed of a Hydra-PP system and a single-channel noncontact microsolenoid dispenser (called the NanoFill). The NanoFill system has a hybrid valve architecture, in which the microsolenoid actuator is isolated from the sample flow, thus preventing clogging issues. This single-channel dispenser transfers between 100 nL and 50 µL of solution with a dispense-precision variation of less than 10% ***(8)***. The Hydra component of the system can successfully dry dispense viscous samples (as little as 200 nL) containing up to 30% PEG 8000 and 50% glycerol, with coefficients of variance (CVs) of less than 10%.

The Hydra-Plus-One system can dispense aqueous and organic solutions onto any surface. This system has gained widespread use for the setup of protein crystallography plates ***(8)*** by the sitting drop, hanging drop, and microbatch methods ***(13,14)***, as well as for the setup of low-volume PCR or sequencing reactions. The reason this system is used in the above applications, in addition to its very low volume dispensing capabilities, is that there is no waste of precious and expensive samples (such as proteins) resulting from virtually full material recovery from the noncontact dispenser. Furthermore, because sample can be aspirated directly from the source tube, the single-channel dispenser eliminates the dead volume associated with aspirating from reservoirs or troughs and, thereby, reduces sample waste. Therefore, this system can be used for the precise low-volume dispensing of valuable reagents.

One of the advantages of noncontact dispensing is the ability it provides to dispense samples without any wash requirement between dispenses of the same sample. When switching from one sample to another, simple wash protocols resident in the PC software facilitate NanoFill fluid-path cleaning.

Fig. 3. The Hydra-Plus-One System. The NanoFill has a dispensing nozzle (internal diameter of 125 μ*M*) connected to a 25-in-long tube. The sample is aspirated from a 0.6 mL microtube into the nozzle and is separated from the system fluid (H_2O) by an air gap of 0.75–1 μL. Therefore, as is the case with the syringes, the sample does not dry or evaporate once inside the noncontact dispenser.

Table 3
The NanoFill Dispenser Throughput

Pipetting function	Time to produce the following number of spots 96	384	1536
Aspirate and dispense 100–500 nL of sample	58 s	2 min 44 s	10 min 30 s

Another advantage of this system is its dispensing speed. The speed of the noncontact dispenser is 0.6 s per spot of the same solution (**Table 3**). As a result, sample evaporation is prevented when necessary. The Hydra part of the system can dispense 96 or 384 different samples (with different viscosities) simultaneously (the Hydra speeds are shown in **Table 2**).

2. Materials

1. Fluorescein (Molecular Probes, Eugene, OR, cat. no. F-1300) was dissolved to a concentration of 10 μg/mL solution using a 0.1 *M* Tris-HCl (pH 8.0) buffer.
2. A Tango liquid handling system and a Hydra-PP microdispenser (Apogent Discoveries, Sunnyvale, CA), equipped with 1, 96, or 384 100-μL stainless steel syringes (with Teflon coating) or syringes with DuraFlex (titanium alloy) needles, and a Hydra-Plus-One system (Apogent Discoveries, Sunnyvale, CA) were used for arraying purposes.
3. Fluorescence detection was performed using a NucleoVISION imaging workstation from Nucleotech™ Corporation, San Mateo, CA.

4. The PCR-amplified DNA samples, which possess an M13 sequence in each fragment, were provided by Genetic-Lab, Sapporo, Japan.
5. PCR core kit, cat. no. 1 578 553, was purchased from Roche, Mannheim, Germany.
6. The amplified samples were purified using a Qiagen QIAquick® PCR-purification kit, cat. no. 28104, Qiagen, Valencia, CA.
7. A 10 m*M* Tris-HCl (pH 7.5) buffer was used as blank in contamination studies.
8. The Biodyne™ Plus membrane, 0.45 μ*M* (8 × 12 cm, cat. no. 60400) was purchased from Pall Corporation, East Hills, NY.
9. CAST™ microarray slides (cat. no. 10484182 and 10484181) were purchased from Schleicher & Schuell, Keene, NH.
10. ArraySlides (silanated) and MaxiSorp 96-well plates (cat. no. 442404) were provided by Nunc, Roskilde, Denmark.
11. Signals were detected using a chemiluminescence kit (Imaging High™, Toyobo, Japan) and a Fluor-S® MAX multiimager system (Nippon Bio-Rad®, Tokyo, Japan).

3. Methods

3.1. Preparation of the Hydra-PP and the Tango Systems

1. Prior to the setup of experiments, the Hydra and the Tango systems' needles and syringes should be washed three times with dH_2O, with each wash composed of three wash cycles ***(10,11)***.
2. If organic solutions are to be dispensed by these systems, the syringes can be washed with methanol, ethanol, or acetone for three full-syringe-volume wash cycles and then with H_2O for a further three wash cycles.
3. After the last use of the dispenser for the day, the syringes are washed three times with Coulter Clenz and then rinsed with H_2O for an additional six wash cycles. Coulter Clenz (Beckman Coulter) is a detergent containing proteases. It is essential to thoroughly rinse this solution out of the syringes if proteins are being dispensed.
4. Finally, the head is washed with 100% ethanol for two cycles and the syringes are emptied and dried overnight.
5. When a Tango or Hydra system is used for dispensing higher concentrations of nucleic acids (≥100 ng/μL), the washing procedures need to be modified to include a one-time rinse with 2% bleach (in a reservoir) followed by multiple rinses in H_2O (in the system's wash reservoir) before the system is used to perform blotting.

3.2. Preparation of the Noncontact 1-Channel Microsolenoid Dispenser

1. Before aspirating samples, the microsolenoid dispenser is automatically washed with 1500 μL of H_2O through the execution of wash commands.
2. An air gap (0.75–1 μL) is then aspirated into the dispenser, followed by the aspiration of the sample.
3. The above wash step is repeated between different samples.
4. After the last use of the system for the day, the microsolenoid dispenser needs to be washed three times with Coulter Clenz and then rinsed with H_2O for an additional six wash cycles. The outside of the microsolenoid dispenser should be rinsed and wiped clean.

3.3. Measuring the Dispensing Precision of the Hydra-PP and Tango Syringes

1. Prior to the use of the Hydra and Tango systems for spotting, the uniformity and consistency of the volumes dispensed across the array of syringes is determined by the CV for specific dispensing volumes ***(7)***.

2. Different volumes of a 10-μg/mL fluorescein solution are dispensed into each well of a 96-well plate containing 0.1*M* Tris-HCL buffer. The final volume in each well after dispensing is 100 μL.
3. Each plate (after an incubation period of an hour) is then read in a TECAN SpectraFluor™ fluorescence plate reader and the CVs are determined across each plate for each of the dispensed volumes. Typical results are shown in **Table 4**. A high uniformity for dispensing volumes of 100 nL used in the arraying assays is evident, with CVs of less than 10%.

3.4. Measuring the Dispensing Precision of the NanoFill Dispenser

The uniformity and consistency of the volumes dispensed by the noncontact microsolenoid dispenser is also determined by the CV for dispensing volumes of 0.1–0.5 μL. The CVs obtained are less than 10% (**Table 5**).

3.5. Setting up the Hydra-PP and Tango Systems

1. The Hydra stage is composed of two nests. For arraying purposes, one nest is dedicated to the source plate and the other nest to a nylon membrane, glass slides, or a plate.
2. When nylon membranes are used, they are placed on a vacuum manifold. The vacuum manifold holds the membrane in position and reduces diffusion, therefore concentrating each sample and reducing its diameter.
3. The Tango stage is composed of 12 nests. During arraying analyses, 1 nest is dedicated to the wash module, 1 nest to a 96-well or 384-well PCR plate, and 10 nests to nylon membranes (placed on a vacuum manifold), slides, or plates. The source plates could be stacked and be removed or placed on the stage by robotic arms.

3.5.1. Arraying Speeds

Using a Tango or a Hydra system equipped with 1, 96, or 384 precision glass syringes, 50–300 nL of a 10 μg/mL fluorescein solution, or PCR-amplified human housekeeping and cancer-related genes and bovine serum albumin (BSA)-flurorescein solution can be spotted onto nylon membranes, glass slides, or plates. The speeds of the arraying process for creating compact-size filters and slides containing up to 6144 spots with the precision glass syringes *(7)* are reported in **Table 2**. The arraying times include the time required for the processes of trial dispensing (priming), spotting, and washing.

To produce a 384-spot surface, for example, using a Hydra-PP equipped with 96 syringes and a 96-well source plate, the source plate needs to be changed four times and the syringes need to be washed four times to prevent carryover contamination. This process takes a total of 11 min. Using a Hydra-PP equipped with 384 syringes, the same procedure takes approx 3 min because only one wash step is required. To produce up to 6144 spots, it is much more efficient to use the fully automated high-throughput Tango system. With the Tango (384 syringes) system, it takes only 13 min to spot a surface with 6144 spots. As the Tango stage is composed of 12 nests, a total of ten 6144-spot surfaces can be produced in 25 min (2.5 min per 6144-spot surface). Furthermore, because samples are generally spotted in duplicates on arrays to validate reproducibility, these spotting times can be reduced by half, as the number of washes required is cut by half. Thus, a 6144-spot surface (3072 samples in duplicate) can be produced in approx 1 min on the Tango system.

Table 4
Typical Dispensing Precision Obtained With the Hydra-PP and the Tango Systems

Volume of fluorescein (μL)	Relative fluorescence units	% CV
2	40535	1.10
1.2	24353	1.28
0.8	16085	1.71
0.4	8079	2.02
0.2	3435	3.83
0.1	1516	7.38

Abbreviations: PP, plate positioner; CV, coefficient of variance.

Table 5
The Dispensing Precision of the NanoFill Dispenser

Volume of fluorescein (μL)	Relative fluorescence units	Relative fluorescence units
0.5	10439	2.39
0.4	8659	3.37
0.3	6497	5.16
0.2	4771	4.36
0.1	2309	9.01

Using the NanoFill dispenser, up to 100 mg/mL of a BSA solution (as well as nucleic acids or organic solvents) (as low as 100 nL) can be spotted onto nylon membranes, glass slides, or plates. Dispensing speed by the noncontact 1-channel microdispenser is 0.6 s per spot (**Table 3**). The NanoFill can aspirate and dispense one solution at a time. Therefore, the speeds shown in **Table 3** are for creating an array of up to 1536 spots of the same sample. For arrays containing spots of many different samples, contact printing with pins and noncontact printing with ink jet technologies offer a faster and more practical solution.

3.5.2. Spot Uniformity and Reproducibility

The uniformity in spot size and the consistency in the quantity of the samples dispensed by the precision glass syringes as well as the noncontact NanoFill dispenser are evident from the chemiluminescence and fluorescence images in **Fig. 4A–D**. Spot reproducibility from membrane to membrane is shown in **Fig. 5**, in which two identical membranes containing 560 different genes in duplicate were created.

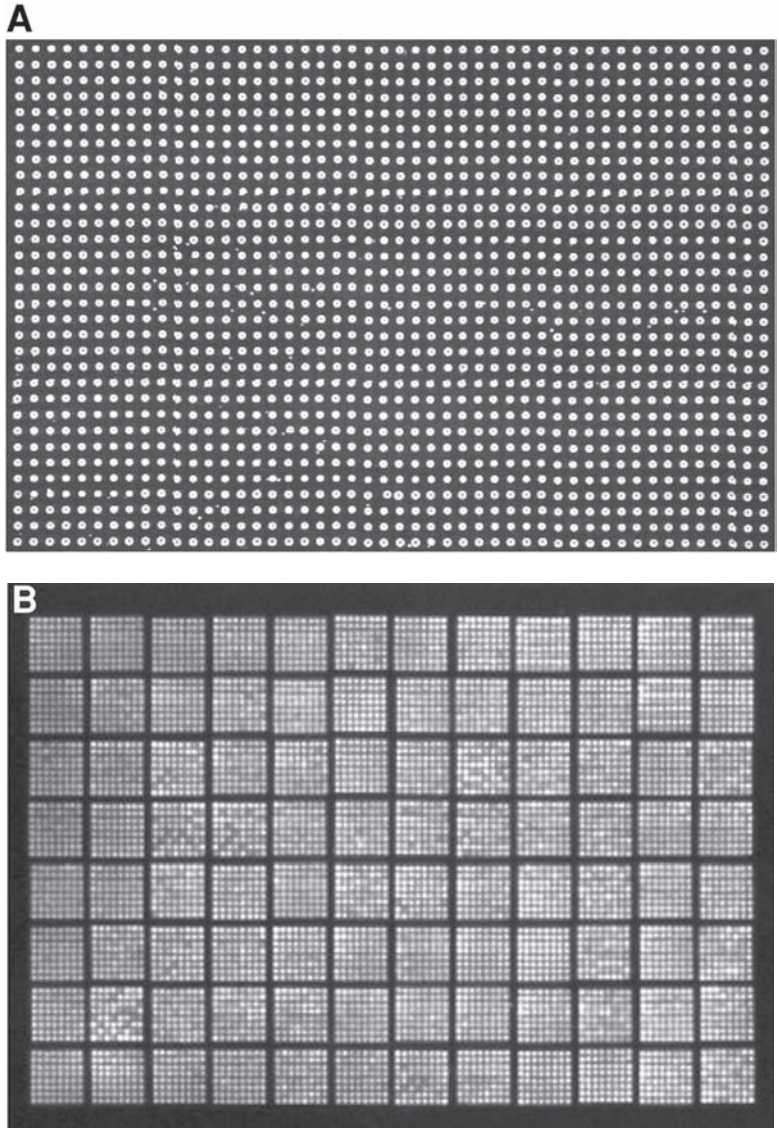


Fig. 4. The uniformity and consistency of dispensing samples with syringes and the NanoFill dispenser. **4A**. Dispensing 1536 spots (100 nL) of a 100 mg/mL BSA-fluorescein solution with the noncontact, 1-channel microsolenoid dispenser. Detection: Fluorescence. **4B**. A 6144-spot DNA membrane prepared by Hydra-PP equipped with 96 syringes (Hydra-PP 96). Gene: Housekeeping gene G3PDH (0.1 ng each spot). Spotting volume: 100 nL. Probe: M13 (the PCR-amplified G3PDH cDNA had a built-in M13 sequence). Detection: Chemiluminescence. (Reproduced with permission from **ref. 7**.) **4C**. A 1152-spot DNA membrane prepared by Hydra-PP 96. Gene: Housekeeping gene β-actin. The spots in the first row contain 5 ng of DNA

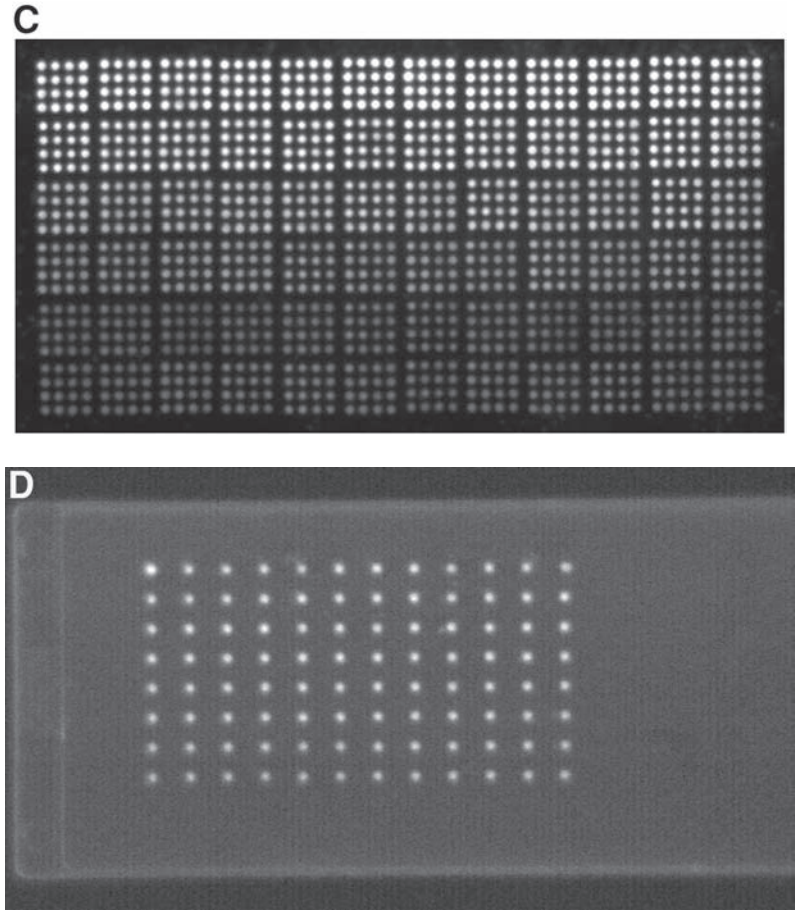


Fig. 4. (*continued*) each. The following rows are at 1/2, 1/4, 1/8, and 1/16 dilutions. The last two rows are at 1/16 dilution. Spotting volume: 200 nL. Probe: M13 (the PCR-amplified β-actin cDNA had a built-in M13 sequence). Detection: Chemiluminescence. (Reproduced with permission from **ref. *7***.) **4D**. A 96-spot fluorescein slide prepared by the Tango 1-channel (single-syringe)-head system. Sample: Fluorescein (10 μg/mL). Spotting volume: 50 nL. Detection: Fluorescence. (Reproduced with permission from **ref. *7***.)

3.5.3. Crosscontamination

When using nondisposable precision glass syringes and the NanoFill system for arraying purposes, following a proper wash procedure ensures the elimination of sample-to-sample carryover contamination. For the NanoFill system this is shown in **Fig. 6A**, in which 100 mg/mL of a BSA-fluorescein solution was used to test the effect of proteins on the NanoFill's contamination. The results indicate that the dispenser can be efficiently washed out with three full-syringe-volume (1500 μL in total) H_2O

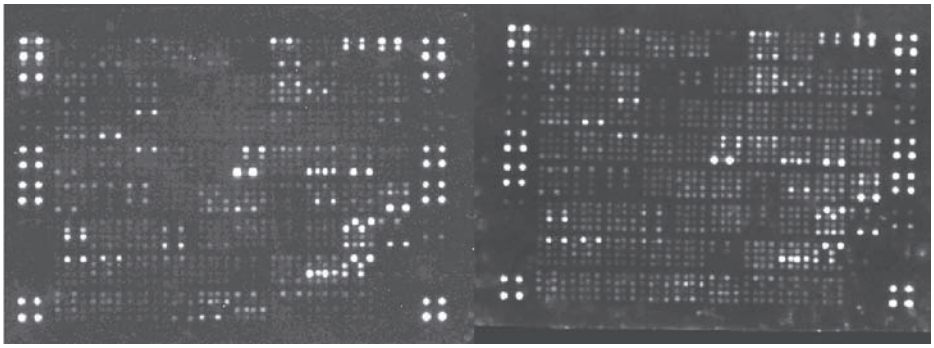


Fig. 5. Membrane-to-membrane reproducibility. Two identical 1536-spot filters prepared by Hydra-PP system. Sample: 560 different genes spotted in duplicate (5 ng). Spotting volume: 100 nL. Probe: Cancer cell line cDNA. Detection: Chemiluminescence. (Reproduced with permission from **ref. *7***.)

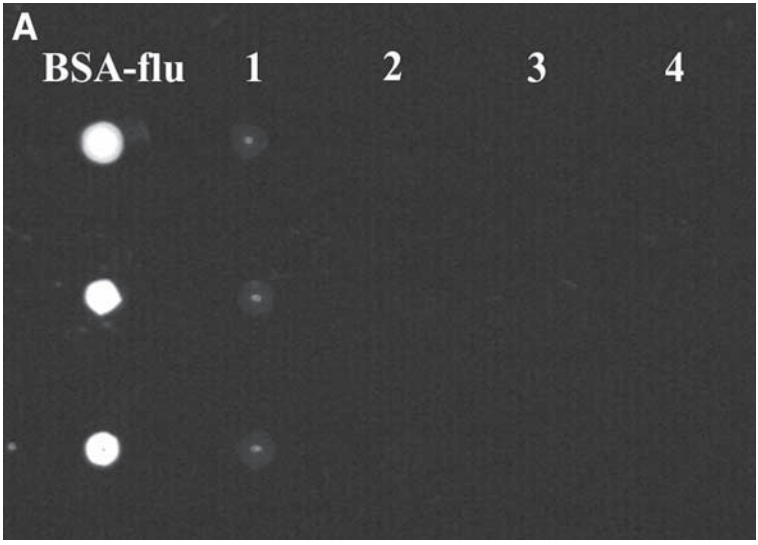
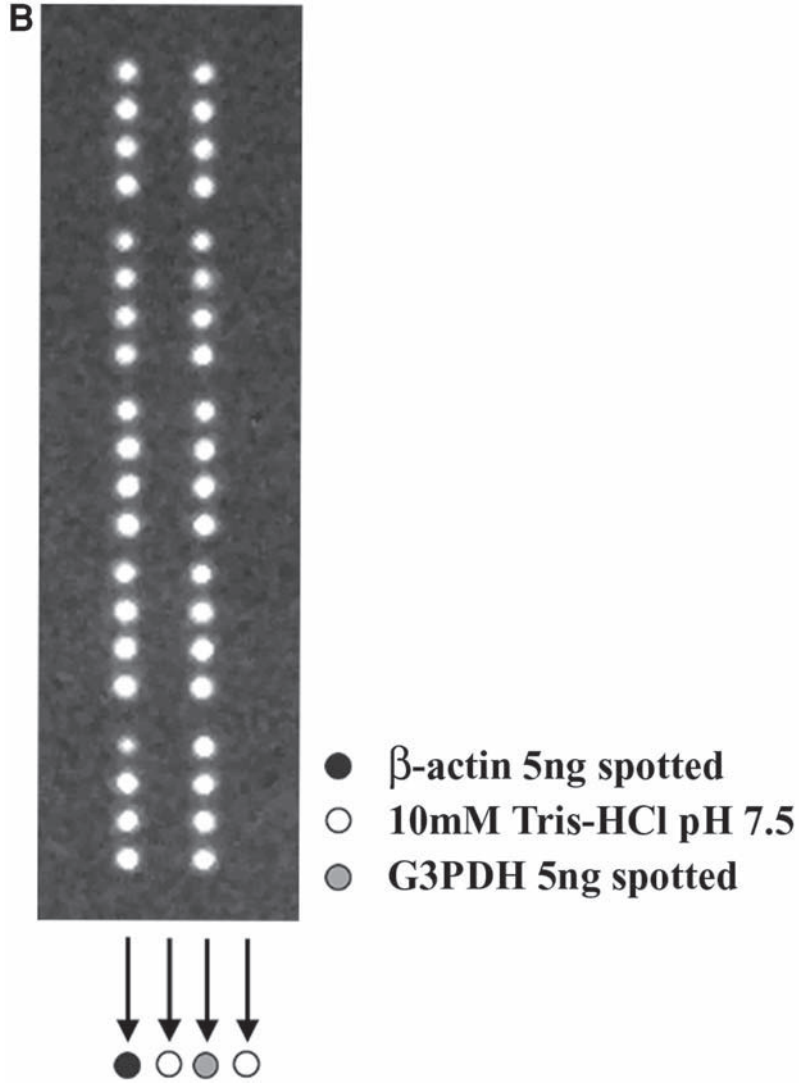


Fig. 6. Elimination of carryover contamination. **6A**. Removal of protein contamination from the NanoFill dispenser. A BSA-fluorescein solution (100 mg/mL) was dispensed, and the dispenser was washed with multiple full-syringe volumes of H_2O (500 μL each). Numbers refer to the number of washes performed. Detection is by fluorescence. (Reproduced with permission from **ref. *8***.) BSA-flu 1 2 3 4. **6B**. Carryover studies on a membrane prepared by the Hydra-PP system. Sample: β-actin (5 ng), 10 m*M* Tris-HCl (pH 7.5) buffer as blank, and G3PDH (5 ng). The pattern is β-actin fragment, 10 m*M* Tris-HCl buffer, G3PDH (5 ng), 10 m*M* Tris-HCl buffer, repeated in 20 rows. Spotting volume: 100 nL. Probe: M13 (the PCR cDNA fragments had a built-in M13 sequence). Detection: Chemiluminescence. (Reproduced with permission from **ref. *7***.)



washes (easily controlled through the Hydra-Plus-One software). In **Fig. 6B**, a membrane containing an alternate of PCR-amplified genes (β-actin and G3PDH) and 10 m*M* Tris-HCl buffer as blank (containing no DNA sample) spotted in 20 rows was produced using nondisposable syringes. As indicated, no carryover contamination is observed between spotted DNA samples and buffer spots following a proper wash protocol (the specific wash protocols are described earlier in this chapter).

4. Conclusions

Nondisposable precision glass syringes in high-throughput liquid dispensers, such as the Hydra and Tango systems and the NanoFill dispenser, offer a precise, fast, reliable, and cost-effective means of creating protein, nucleic acid, and compound arrays.

Precision glass syringes are very efficient in creating low-density arrays containing up to 6144 spots. However, for arrays containing a larger number of spots, contact printing with pins and noncontact printing with ink jet technologies offer a faster and more practical solution.

The NanoFill system can aspirate and dispense one solution at a time and is therefore used for arraying applications, such as protein crystallography, in which one protein is dispensed with the NanoFill dispenser into crystallography plates. The plates contain up to 1536 different crystallography buffers, which were dispensed with the Hydra-PP part of the Hydra-Plus-One system. For arrays containing spots of many different samples, contact printing with pins and noncontact printing with ink jet technologies should be used.

NanoFill system offers the best method of spotting resulting from its full material recovery capabilities.

Complications, such as difficulties in thoroughly cleaning the spotting devices, the drying of samples during the process of delivery, and shearing of samples are not observed when precision glass syringes and the NanoFill are used for arraying.

Following the proper syringe and NanoFill washing procedures ensures the prevention of both cross-over contamination and clogging.

Acknowledgments

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14

A Protein Microarray ELISA for Screening Biological Fluids

Susan M. Varnum, Ronald L. Woodbury, and Richard C. Zangar

Summary

Protein microarrays permit the simultaneous measurement of many proteins in a small sample volume and therefore provide an attractive approach for the quantitative measurement of proteins in biological fluids, including serum. This chapter describes a microarray enzyme-linked immunosorbent assay (ELISA). Capture antibodies are immobilized onto a glass surface; the covalently attached antibodies bind a specific antigen from a sample overlaying the array. A second, biotinylated antibody that recognizes the same antigen as the first antibody, but at a different epitope, is then used for detection. Detection is based on an enzymatic signal-enhancement method known as *tyramide signal amplification* (TSA). By coupling a microarray-ELISA format with the signal amplification of tyramide deposition, the assay sensitivity is as low as sub-pg/mL.

Key Words:

Microarray; ELISA; tyramide; antibody; biological fluids; serum.

1. Introduction

The use of protein microarrays allows for a significant reduction in the amount of sample required and an increase in the number of analytes that can be simultaneously measured. These qualities provide an increased throughput potential over existing methodologies. The fundamental principals of miniaturized ligand-binding assays have been described previously by Ekins et al. ***(1,2)***. They theoretically and empirically demonstrated that an assay that uses a small quantity of capture molecules and a small sample volume can be more sensitive than an assay that uses 100 times more material. These principals have been applied in numerous types of protein microarrays using various different support substrates including polyvinylidene difluoride, gel-coated, and glass surfaces ***(3–14)***.

ELISAs have been the mainstay of the clinical laboratory because of their high sensitivity and specificity. In this chapter, the authors describe a microarray technology that couples an ELISA format with the signal amplification of tyramide deposi-

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tion ***(15)*** (*see* **Fig. 1**). This "sandwich" ELISA uses a "capture" antibody that is immobilized to the glass support and a "detection" antibody that is biotinylated. The tyramide amplification step improves the assay sensitivity to sub-pg/mL when high-affinity antibodies are available. Because of the assay's sensitivity and ability to measure a large number of antigens simultaneously in a small volume, the microarray ELISA is ideal for measuring analytes in precious biological samples such as serum or nipple aspirate fluid.

This protocol was devised to use equipment that is currently found in many laboratories performing complementary DNA (cDNA) microarray experiments. Furthermore, all reagents are inexpensive and commercially available. As such, the microarray ELISA methodology described in this chapter can be adapted by many laboratories without large capital investments or other expenditures.

2. Materials

1. Silane-Prep slides (Sigma-Aldrich, St. Louis, MO)—aminoalkylsilanated slides. The authors have experimented with more expensive amine-reactive slides but have found no significant differences in the immobilization of the capture antibody.
2. BS^3 (Pierce, Rockford, IL)—a homobifunctional crosslinker that links amino groups.
3. Hygroscopic compound: store under N_2 and warm to room temperature before opening. Suspend at 0.3 mg/mL in phosphate-buffered saline (PBS) immediately prior to use.
4. Antibodies as required.
5. Antigen standards—diluted in Tris-NaCl Block (TNB) blocking buffer.
6. Serum samples—diluted in 3 vol of TNB blocking buffer.
7. PBS.
8. TNB blocking buffer: 20 m*M* Tris-HCl, pH 7.5, 0.15 *M* NaCl, 0.5% blocking reagent (supplied in TSA biotin kit). To prepare blocking buffer, the blocking reagent is added slowly in small increments to buffer while stirring. Heat to 60°C to completely dissolve. Aliquot and store at –20°C.
9. Tris-NaCl Tween (TNT) wash buffer: 20 m*M* Tris-HCl, pH 7.5, 0.15 *M* NaCl, 0.05% Tween-20.
10. PAP pen (Sigma-Aldrich Inc., St. Louis, MO).
11. TSA biotin system, including blocking reagent, streptavidin-horseradish peroxidase (HRP) conjugate, biotinyl-tyramide (PerkinElmer, Boston, MA).
12. Streptavidin-Cy3 conjugate (Amersham Biosciences, Piscataway, NJ).
13. Biotinylation kit: EZ-Link Sulfo-*N*-hydroxysuccimimide (NHS)-LC-Biotinylation Kit (Pierce, Rockford, IL).
14. N_2 gas with pistol grip nozzle (Ted Pella, Inc., Redding, CA).
15. Orbital shaker (e.g., Belly Dancer, Stovall Life Science, Greensboro, NC).
16. Humidity chamber—a small food storage container with tight-fitting lid and lined with a moistened paper towel at the bottom of the container.
17. Microspotter: robotic printers equipped with split pins, blunt pins, or ink jet printing have been used to construct microarrays of proteins. The split pin and blunt pin robotic systems are widely used to construct DNA microarrays and are commercially available. The authors use a PixSys 5000 robot from Cartesian Technologies with ChipMaker2 split pins from TeleChem (Sunnyvale, CA). It is important that the printer be equipped with a humidified chamber to prevent dehydration of arrays. A partial list of commercial sources of robotic printers includes: (a) BioRobics, Woburn, MA; (b) Cartesian Technologies,

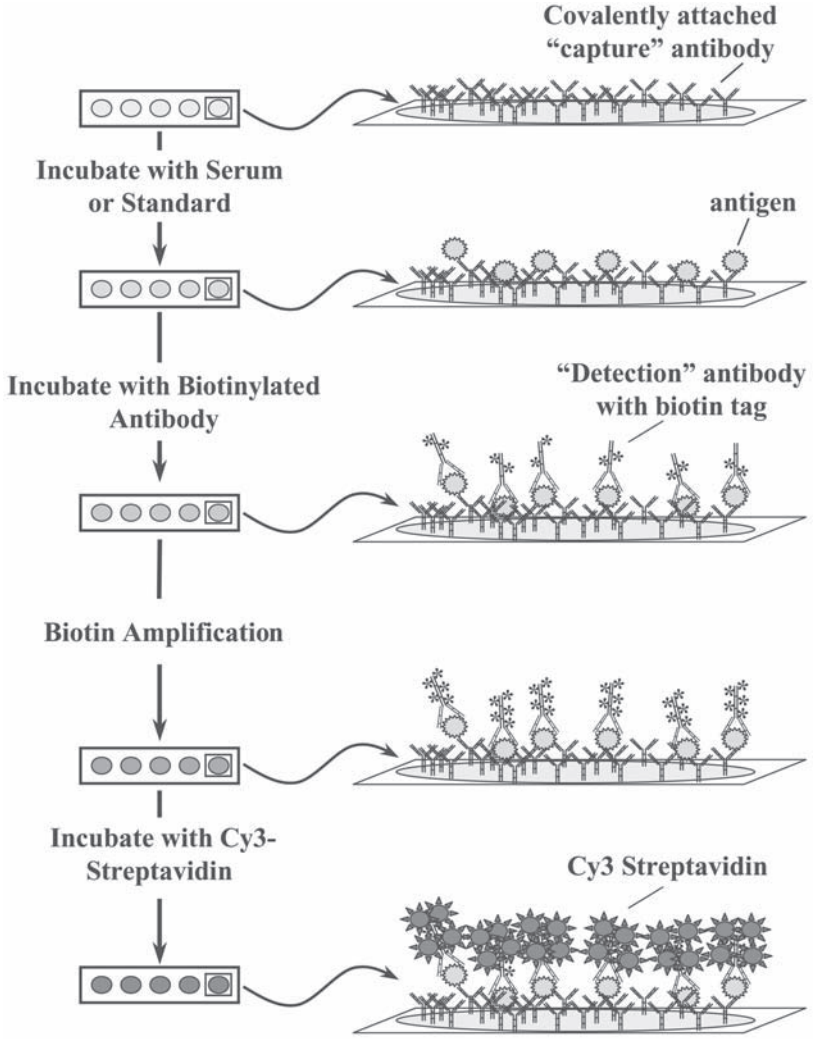

Fig. 1. Schematic representation of the microarray ELISA. (Reprinted by permission from **ref. *15***. Copyright 2002 American Chemical Society.)

Irvine, CA; (c) Packard Instrument Co., Meriden, CT; and (d) TeleChem International, Inc., Sunnyvale, CA.

18. Scanner: Typically any fluorescence scanner that can be used in the detection of cDNA microarrays can be employed to analyze protein microarrays. The authors use a GSI ScanArray3000 for fluorescence detection and ImaGene software for quantitation. A partial list of commercial sources of fluorescence scanners and analysis software includes:
    (a) Agilent Technologies, Palo Alto, CA; (b) Applied Precision, Issaquah, WA; (c) Axon Instruments, Union City, CA; and (d) PerkinElmer (Boston, MA).

3. Methods

3.1. Antibody Selection

The first criteria for choosing antibodies for both capture and detection are the antibody's affinity and specificity. Second, the composition of the antibody solution is important in choosing the capture antibody. Finally, the detection antibody must be biotinylated, and, when possible, researchers may prefer to purchase a labeled antibody rather than label it themselves. These criteria are discussed in more detail in the following paragraphs. Linscott's Directory (http://www.linscottsdirectory.com/) is an excellent source for locating commercial sources of both antibodies and antigens. For both the capture and the detection antibody, either monoclonal or polyclonal antibodies can be used. In some cases, a single polyclonal antibody can be used both as the capture and the detection antibody.

3.1.1. Affinity and Specificity

The most important criteria for choosing antibodies for microarray ELISA purposes are the affinity and specificity of the capture and detection antibodies. However, these criteria are often unknown for commercial antibodies. Therefore, other indicators are examined, such as the demonstrated use of the antibody in immunoprecipitation, immunohistochemistry, or Western blotting. The ability to use an antibody in immunoprecipitation is the best indicator, because this demonstrates that the antibody will bind to the native protein, as is required in this microarray ELISA.

If the analyte is present in the biological fluid at a high concentration ($10^{-8}M$), it will saturate a capture antibody with a high affinity ($K_d \leq 10^{-9}$), as diagrammed in **Fig. 2**. To generate a linear curve with such an analyte, either a lower affinity capture antibody should be employed (affinity of $K_d \geq 10^{-8}$), or the analyte must be diluted to a lower concentration.Often, dilution of the analyte is not feasible in multiplex experiments in which the concentrations of some analytes are near the detection limit. In many cases, antigen concentration will be so low that the highest affinity capture antibody available will be preferred. For example, the authors have measured hepatacyte growth factor (HGF) concentrations that are far below the typical K_d range of antibodies ***(15)*** (*see* **Fig. 2**). Under these circumstances, detection sensitivity is the major concern, which will improve with decreasing K_d of the capture antibody.

Ideally, a customized library of antibodies will be available for screening, either from a phage-display library or a yeast single-chain antibody library. If this is the case, then the K_d of the capture antibodies can be chosen based on expected concentrations of the antigen. In the detection antibody, the lowest K_d antibody available is generally preferred. A low K_d detection antibody will minimize the required antibody concentration and, thereby, also minimize nonspecific binding.

Affinity-purified antibodies are the best choice for both the capture and detection antibody. IgG-purified antibodies are also adequate; however, they should be purified by chromatography.Generally, antibodies that have not been purified in some way (i.e., serum) should not be employed.

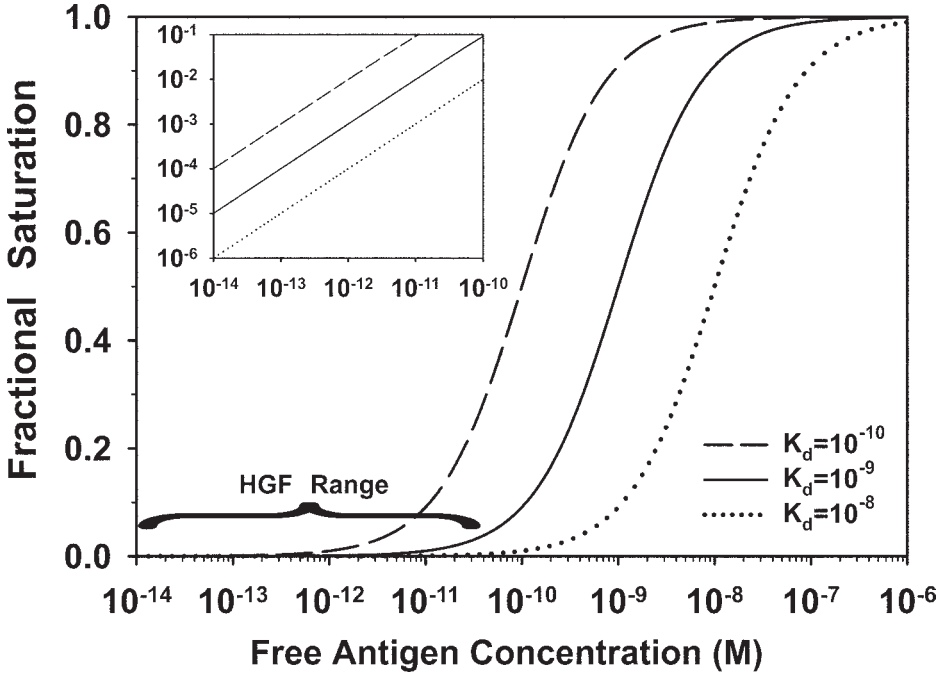

Fig. 2. Diagram representing the binding kinetics of antigen with three capture antibodies with different binding affinities. The *bracket* indicates the concentration range of HGF detected using the microarray ELISA ***(15)***. The inset graph is a detailed expansion of the bracketed region except that a log-log scale is used.

3.1.2. Antibody Solution

Two different antibodies—a capture antibody and a detection antibody—are required for the protein microarray ELISA described here. As stated above, either monoclonal or polyclonal antibodies can be employed for the capture and the detection antibody. However, it is important that the capture antibody solution is not contaminated with any carrier protein, such as bovine serum albumin (BSA).Usually, BSA is present at such a high concentration relative to the antibody that it will essentially block antibody immobilization. The authors have not found any advantage to using other stabilizers of protein structure, such as glycerol and polyethylene glycol. It is advantageous to have the capture antibodies in a solution with similar physical properties (i.e., viscosity) to ensure uniformity between the different antibody spots. Finally, the capture antibody should not be suspended in buffers containing free amines (such as Tris-HCl), which compete for reaction with the NHS-esters contained on the BS^3 carboxylates.

3.1.3. Biotinylation

For detection of low concentration of antigens, the detection antibody must be biotinylated. If a biotinylated antibody is not commercially available, then the antibody must be biotinylated in the laboratory (*see* **Subheading 3.4.**). If it is necessary to biotinylate the detection antibody, it is important that the antibody not be contaminated with BSA or other carrier proteins, and the antibody buffer should not contain free amines.

3.2. Microarray Printing

1. Apply 200 μL of BS^3 to aminosilanated slide, cover with a 22 × 5 mm cover slip, and incubate for 5 min.
2. Rinse slides briefly with a stream of 70% ethanol.
3. Dry slides under a stream of N_2 gas.
4. Immediately print capture antibodies onto the slide. The authors typically use the capture antibody at a concentration between 0.5 mg/mL to 1.0 mg/mL (*see* **Note 1**).
5. Spots can be printed in many different configurations. The authors typically print four or five replicates of each antibody reagent as shown in **Fig. 3**. Each array also contains an appropriate negative and positive control (*see* **Note 2** and depicted in the top left spot and the surrounding spots of **Fig. 3**).
6. To immobilize the capture antibody to the glass surface, incubate the slides in a humidified chamber for 30–60 min. Use immediately or *see* **Note 3**.

3.3. Antigen Hybridization and Detection

All incubations are at room temperature with gentle agitation on an orbital shaker; *see* **Note 4**.

1. Circle each array with a pap-pen creating a hydrophobic barrier that will retain small volume samples directly over the spotted antibodies. Air-dry slides for 5 min (*see* **Note 5**). Prior to scanning, slides should not be permitted to dry completely.
2. Block each array with 50 μL of TNB.
3. Incubate slides in a humidified chamber for 1 h.
4. Rinse with ddH_2O and wash once with a volume of TNT sufficient to cover the array.
5. Incubate with 50 μL antigen solution (diluted in TNB) overnight in a humidified chamber with gentle agitation.
6. Decant and blot excess without touching the surface of the array. Rinse slides individually with a gentle stream of ddH_2O.
7. Wash with TNT buffer three times for 3–5 min.
8. Incubate each array with 50 μL of detection antibody diluted in TNB blocking buffer at appropriate dilution (*see* **Subheading 3.5.** for optimization) for 2 h in a humidified chamber (for sequential tyramide amplification, *see* **Note 6**).
9. Decant and blot excess without touching the surface of the array. Wash with TNT buffer three times for 3–5 min.
10. Incubate each array with 50 μL streptavidin- HRP conjugate diluted 1:100 in TNT for 30 min.
11. Remove excess as in **step 9** and wash with TNT buffer three times for 3–5 min.
12. Incubate each array for 10 min with 50 μL biotinyltyramide diluted 1:100 in the supplied reaction diluent (or, alternatively, in 100 m*M* borate pH 8.5, 0.001% H_2O_2).
13. Remove excess as in **step 9** and wash with TNT buffer three times for 3–5 min.

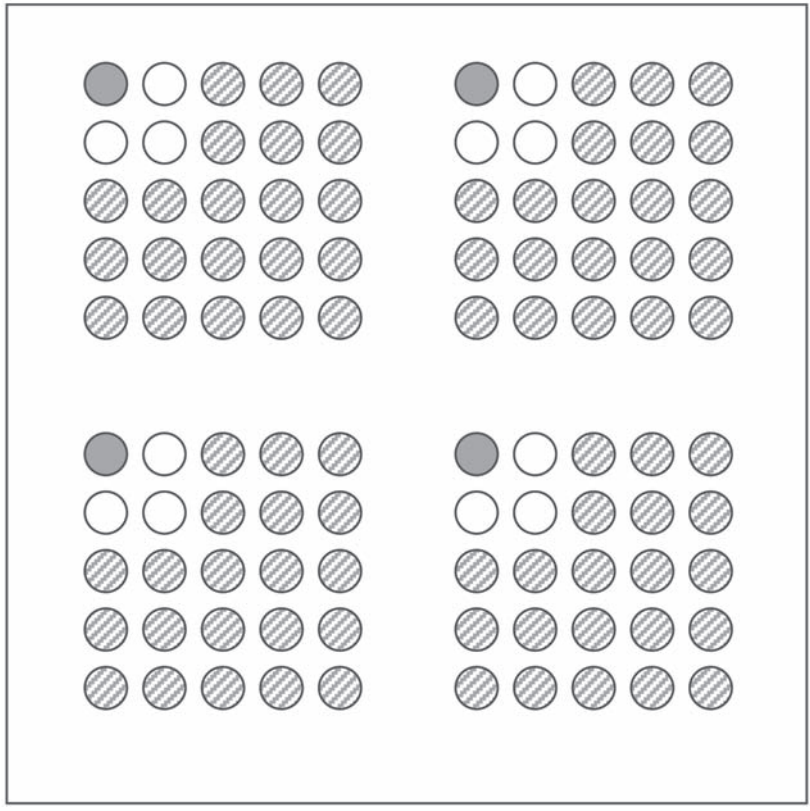

Fig. 3. Example of four replicates of a microarray for the detection of 21 different antigens. A positive control of streptavidin-Cy3 is located in the *upper-left corner* of each array. Negative PBS-only controls surround the positive control. The *stippled spots* correspond to the individual antigens assayed.

14. Incubate each array with 50 µL of streptavidin-Cy3 diluted to 1 µg/mL in TNT buffer for 30 min to 1 h in the dark.
15. Remove excess as in **step 9** and wash with TNT buffer three times for 3–5 min.
16. Rinse with ddH_2O twice, air-dry and proceed to scan slide.

3.4. Biotinylation of Antibodies

1. If necessary, dialyze or concentrate antibody into PBS.
2. Immediately before use, add 2 mg of Sulfo-NHS-LC-Biotin to 200 µL of H_2O (18 m*M*).
3. If antibody is 10 mg/mL or greater, add a volume of Sulfo-NHS-LC-Biotin to give a 12-fold molar excess of Sulfo-NHS-LC-Biotin. If antibody is less than 10 mg/mL, then add a sufficient volume of Sulfo-NHS-LC-Biotin to give a 20-fold molar excess of Sulfo-NHS-LC-Biotin to antibody. The authors have been able to successfully biotinylate an antibody solution as dilute as 1 mg/mL.

4. Incubate at room temperature for 30 min and then on ice for an additional hour. The additional incubation on ice is not required if the antibody concentration is above 2 mg/mL.
5. Quench reaction by adding 1.5 *M* Tris-HCl, pH 8.8, to give an approx 100-fold excess of Tris-HCl to biotin.
6. Equilibrate a 10-mL dextran desalting column (supplied with biotinylation kit) with 30 mL of PBS buffer. Load quenched reaction onto column. Apply PBS to column in 1-mL steps and collect 8–10 1-mL fractions.
7. Sample 8 µL from each fraction and analyze which fraction contains detection antibody either by sodium dodecyl sulfate-polyacrylamide gel electrophoresis electrophoresis followed by Western blot analysis or by determining protein content of fractions with a protein assay.
8. Store biotinylated antibody either at 4°C in 0.1% sodium azide or frozen in aliquots until ready for use.

3.5. Microarray ELISA Validation and Optimization

To validate the antibodies in the microarray, a standard curve is established for each antibody individually, determining both the upper and lower limits of detection. An example of a standard curve is shown for HGF in **Fig. 4**. Serial dilutions of antigen are used as standards in the assay. These standards are used to generate a curve representing fluorescence intensity as a function of antigen concentration. To control for background noise, a no-antigen sample, or zero, is included. In general, a linear or two-parameter power function will provide a good fit for most standard curve data ***(13)***. These functions may provide a better overall fit when the data are weighted by the inverse of the fluorescence (1/y) or the inverse of the fluorescence squared ($1/y^2$) in the regression.

If the standard curve generated is not in the appropriate range for the biomarker of interest, the quantitative range of the assay can be shifted by either diluting the detection antibody, which shifts the curve to the lower concentration range, or by using an increased concentration of the detection antibody, which shifts the curve to the higher concentration range. In addition, the range of the assay can be increased by using higher or lower laser power settings on the fluorescence scanner (*see* **Note 7**).

After optimizing the assay for each individual biomarker, the different biomarkers are combined into a multiplex microarray. Arrays are prepared containing a mixture of all antigens; the percentage coefficient of variation (% standard deviation/mean) between slides is determined for each assay as an indication of interassay reproducibility. Additional arrays are prepared where each individual antigen is omitted. If removal of an antigen alters the fluorescence of another antigen spot, then there is a problem with nonspecific interactions between the antibodies and/or antigens.

To further validate the microarray, it is useful to compare the standard curve and biological measurements for some biomarkers vs a commercially available ELISA kit. **Figure 5** shows a comparison of microarray data vs a commercial 96-well ELISA kit for the measurement of HGF.

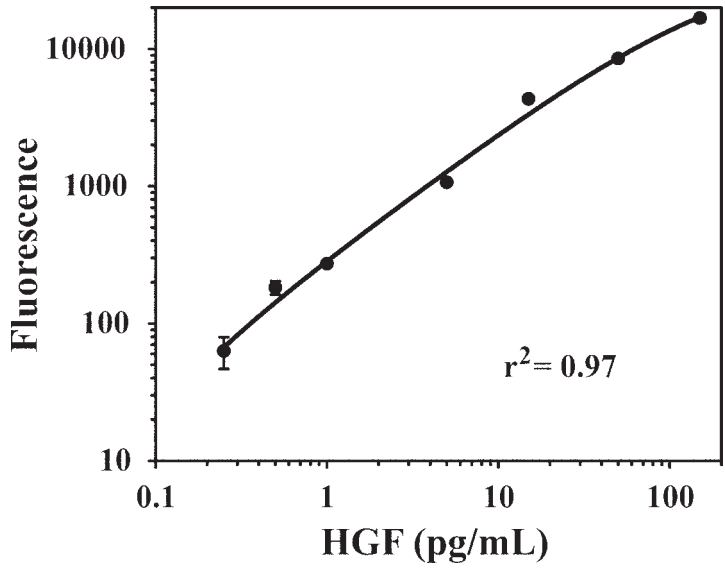

Fig. 4. A standard curve for the HGF microarray. Each data point represents the mean +/– SE of five fluorescent spots for each HGF concentration. The curve was calculated using a four-parameter logistic curve. Each data point was weighted by the inverse of the square of fluorescence intensity ($1/y^2$). (Reprinted by permission from **ref. *15***. Copyright 2002 American Chemical Society.)

4. Notes

1. Capture antibody is used in 8–30 times the maximum calculated amount that can bind in a 125–200 μ*M* diameter spot. The maximum calculated amount that can bind this area was based on the following assumptions and calculations:
   - The diameter of the antibody is 11 nm ***(16)***, thus the Ab covers an area equal to 9.5×10^{-11} mm^2.
   - A 200-μ*M* diameter spot has an area equal to 3.14×10^{-2} mm^2, an area that will accommodate 3.3×10^8 molecules of antibody if the antibodies are aligned in an upright manner.
   - 3.3×10^8 molecules of antibody per spot are equivalent to 77 pg, 18 fmol/mm^2, or 4.4×10^{-11}*M* for a 50-μL array.
2. An antibody that does not recognize any of the antigens can serve as a negative control for the microarray assay. To control for background signal, the standard curve consists of antigen dilutions including a no-antigen control. As a positive control, the authors use a Cy3-labeled protein. A positive control for the tyramide amplification can also be added by spotting a biotin-labeled protein.

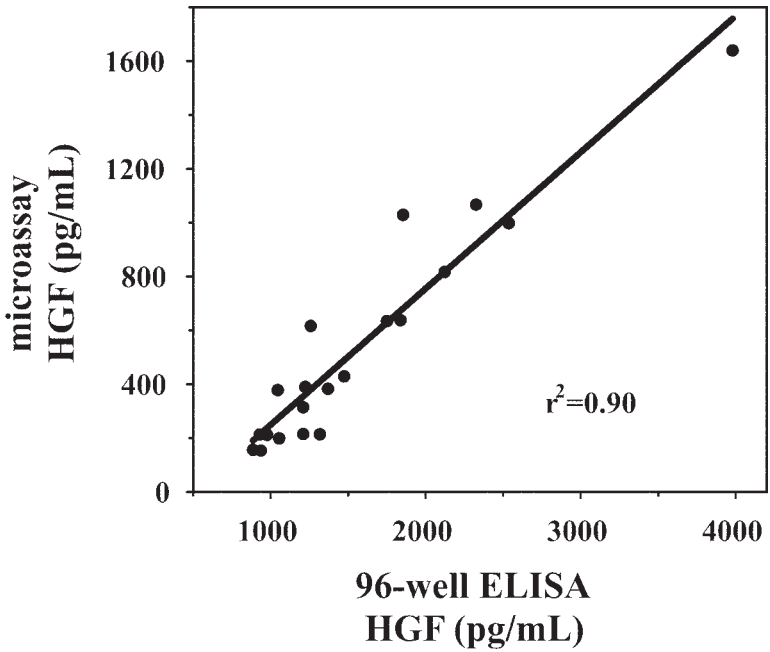

Fig. 5. Comparison of HGF values obtained with the microarray ELISA and a commercial 96-well ELISA. HGF concentration was measured by both methods in sera from 10 breast cancer patients and 10 age-matched controls. (Reprinted by permission from **ref. *15***. Copyright 2002 American Chemical Society.)

3. The storage of microarrays containing only capture antibodies immobilized to the slide surface has been described ***(17)***.
4. It is important to perform the assay in a draft-free environment. Otherwise, increased variability in spot fluorescent intensity between slides is observed, probably resulting from differential drying. As stated earlier, do not allow arrays to dry out after the blocking step.
5. The authors have found that problems with spot smearing or "comet tails" are greatly reduced when we proceed directly to the blocking step after air-drying the capture antibody spots for 5 min. Presumably, this drying step prevents unbound antibody in the spots from quickly moving with the flow of blocking solution as it is applied to the array, thereby giving the blocking reagent time to interact with reactive sites on the array surface outside the spots.
6. In multiplex experiments where a few antigen signals may be weak, the authors have found that these signals can be increased by using two sequential tyramide amplification steps for the specific antigens. To accomplish this, the microarray is incubated with the antigen mixture followed by incubation with biotinylated antibodies to the "weaker signal" antigens. The biotin is then amplified using the tyramide deposition procedure. Then, the microarray is incubated with a mixture of the remaining biotinylated antibodies.Therefore, the subsequent tyramide amplification step is a second amplifica-

tion for the "weaker signal" antigens but is a first amplification step for the rest of the antigens.

7. In some cases, the concentration range of an antigen standard may exceed the practical dynamic range of the fluorescence scanner. Legitimate data at both ends of the concentration range may be gathered simply by scanning the arrays at two laser power settings. The two sets of data representing the low-to-mid and mid-to-high concentration ranges of the antigen can be treated separately, data from each being fit to two separate curves. The authors have found that when the photomultiplier tube gain is kept constant, the two curves are parallel through the mid-concentration range where viable data is collected at both laser power settings. Thus, when the data are normalized based on these mid-range data points, the derived curve appears to be contiguous.

Acknowledgments

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15

Protein-Domain Microarrays

Alexsandra Espejo and Mark T. Bedford

Summary

Protein domains are independently folded regions of proteins that are often involved in protein–protein interactions. They are good candidates for the generation of protein microarrays because of their small size, their globular structures, and the fact that they are the protein-interacting workhorses of the cell. In addition, arayed recombinant protein domains retain much of their binding specificity. Such microarrays can be probed with other recombinant proteins or fluorescently labeled peptides to identify potential binding partners and also determine how posttranslational modifications influence specific interactions. Thus, protein domains provide us with a system-oriented array that is focused on elucidating signal transduction pathway specificity and regulation.

Key Words:

Signal transduction; proline-rich motif; phospho-tyrosine; WW domain; SH3 domain; PDZ domain; 14-3-3; SmB and FAST slides.

1. Introduction

Protein–protein interactions often occur between a noncatalytic domain in one protein and a specific motif in the ligand. Protein domains vary in size from approx 35 to greater than 150 amino acids and are often found in signal transduction molecules. Protein domains can be repeated within proteins, and individual domains form compact fold structures with the N- and C-terminal regions in close proximity—thus functioning as a plug-in module within larger proteins. Protein domains usually bind a protein motif of less than 10 amino acids in size, and this interaction can be regulated by posttranslational modification, including phosphorylation, acetylation, and methylation. Protein domains can be classified broadly as phospho-tyrosine binding domains (SH2, PTB) ***(1)***; phospho-serine/threonine binding domains (14-3-3, FHA, WW) ***(2)***; proline-rich binding domains (SH3, WW, EH); phospho-lipid binding domains (PH, PX, FYVE, ENTH) ***(3)***; and free C-terminal binding domains (PDZ) ***(4)***. The authors have generated protein-domain microarrays with representative domains from all five

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groups *(5)*. Protein-domain microarrays can be used for elucidating signal-transduction pathway specificity and regulation.

2. Materials

1. pGEX-6P1 expression vector (Amersham Biosciences, Piscataway, NJ).
2. *Escherichia coli* strains *DH5α* (Invitrogen Life Technologies, Carlsbad, CA) or *BL21* (Stratagene, La Jolla, CA).
3. Luria Bertani (LB) broth and agar (GIBCO, Carlsbad, CA).
4. Isopropyl-β-D-thiogalactopyranoside (IPTG) (Sigma, St. Louis, MO).
5. Ampicillin (Sigma, St. Louis, MO).
6. Phosphate-buffered saline (PBS) (GIBCO, Carlsbad, CA).
7. Glutathione Sepharose beads (Amersham Biosciences, Piscataway, NJ).
8. Elution buffer: 100 m*M* Tris-HCl pH 8.0, 120 m*M* NaCl and 30 m*M* reduced glutathione.
9. Acrylamide (Roche, Indianapolis, IN).
10. Coomassie blue staining solution: 0.5 g Coomassie blue and 200 mL methanol).
11. Destaining solution: 30% ETOH and 10% glacial acetic acid.
12. Blocking buffer: 1X PBS pH 7.2, 10% Tween-20, 3% dried nonfat milk and 3% bovine serum albumin (BSA).
13. Biotin-labeled peptides (W. M. Keck Biotechnology Resource Center; New Haven, CT).
14. Biotin 6% agarose beads (Sigma, St. Louis, MO).
15. FluoroLink Cy3 and Cy5 (Amersham Biosciences; Piscataway, NJ).
16. Sodium vanadate Na_3VO_4 (Sigma, St. Louis, MO).
17. Methanol (Sigma, St. Louis, MO).
18. Glacial acetic acid (Sigma, St. Louis, MO).
19. Hydrochloric acid (Sigma, St. Louis, MO).
20. 6X protein loading buffer: 0.5% bromophenol blue, 250 m*M* Tris-HCl, pH 6.8, 10% sodium dodecyl sulfate (SDS), 50% glycerol, and 0.1 *M* dithiothreitol (DTT).
21. Tris-HCl glycine electrophoresis buffer: 25 m*M* Tris-HCl base, pH 8.3, 0.2 *M* glycine, and 0.1% SDS.
22. Electrophoresis apparatus (Bio-Rad, Hercules, CA).
23. 386-well plates (Marsh, Rochester, NY).
24. Flexys® robotic workstation (Genomic Solutions, Ann Arbor, MI).
25. FAST™ slides (Schleicher & Schuell BioScience, Keene, NH).
26. Hybridization chamber (Clontech, Palo Alto, CA).
27. Slide-staining jar (Wheaton Science Products; Millville, NJ).
28. Gene Tac™ LS IV Microarray analyzer (Genomic Solutions, Ann Arbor, MI).
29. Analysis program, ArrayVision™ (Imaging Research, St. Catharines, Ontario, Canada).

3. Methods

Recombinant protein domains can be generated as fusion proteins to glutathione-*S*-transferase (GST), maltose-binding protein (MBP), or His. The authors use the GST-fusion system because of the ease with which GST-fusion proteins can be produced and the accessibility of a large number of GST/protein domains that have already been generated by fellow researches. Protein domains were cloned into pGEX vectors using standard molecular techniques. The resulting constructs were transformed into competent bacterial cells (*DH5α* or *BL21*). The following methods outline (a) the generation of recombinant proteins to be arrayed, (b) the making of the protein-domain arrays,

(c) the probing of the arrays with labeled peptides, recombinant proteins, and total cell lysates, and (d) analyzing the results.

3.1. Purification of Recombinant Proteins

1. Inoculate 20 mL of LB broth (50 μg/mL ampicillin) with a single colony of transformed *E. coli.* Grow overnight at 37°C with shaking (250 rpm).
2. The following morning, add 80 mL LB broth (50 μg/mL ampicillin) to the overnight culture. Culture 1 h at 37°C shaking (250 rpm) (*see* **Note 1**).
3. Add 0.1 *M* IPTG to a final concentration of 0.1 m*M*.
4. Culture an additional 4 h shaking at 37°C.
5. Centrifuge at 2,500*g* for 10 min at 4°C, discard the supernatant, and store the pellet at –70°C overnight.
6. Resuspend pellets in 500 μL of chilled 1X PBS.
7. Sonicate 30 s repeating the ultrasonic vibrations with pulses of 0.5 s on and off (amplitude 30%).
8. Spin down at 10,000*g* at 4°C for 10 min; keep the supernatant.
9. Wash glutathione Sepharose beads once with chilled 1X PBS, then put 20 μL of rinsed beads into a 1.5-mL microcentrifuge tube and add the supernatant from **step 8**. Rock for 3 to 5 h at 4°C.
10. Wash the beads from the previous step twice with chilled 1X PBS.
11. Prepare fresh elution buffer.
12. Add 200 μL of elution buffer and rock the beads overnight at 4°C.
13. Spin down beads at 10,000*g* for 5 min at 4°C; carefully save the supernatant and keep it at –70°C.
14. Add 2 μL of 6X protein loading buffer to 10 μL of sample, boil for 3 min, and run on a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel at 100 V for 1 h using Tris-HCl/glycine running buffer. Stain with Coomassie blue staining solution for 30 min, and de-stain for 1 h (*see* **Fig. 1**).

3.2. Generating Protein-Domain Microarray

The next step is to array the recombinant protein onto glass slides. The authors have used a complementary deoxyribonucleic acid (cDNA) microarraying workstation to generate their arrays. The GST-fusion proteins were arrayed from a 384-well plate, which contained 20 μL of each protein at a concentration of 1 μg/μL. The protein stocks were in elution buffer (no glycerol was added) (*see* **Note 2**). A Flexys® robotic workstation (Genomic Solution, Ann, Arbor, MI) was used to spot the proteins onto a glass slide using a high-density arrayer 48-pin head (FLX 12021). The glass slides are precoated with a nitrocellulose polymer (FAST™ slide; Schleicher & Schuell, Keene, NH). Nitrocellulose has a high protein-binding capacity and the protein is bound in a noncovalent but irreversible manner (*see* **Note 3**). The protein-domain array is composed of 20 grids each, in a 5 row × 5 column format, with a distance of 700 μ*M* between each spot (*see* **Fig. 2A**). Each protein was arrayed five times onto the same spot to increase the local concentration of protein. Each grid thus contains 12 fusion proteins arrayed in duplicate, with GST alone spotted in the middle of the grid as a control. Within a grid of 25 spots, the duplicates are arrayed at unique angles to allow easy identification of protein domains that bind the analyte (*see* **Fig. 2B**). A 384-well master plate containing 10 μL of each protein (1 μg/μL) was sufficient for arraying

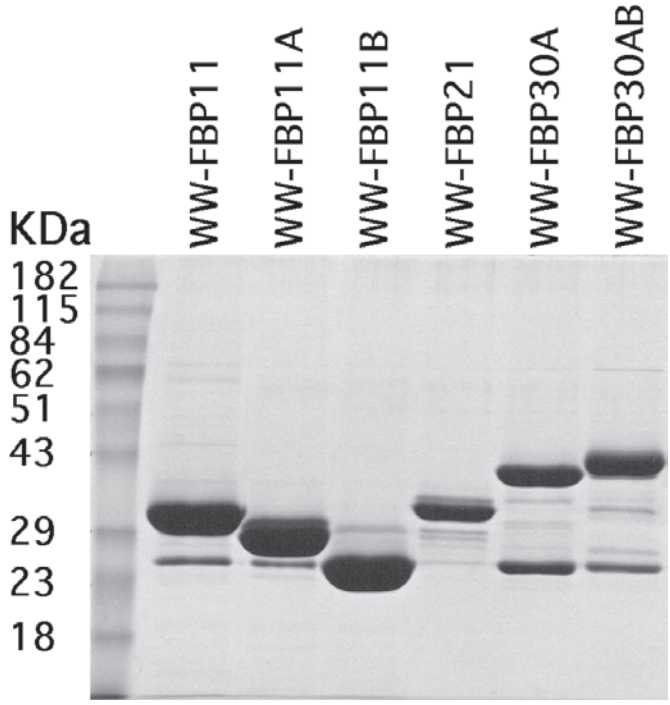

Fig. 1. Purified GST-fusion proteins harboring WW domains. Cells were induced (with IPTG) to express recombinant fusion protein. The fusion protein was affinity purified on glutathione Sepharose and eluted with free reduced glutathione. A fraction of the eluate (10 μL/200 μL) was resolved on a 10% SDS-PAGE gel and stained with Coomassie blue.

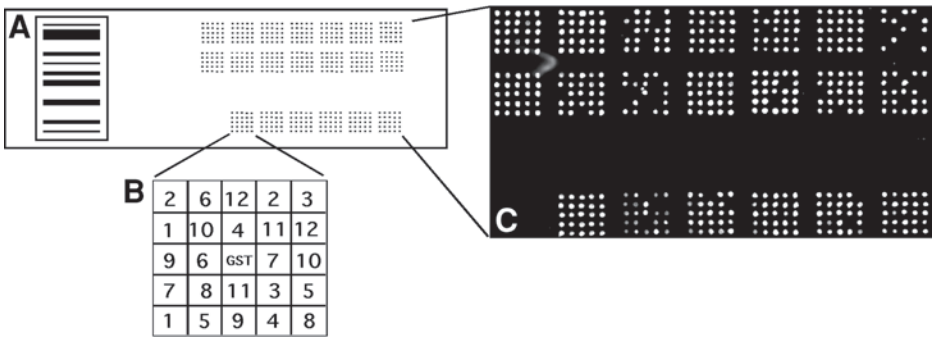

Fig. 2. The format of the arrayed protein domains. (**A**) Diagrammatic representation of an arrayed FAST™ slide. Each grid contains 12 fusion proteins arrayed in duplicate as shown in (**B**). In the center position GST alone is placed as a control for nonspecific binding to the GST moiety. (**C**) An arrayed FAST™ slide probed with an αGST antibody followed with a FITC-conjugated secondary antibody and scanned using the Gene Tac™ LS IV Microarray analyzer. This shows the format of a set of protein domains arrayed on a FAST™ slide.

35–40 slides. Therefore, each spot contains approx 200 ng of fusion protein. The arrayed proteins were then air-dried for 10 min and kept at 4°C (*see* **Note 4**). The quality of the array should be tested by probing with an anti-GST antibody (*see* **Fig. 2C**), which, in turn, is detected with a fluorescein isothiocyanate (FITC)-conjugated secondary antibody as described in **Subheading 3.3.**

3.3. The Probing of Protein-Domain Microarray

Protein-domain microarrays can be probed with peptides corresponding to predicted motifs in potential interacting proteins, with recombinant proteins, or with total cell lysates. These different screening approaches are described in **Subheadings 3.3.1.–3.3.3.**

3.3.1. Peptide Probes

Peptides are synthesized with an N-terminal biotin moiety to allow precoupling to streptavidin-flour conjugates. Cy3 and Cy5 fluorescent dyes are used. A spacer is engineered between the biotin moiety and the protein motif used to probe the array (*see* **Note 5**).

1. Before probing the slide with a peptide, incubate the slide in blocking buffer for 1 h at room temperature (or overnight at 4°C; *see* **Note 6**).
2. Prebind the peptide probe as follows:

Biotinylated peptide (5 μg/μL)	2 μL
FluoroLink™ Cy3 or 5 (1 μg/μL)	5 μL
1X PBS	13 μL
Total volume	20 μL

3. Incubate on ice for 30 min.
4. To remove the unbounded FluoroLink™, it is necessary to incubate the peptide probe with biotin agarose beads. During the prebinding step prepare the beads by taking 60 μL of biotin agarose beads and washing with 1X PBST (1X PBS pH 7.2; 10% Tween-20), then separate into three clean microcentrifuge tubes. Briefly spin down the tubes and discard the supernatant. Place the three tubes with the bead pellet on ice for the following step.
5. Add the peptide mix to one of the previously prepared tubes containing biotin beads, and rock for 10 min at 4°C.
6. Add 500 μL 1X PBST and rock again for 10 min at 4°C. Centrifuge at 10,000*g* for 30 s with a "soft" stop, at room temperature.
7. Transfer the supernatant to the clean tube from **step 4**. Repeat **steps 4–7** two times to remove all unbound FluoroLink™.
8. Save 500 μL of supernatant from the third tube and mix with 1.3 mL of blocking buffer.
9. Take the slide from the blocking buffer (**step 1**). Do not allow the slide to dry, and place in a hybridization chamber. Add the peptide probe and incubate at room temperature for 1 h.
10. Wash the probed slide three times by placing it in a glass slide-staining jar with 1X PBST. Shake for 30 min. at 60 rpm at room temperature. Change the buffer every 10 min.
11. Centrifuge the slide at 500*g* for 1 min at room temperature in an appropriate container to remove excess PBST.
12. Scan the slide with Gene Tac™ LS IV Microarray analyzer (Genomic Solution).

Results of a peptide-probed microarray are depicted in **Fig. 3**.

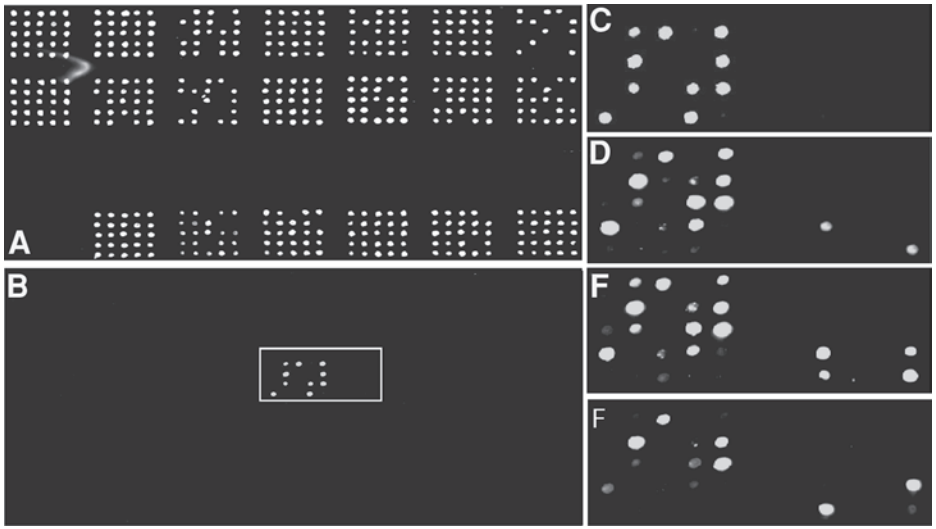

Fig. 3. Probing the protein-domain array with peptide probes. (**A**) Complete protein-domain array probed with αGST primary antibody and detected with a FITC-conjugated secondary antibody. (**B**) Protein-domain array probed with Cy3 labeled KV1.4 peptide (biotin—SGSGSNAKAVETDV-COOH). (**C**) Detail of PDZ domain grids highlighted in (**B**). (**D**) The array was probed with Cy5-labeled Kir 2.1 peptide (biotin—SGSGPRPLRRESEI-COOH). (**E**) The array was probed with Cy5-labeled Star peptide (biotin—SGSGNTANRRTTPV-COOH). (**F**) The array was probed with Cy5-labeled KNCK peptide (biotin—SGSGRGLM-KRRSSVCOOH).

3.3.2. Recombinant Protein Probes

Recombinant proteins can be used as probes if they are tagged (His, HA, or Flag tags can be used) or if a specific antibody is available to the recombinant probe (*see* **Note 7**). GST-fusion proteins cannot be used as probes, as GST can form homodimers with the arrayed proteins ***(6)***, resulting in nonspecific binding (*see* **Note 8**).

1. Isolate recombinant protein for use as a probe.
2. Block the slide for 1 h at room temperature with blocking buffer.
3. Dilute the recombinant protein in 1X PBS to a concentration of 0.5 mg/mL and to a final volume of 2 mL. Add BSA and nonfat milk to obtain a final concentration of 3% of each reagent.
4. Remove the slide from the blocking buffer (do not let it dry) and place it in the chamber. Incubate the slide with the cell lysate mix for 2 h at room temperature.
5. Wash the slide twice in a glass slide-staining jar with 1X PBST washing buffer, shaking at 60 rpm at room temperature and changing the buffer every 10 min.
6. Dilute the 1° antibody in 2 mL of 1X PBST 3% milk (*see* **Note 9**).
7. Place the washed slide in a clean chamber and add the 1° antibody solution to the slide; incubate for 1 h at room temperature.
8. Wash two times with 1X PBST as in **step 5**.

9. Dilute the FITC-labeled 2° antibody in 2 mL of 1X PBST 3% milk.
10. Place the washed slide in a clean chamber, add the 2° antibody solution to the slide, and incubate for 1 h at room temperature.
11. Wash two times with 1X PBST as in **step 5**.
12. Centrifuge at 500*g*for 1 min at room temperature.
13. Scan the slide with Gene Tac™ LS IV Microarray analyzer (Genomic Solution).

3.3.3. Cellular Protein Enzyme-Linked Immunosorbent Assay (ELISA)

In addition to using peptides and recombinant proteins as probes, binding proteins have been "fished out" of total cell lysates or tissue extracts. In this case, after probing, each immobilized protein domain will have a unique spectrum of cellular protein bound to it. Using a specific antibody for the protein of interest (*see* **Note 7**), it can be determined if that protein is bound to any of the arrayed domains, thus generating a binding profile for cellular proteins. This approach works best for proteins that are highly expressed (or overexpressed) in the cell.

1. Culture MCF7 cells to 80% confluency.
2. Wash the cells once with 1X PBS and collect them by scraping with l mL of 1X PBS supplemented with 0.1 *M* NaCl and protease inhibitors.
3. Sonicate 30 s repeating the ultrasonic vibrations with pulses of 0.5 s on and off (amplitude 30%) on ice.
4. Spin down at 10,000*g* at 4°C for 10 min, save the supernatant, and quantitate the protein using standard methods.
5. Block the slide for 1 h at room temperature with blocking buffer.
6. Dilute cell lysate from **step 4** with 1X PBS to reach a concentration of 1mg/mL and a final volume of 2 mL. Add BSA and nonfat milk to obtain a final concentration of 3% of each reagent.
7. Remove the slide from the blocking buffer (do not let it dry) and place it in the chamber. Incubate the slide with the cell lysate mix for 2 h at room temperature.
8. Wash the slide twice in a glass slide-staining jar with 1X PBST washing buffer, shaking at 60 rpm at room temperature and changing the buffer every 10 min.
9. Dilute the 1° antibody in 2 mL of 1X PBST 3% milk (*see* **Note 9**).
10. Place the washed slide in a clean chamber and add the 1° antibody solution to the slide; incubate for 1 h at room temperature.
11. Wash two times with 1X PBST as in **step 8**.
12. Dilute the FITC-labeled 2° antibody in 2 mL of 1X PBST 3% milk.
13. Place the washed slide in a clean chamber, add the 2° antibody solution to the slide, and incubate for 1 h at room temperature.
14. Wash two times with 1X PBST as in **step 8**.
15. Centrifuge at 500*g* for 1 min at room temperature.
16. Scan the slide with Gene Tac™ LS IV Microarray analyzer (Genomic Solution).

Results of a cell lysates-probed microarray are depicted in **Fig. 4**.

3.4. Reading Slides

The authors use ArrayVision™ software (version 6.0; Imaging Research) for array analysis. They have probed arrays with a mixture of labeled peptides. For example, the same sequence unphosphorylated (Cy3-labled) and phosphorylated (Cy5-labled) can be used to screen a domain microarray for phosphorylation-regulated protein

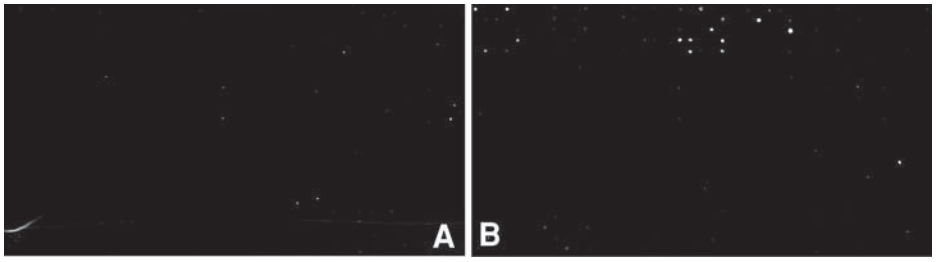

Fig. 4. Protein-domain array probed with cell lysate identifies binding profiles for endogenous cellular proteins. (**A**) The protein-domain array was probed with an antibody to the SmB splicing factors (Y12) and detected with a FITC-conjugated secondary antibody. (**B**) The array was first incubated with 2 mg/mL of MCF7 cell lysate and then probed with an antibody to the SmB splicing factors (Y12) and detected with a FITC-conjugated secondary antibody. Panel **A** depicts nonspecific interactions that are detected by the Y12 primary antibody in the absence of cell lysate.

interactions. Accurate spot quantification of multilabeled fluorescent arrays can be obtained using ArrayVision™.

4. Notes

1. Certain fusion proteins are produced better than others. The induction volume should be adjusted according to the efficiency of recombinant protein production and has to be determined empirically to obtain a final concentration of 1 μg/μL in 200 μL of elution buffer. The authors divide their constructs into "good" expressers and "bad" expressers. For "good" expressers, they follow the protocol described here. For "bad" expressers, they inoculate a 50-mL LB culture for overnight growth and the next morning add 450 mL 1 h prior to IPTG induction. About 70% of the authors' constructs are in the "good" expresser category.
2. In some studies, glycerol is added to the protein stocks that are arrayed. This is done to increase the amount of protein transferred by a pin arrayer and to prevent the arrayed protein from drying out. The authors have found that a 10% glycerol solution prevents efficient protein binding to FAST™ slides, that the features arrayed with glycerol are not sharp, and finally that the protein domains retain their binding integrity in the absence of glycerol.
3. Many different surfaces have been used to immobilize proteins. Initial studies arrayed cDNA expression libraries on polyvinylidene difluoride membranes ***(7)***. MacBeath and Schreiber arrayed proteins on aldehyde slides that were probed with fluorophore-tagged proteins to identify protein–protein interactions ***(8)***. Nickel-coated slides were used to array GST-6XHis-tagged yeast proteins ***(9)***. The group led by Pat Brown has also made inroads into the protein microarray field ***(10)***, using poly-L-lysine slides. The authors have compared nitrocellulose-coated FAST™ slide side-by-side with aldehyde, poly-L-lysine, and HydroGel™ (PerkinElmer Life Sciences, Boston, MA) slides and found the FAST™ slide to be far superior.
4. Six-month-old slides have been used and were found to have exactly the same selectivity as slides only 1 wk old.

5. To prevent steric interference of the attached fluorescent dyes, a four-amino-acid spacer is inserted between the biotin moiety and the 10-mer peptide motif. This linker (SGSG) is made up of two small fairly inert amino acids, serine and glycine.
6. If your experiments result in high background staining, you can increase the concentration of the BSA and milk in the blocking buffer.
7. The specific antibody must not have been raised against a GST-fusion protein, as it will crossreact with all the protein domains on the array.
8. GST-fusion proteins can be used as probes if they are liberated from the GST moiety, prior to binding, by thrombin or PreScission proteases.
9. The dilutions of 1° and 2° antibodies used in the ELISA approach have to be determined empirically, as each antibody is a unique reagent. A good starting point is to use dilutions that work well for Western blot analysis.

Acknowledgments

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16

SH3 Domain Protein-Binding Arrays

Sangpen Chamnongpol and Xianqiang Li

Summary

First identified as part of the Rous sarcoma oncogene product Src, SH3 (Src Homology 3) domains play an important role in intercellular communication and intracellular signal transduction. A high-throughput assay for ligand binding to SH3 domains—SH3 domain proteins immobilized on a membrane—allows rapid visualization of numerous SH3 domain protein–protein interactions with no expensive equipment or radioactivity required. Once the array is constructed or obtained commercially, the procedure is straightforward: The protein of interest is cloned into a fusion-tagged expression vector and expressed in bacteria, the prepared bacterial extract is incubated with the array membrane, and the signal is measured using a chemiluminescence detection system.

Key Words:

Src; SH3 domain; protein array; ligand binding; high-throughput screening.

1. Introduction

A key to understanding cellular signal transduction is clarifying how proteins interact with one another. Protein–protein interactions are often mediated by noncatalytic, conserved domains. One of these domains is the SH3 domain ***(1)***, which was first identified as part of the Rous sarcoma oncogene product Src. Each SH3 domain is a small, conserved sequence of about 60 amino acids that interacts with proline-rich binding sites known as SH3 ligands. These sites contain 6–12 residues, with a conserved Pro-Xaa-Xaa-Pro motif ***(1,2)***.

SH3 domains play an important role in intercellular communication and intracellular signal transduction. SH3 domains act as part of an adapter molecule of signaling proteins and enzymes, recruiting downstream proteins in a signaling pathway. SH3 domains mediate assembly of specific complexes in many key signaling pathways, including epidermal growth factor receptor signaling ***(3)***, cellular localization of cytoplasmic proteins ***(4)***, up-regulation of the GTPase activity of dynamin ***(5)***, activation

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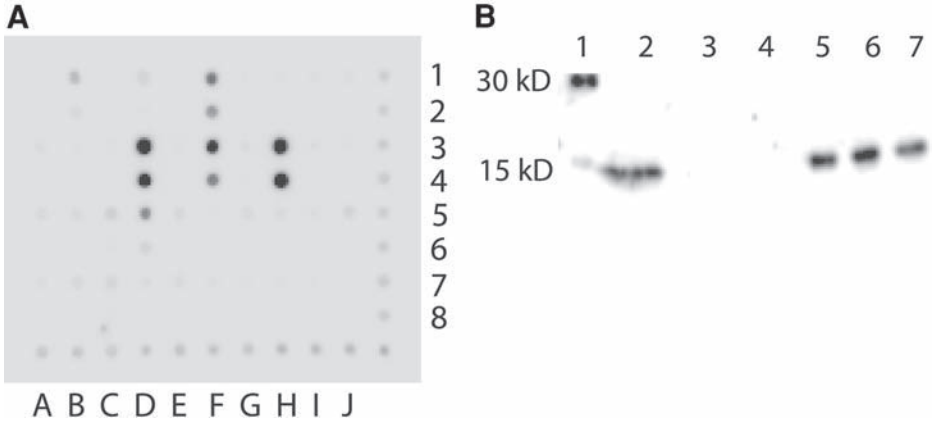

Fig. 1. Typical results obtained with the Panomics TranSignal SH3™ Domain Array I. **Panel A.** Class Ib SH3 ligand from bacterial extract specifically interacts with corresponding SH3 domains. Class Ib encoding sequence was inserted into the ligand expression vector (pEXP), and transformed into DH5α. Bacterial extract from the transformed cells was hybridized with the TranSignal SH3™ Domain Array I, and the image was acquired using FluorChem imager (Alpha Innotech). Spots with stronger intensities indicate higher binding affinity with the ligand of interest to SH3 domain(s).

Note: For the schematic diagram of the Panomics TranSignal SH3™ Domain Array, visit www.panomics.com.

Panel B. Class Ib SH3 ligand specifically "pulls down" its corresponding SH3 domains. *Lane 1*: marker. *Lane 2*: ligand. *Lane 3*: negative control. *Lane 4*: EMP55. *Lane 5*: TXK. *Lane 6*: c-Src. *Lane 7*: Yes1.

of phosphatidylinositol 3-kinase in response to IgM crosslinking ***(6)***, and SH3 domain activity has been implicated in both cancer and AIDS. In recent years, the SH3 domain has been a promising target in the search for novel therapeutic agents that are highly specific to a given signaling pathway. Most inhibitors of the SH3 domain act through interference with its binding ability ***(7–9)***.

An important step toward characterizing the function of an SH3 domain-interacting ligand and/or protein is to identify to which SH3 domain it binds, and hence determine in which signaling pathway it is involved. Traditional methods for detecting protein–protein interactions, such as co-immunoprecipitation, are arduous and time-consuming. A high-throughput assay consisting of SH3 domain proteins immobilized on a membrane can save a vast amount of time by testing numerous interactions simultaneously in a single simple experiment (*see* **Figs. 1** and **2**).

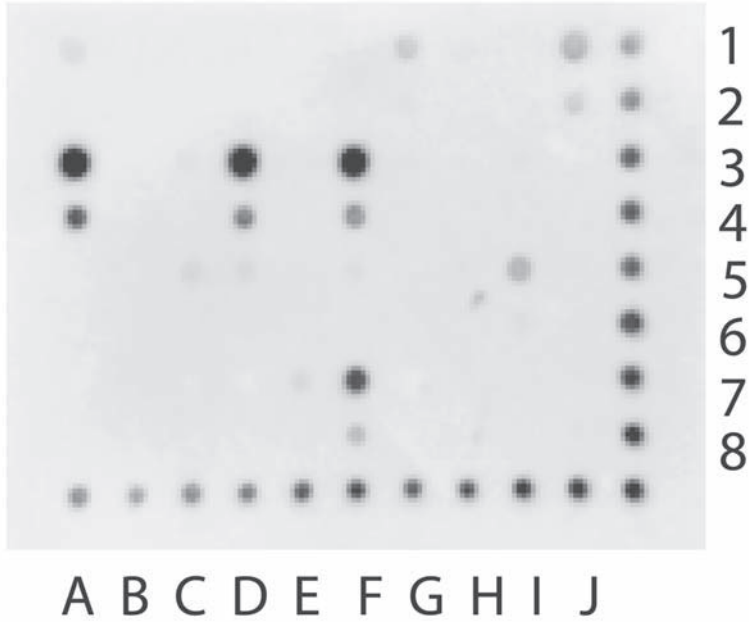

Fig. 2. Typical results obtained with the Panomics TranSignal™ SH3 Domain Array II. Class Ib SH3 ligand from bacterial extract specifically interacts with corresponding SH3 domains. Class Ib encoding sequence was inserted into the ligand expression vector (pEXP), and transformed into DH5α. Bacterial extract from the transformed cells was hybridized with the TranSignal SH3 Domain Array II, and the image was acquired using FluorChem imager (from Alpha Innotech). Spots with stronger intensities indicate higher binding affinity with the ligand of interest to SH3 Domain(s).

2. Materials

1. pGEX-4T expression and purification systems (Amersham Biosciences, Piscataway, NJ).
2. Human SH3 domain complementary DNA (cDNA) (Panomics, Redwood City, CA).
3. pEXP ligand expression vector (Panomics).
4. Restriction enzymes and T4 DNA ligase.
5. *Escherichia coli* strains DH5α, and/or BL21.
6. Luria-Bertani (LB) broth:1% tryptone, 0.5% yeast extract, 1.0% sodium chloride, and/or 1.5% agar.
7. 2YT broth: 1.6% tryptone, 1.0% yeast extract, and 0.5% sodium chloride.
8. Ampicillin.
9. Isopropyl β-D-thiogalactopyranoside (IPTG).
10. Incubator.
11. Sonicator.
12. Orbital shaker.
13. Resuspension buffer (Panomics).

14. Polyvinylidene difluoride membrane, such as Immobilon-P (Millipore, Bedford, MA).
15. TranSignal™ SH3 domain array (Panomics).
16. Blocking buffer, such as SuperBlock (Pierce, Rockford, IL).
17. Wash buffer (Panomics).
18. Antihistidine horseradish peroxidase (HRP) conjugate (Panomics).
19. Detection buffers: peroxide solution and luminol enhancer (Panomics).
20. Film and developer, such as Hyperfilm ECL (Amersham Biosciences) or a chemiluminescence imaging system, such as the FluorChem imager (Alpha Innotech, San Leandro, CA).

3. Methods

The methods described in this section outline (a) the preparation of recombinant SH3 domain proteins in *E. coli* (**Subheading 3.1.**), (b) the preparation of an SH3 domain array (**Subheading 3.2.**), (c) the preparation of the bacterial extracts expressing ligand in *E. coli* (**Subheading 3.3.**), (d) incubation of the ligand extracts with the SH3 domain array (**Subheading 3.4.**), and (e) detection (**Subheading 3.5.**). If a prepared array is purchased, then start at **Subheading 3.3.**

3.1. Preparation of the Recombinant SH3 Domain Proteins in E. coli

The preparation of the recombinant glutathione-*S*-transferase (GST)-tagged SH3 domain proteins follows standard molecular biology cloning techniques ***(10)***. A brief summary follows.

1. Insert human SH3 domain cDNAs (Panomics) into the multiple cloning sites of pGEX-4T (Amersham Biosciences). This will express GST-fusion proteins that are transcriptionally initiated from the P*lac*-IPTG-inducible promoter.
2. Transform the recombinant clones into *E. coli* DH5α or BL21.
3. Prepare a bacterial culture expressing recombinant proteins by diluting an overnight culture 1:100 with 2YT medium (plus ampicillin at 100 μg/mL), growing at 30°C until an optical density $(OD)_{600}$ is reached, 0.5–1, and then adding IPTG to a final concentration of 0.1–1.0 m*M* and continuing incubation for 12 h at 20°C.
4. Collect cells by centrifugation, disrupt by sonication, then purify the recombinant proteins according to the supplier's recommendation.

3.2. Preparation of SH3 Domain Array

The proteins are purified using standard methodology ***(10)*** (*see* **Note 1**) or the supplier's recommendation.

1. Determine the amount of purified recombinant protein by a colorimetric assay.
2. Dilute the protein to the same concentration, and resolve by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) to determine protein quality and confirm quantity.
3. Immobilize the purified proteins at a standard amount onto a nylon membrane that has been pretreated according to supplier's recommendation.

3.3. Preparation of Bacterial Extracts Expressing Ligand in E. coli

Insert the sequence of the SH3 ligand of interest into the ligand expression vector (pEXP) using standard molecular cloning techniques ***(10)***. pEXP is used so that the fusion proteins have polyhistidine tags (*see* **Notes 2** and **3**).

Then prepare the bacterial extract containing the ligand of interest for hybridization with the array membrane (*see* **Note 4**). It is recommended to prepare lysate from *E. coli*-expressing pEXP vector without an insert when using the extract for a negative control experiment.

1. Inoculate the transformed bacteria in 1 mL of LB broth with 100 μg/mL ampicillin.
2. Grow bacteria overnight at 37°C.
3. Transfer 80 μL of the overnight culture to a tube containing 4 mL of LB broth with 100 μg/mL ampicillin.
4. Grow bacteria at 37°C until OD_{600} readings are approx 0.5–0.8.
5. Add IPTG to a final concentration of 100 μ*M*.
6. Continue to grow for an additional 3–4 h at 37°C.
7. Collect cells by centrifugation. Decant supernatant.
8. Resuspend the pellet in 750 μL of ice-cold resuspension buffer.
9. Lyse cells using a sonicator.
10. Centrifuge at 10,000*g* for 5 min at 4°C.
11. Transfer supernatant into a clean microcentrifuge tube.
12. Store on ice until further use. For longer storage, keep at –20°C.

3.4. Incubation of the Ligand Extracts to SH3 Domain Array

In this section, the bacterial extract containing the SH3 ligand of interest (*see* **Subheading 3.3.**) is incubated with and bound to the array membrane prepared in **Subheading 3.2.** or purchased (Panomics) (*see* **Note 5**). In each step, use enough buffer to fully submerge the membrane. Never let the membrane dry out.

1. Preequilibrate membranes by placing each one into a small tray containing wash buffer, and incubate for 20 min at room temperature.
2. Remove the wash buffer, and add blocking buffer.
3. Place the tray on shaker and incubate, gently rocking, for 1 h at room temperature or overnight at 4°C.
4. Remove blocking buffer, and briefly rinse membrane twice with wash buffer.
5. Dilute the bacterial extract described in **Subheading 3.3., step 12** to a final concentration of 0.1 mg/mL in resuspension buffer, at a volume large enough to submerge the membrane.
6. Incubate the membrane with the diluted bacterial extract at room temperature for 1 h.
7. After incubation, wash the membrane three times with wash buffer for 10 min (each wash) at room temperature.
8. Incubate with antihistidine HRP conjugate diluted in wash buffer (at the manufacturer's suggested working concentration) for 1 hr at room temperature.
9. Wash three times with wash buffer for 10 min (each wash) at room temperature.

3.5. Detection

Do not let the membrane dry out during detection.

1. Prepare the detection solution immediately before use by mixing equal amounts of detection buffers (peroxide solution and luminol enhancer)—for example, 1 mL of peroxide solution and 1 mL of luminol enhancer.
2. Using forceps to hold the membrane at the corner, carefully remove membrane from its tray. Drain the excess wash buffer from the membrane by touching the edge against tissue. Place protein-side up.

3. Pipet the mixed detection buffers onto the membrane. Ensure that the buffer mixture is evenly distributed over the membrane without air bubbles.
4. Incubate at room temperature, uncovered, for 5 min.
5. Remove excess substrate by holding the membranes with forceps and touching the edge against tissue. Place the membrane between two plastic sheets, and gently press on the top sheet to remove air bubbles.
6. Expose the membranes using either Hyperfilm ECL or a chemiluminescence imaging system, such as the FluorChem imager (Alpha Innotech). In either case, try several different exposures of varying lengths of time (e.g., 30 s–5 min) (*see* **Note 6**).

4. Notes

1. Some protein arrays are prepared from whole bacterial lysate, but it is not the method of choice for the SH3 domain array. Although the sequence of SH3 domains are conserved in humans, expression levels of each clone in *E. coli* vary. To generate array membranes for a binding assay that results in a semiquantitative or comparative data, it is better to make membranes from purified proteins.
2. This system is designed for using a polyhistidine-tagged fusion protein expressed in *E. coli.* Biotinylated ligand or purified protein can also be used with appropriate secondary antibody for detection. The Panomics system uses antihistidine-HRP, biotin uses antistreptavidin conjugate, and purified protein uses antibody specific to the protein followed by a corresponding HRP- or biotin-conjugated secondary antibody.
3. The ligand used for this experiment can be in the form of a full-length protein or a short peptide (10–15 amino acids). Using a short ligand peptide tagged to polyhistidine molecules may sometimes reduce accessibility of the antihistidine-HRP conjugate after the ligand binds to SH3 domain on the array, resulting in a low signal. Increased accessibility may be achieved by adding a polyglycine linker of nine residues between the polyhistidine residues and the ligand.
4. The protocol described for preparing the bacterial extract containing the ligand of interest for hybridization with the array membrane is only a guideline for establishing induction conditions. Growth conditions in *E. coli* should be optimized for each ligand construct to obtain its maximal expression level by varying the amount of IPTG, temperature, and/or culture volume.
5. Membranes can be stripped and reused, but it is not recommended. Stripping may cause damage to proteins on the array membrane, thus affecting the results of the binding assay.
6. Signal intensities can be quantified by densitometry or by chemiluminescence imaging. Results can be interpreted by comparing signal intensities to the suggested negative control. When the SH3 domains that interact with the test protein or ligand are identified by the array, results can easily be confirmed via coimmunoprecipitation or a pull-down assay.

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Site-Specific Peptide Immobilization Strategies for the Rapid Detection of Kinase Activity on Microarrays

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Summary

The massive throughput offered by array-based technologies can only be realized with the development of equally powerful strategies that offer reproducible consistency. The competence of arrays and efficacy of screening come under scrutiny, with most existing immobilization schemes that do not site-specifically ligate peptides on the arrays. Thus, it is crucial in array-based experiments to orientate peptides in an ordered and uniform fashion. Two new approaches were developed for the directed immobilization of peptides on a microarray, by exploiting measures involving native chemical ligation reactions as well as biotin–streptavidin interactions. This makes it possible to stably immobilize peptides in a consistent manner and in a predetermined orientation on the microarray. The first scheme employs glass slides that are functionalized with avidin for attachment of terminally biotinylated peptides. The second uses slides containing thioester moieties to ligate N-terminal cysteine containing peptides. The authors successfully immobilized peptides on chip using these strategies, and, in extending their method to the study of kinase activity on microarrays, they also developed a novel detection scheme that abrogates the dependence on traditional radioactivity-based kinase screening assays. This method employs fluorescently labeled antiphosphoserine and antiphosphotyrosine antibodies in assessing and monitoring kinase activity on arrays. The above methodologies provide for a fast and sensitive approach with which to conveniently assess kinase activity using peptide microarrays.

Key Words:

Peptide microarray; high-throughput screening; kinase activity; fluorescence-based antibody detection; native chemical ligation; biotin–streptavidin interaction.

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1. Introduction

Peptide, protein, and small molecule arrays are becoming increasingly important in the field of proteomics by offering a high-throughput means of discovering novel protein function and interactions ***(1,2)***. This great benefit of microarray technology comes from its provision of a miniaturized platform on which a whole spectrum of targets may be screened rapidly in a massively parallel format. This chapter dicusses peptide arrays, which facilitate the addressable immobilization of a repertoire of peptides in high-density grids. Peptide arrays provide the technological means for various high-throughput studies, thus considerably ameliorating research targeted at profiling enzymatic activity. In addition, such arrays may also be employed for the determination of ligand–receptor interactions, assessment of antigen–antibody affinities, and establishment of other similar interactions. Usefulness in these areas has made peptide arrays a valuable and promising tool for the rapid discovery of drug candidates as well as in the design and selection of effective substrates, ligands, or inhibitors for various enzymes and antibodies.

Before microarray technology can be fully appreciated and ubiquitously applied, it is critical to establish methods to uniformly immobilize peptides on the array surface. Conventional immobilization schemes exploit common nucleophilic groups (-NH_2, -SH, -OH), where any one of these functionalities present along the peptide may react with *N*-hydroxysuccinimide (NHS) or epoxide-derivatized slides. This heterogeneously presents these substrates on the slide surface, where not all immobilized peptides are oriented suitably for subsequent screenings. Falsey et al. conceived a method using N-terminal cysteine-containing peptides immobilized on glyoxylic acid-functionalized slides via the thiazolidine ring ligation reaction. ***(3)*** However, there is limited value of employing the thiazolidine ring as a peptide immobilization tool, as the linkage is unstable and may restrict the orientation of the peptides on the slides, thereby impinging on their ability to freely interact with the targeting proteins. Another group exploited the Diels-Alder reaction that also allows for site-specific ligation of peptides onto slides ***(4)***, but the method requires that peptides be first conjugated with an unnatural cyclopentadiene moiety, a step that is synthetically challenging.

The authors offer two approaches for site-specifically immobilizing peptides on an array. The first uses a native chemical ligation reaction in which the N-terminal cysteine residue engineered on the peptide substrate reacts chemoselectively with thioester moieties on the array platform ***(5)***. This method is advantageous, as peptides with an N-terminal cysteine may be readily synthesized using established 9-fluorenylmethoxycarbonyl (Fmoc) strategies. The reaction also produces a stable, native peptide bond (*see* **Fig. 1**) with absolutely no crossreactivity with other cysteine residues that may be present along the peptide chain.

The second approach exploits the strong noncovalent interaction of biotin–avidin ($K_d = 10^{-15}$ *M*) to immobilize peptides constructed with an N-terminal biotin onto avidin-functionalized surfaces ***(5)***. Avidin is an extremely stable protein, making it an excellent candidate for slide derivatization and immobilization. The reaction with its natural ligand takes place almost instantaneously, thus doing away with the long incubation time that alternative methods require for the critical immobilization step. Avi-

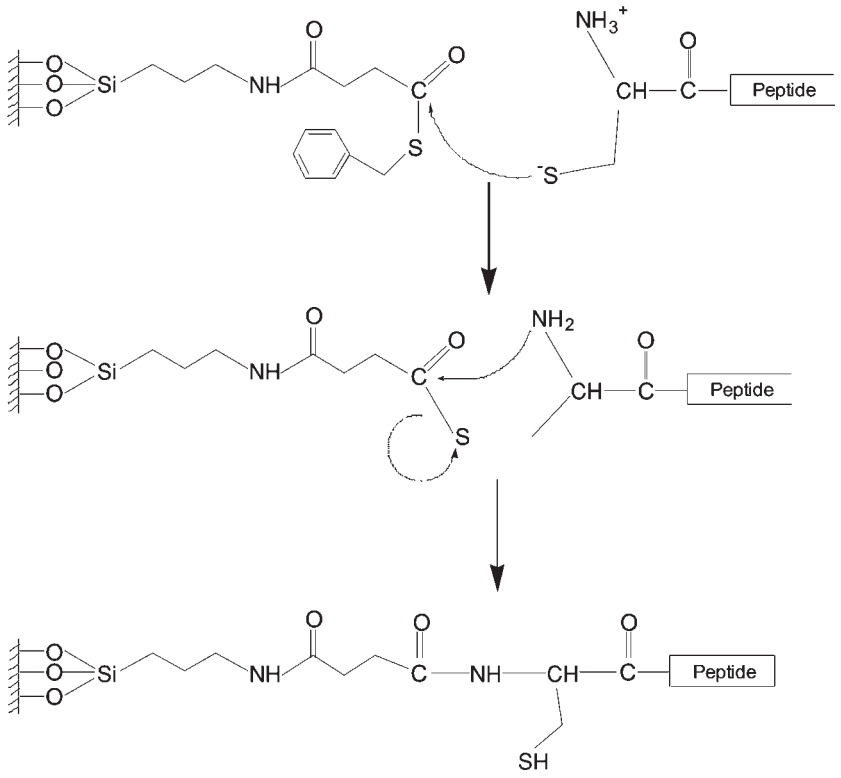

Fig. 1. Native chemical ligation. The thioester functionality on the slide reacts with an N-terminal cysteine residue on a peptide sequence to produce a stable amide linkage.

din also acts as a molecular layer that minimizes nonspecific binding on the slide surface (*see* **Fig. 2**).

The authors tested the efficacy of the above methods by site-specifically arraying substrates for kinases, a key group of enzymes that plays a crucial role in signal transduction. Kinases are an avidly studied group of enzymes, with only a few of these enzymes having been fully characterized. Pioneering work in this field has been done with the aid of platform strategies including SPOT™ technology ***(6)***, one-bead-one compound peptide libraries ***(7)***, position-scanning combinatorial libraries ***(8)***, and peptide libraries using affinity-column selection ***(9)***. Assessing kinase activities on arrays is the latest introduction to this host of strategies ***(10,11)***. Now it is possible to screen thousands of potential kinase substrates on a single glass slide, with the use of only minute quantities of both enzyme and substrate.

Kinases phosphorylate specific target amino acid residues in proteins, thereby regulating the latter's activity. In eukaryotic systems, three amino acid residues are potentially phosphorylated, namely serine, threonine, and tyrosine. Kinases are classified according to the types or combinations of these target residues that they are able to

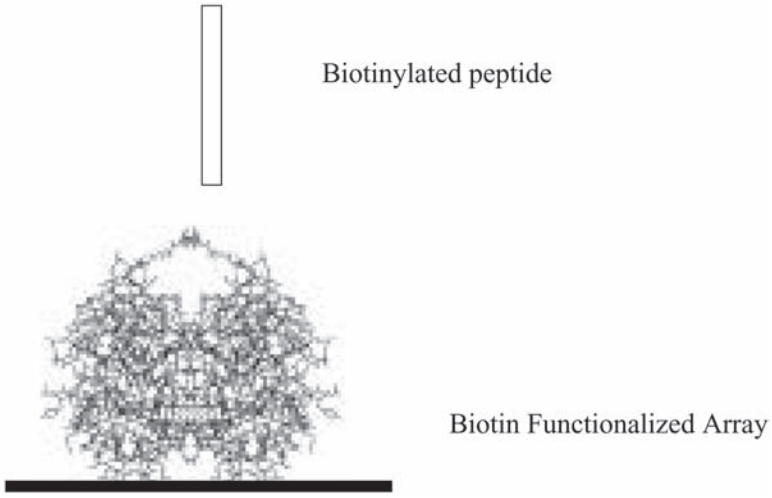

Fig. 2. Immobilizing biotinylated peptides on avidin-functionalized slides on a microarray.

phosphorylate. It is important to note that the specificity of kinases is dependent on their ability to recognize short sequences of amino acids flanking the phosphorylation site. Peptides immobilized on arrays may thus be designed to contain unique target residues, thereby serving as substrates with which to assess the specificity and activity of kinases. Established techniques apply radiolabeled adenosine triphosphate (ATP) for monitoring kinase activity on microarrays. However, radioactivity-based detection systems using ^{32}P are generally unsafe to work with. In recent years, these have become less popular, in light of newer fluorescence-based technologies that offer safe and nearly equivalent sensitivities to most traditional radioactivity-based techniques. Fluorescence also does away with the dependence on long exposure time that may be required when using radioisotopes, but it retains the ability to obtain quantifiable measurements of enzyme kinetics. Radioactivity-based strategies also make it impossible to obtain real-time measurements of activity. With these considerations, the authors developed a novel fluorescence-based detection system that enables one to assess and detect the activity of kinases on an array platform, eliminating the hitherto dependence on radioactivity as a means to screen for kinase activity on microarrays ***(11)***. Antibodies have been established to specifically target only phosphorylated amino acids that can detect as little as a few femtomoles of their binding epitopes ***(12)***. The authors reasoned that they could exploit these commercially available antibodies to detect the activity of kinases in a high-throughput fashion on peptide microarrays. They demonstrated that this detection system is able to efficiently detect phosphorylated peptides with negligible crossreactivity on arrays.

The methodologies the authors have described may be used to immobilize peptides and even proteins or small molecules site-specifically on arrays. With the use of microarrays as a rapid screening tool, fluorescence-based detection of kinase activity would enhance the understanding of the role kinases play in complex biological sys-

tems, as well as cater to the provision and design of drugs that specifically intervene in kinase-mediated signaling pathways.

2. Materials

2.1. Chemicals

2.1.1. Standard Reagents for Peptide Synthesis

1. Rink amide resin (0.7 mmol/g, 100–200 mesh) may be obtained from Novabiochem/Advanced ChemTech.
2. The synthesis employs Fmoc-protected amino acids with the following acid-labile side-chain protecting groups: Boc for Lys and Trp; *t*-butyl for Ser, Tyr, Glu, Asp, and Thr; trityl for Cys, Asn, and Gln; and 4-methoxy-2,3,6-trimethylbenzenesulphonyl (Mtr) or 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl for Arg.
3. The solvent used for all coupling reactions is dimethyl formamide (DMF), high-pressure (performance) liquid chromatography (HPLC) grade.
4. Fmoc cleavage is performed with 20% piperidine in DMF.
5. 0.5 *M O*-benzothiazol-1-yl-*N,N,N',N'*-tetramethyluronium tetrafluoroborate (TBTU) or equivalent esterifying agent in DMF.
6. 0.5 *M N*-hydroxybenzotriazole (HOBt) in DMF.
7. 1 *M N,N*-diisopropylethylamine (DIEA) in DMF.
8. Reagent R cleavage cocktail contains trifluoroacetic acid/thioanisole/anisole/ethanedithiol, 90:5:3:2 (v/v/v/v).
9. Cold diethylether.

2.1.2. Slide Derivatization

2.1.2.1. Avidin Slides

1. Piranha solution: 70% sulfuric acid, 30% hydrogen peroxide.
2. 1% 3-glycidopropyltrimethoxysilane in 95% ethanol with 16 m*M* acetic acid.
3. 1 mg/mL avidin in 10 m*M* sodium bicarbonate buffer (pH 9.0).
4. 2 m*M* aspartic acid in 0.5 *M* sodium bicarbonate buffer (pH 9.0).

2.1.2.2. Thioester Slides

1. 3% aminopropyltriethoxysilane (APTES) in 2% H_2O and 95% ethanol.
2. 180 m*M* succinic anhydride in DMF.
3. TBTU, DIEA, *N*-hydroxysuccinimide (1:2:1 molar equivalents) in DMF.
4. 100 m*M* benzyl mercaptan and 120 m*M* DIEA in DMF.
5. Diamine polyethylene glycol (PEG) (Shearwater, Birmingham, AL).
6. PEG-succinimidyl propionate (SPA-PEG) (Shearwater, Birmingham, AL).

2.1.3. Printing Slides

1. 3–5 mg/mL of peptides in phosphate-buffered saline (PBS) (pH 7.4).

2.1.4. Kinase Assay

2.1.4.1. Kinase Preparations

1. 2 U protein kinase A (PKA) (Upstate Biotechnology, Lake Placid, NY) in 25 m*M* Tris-HCl (pH 7.4), 15 m*M* magnesium chloride, 1 m*M* dithiothreitol, 2 m*M* ethylene glycol *bis* (2-aminoethyl ether)-*N,N,N'N'*-tetraacetic acid (EGTA), 100 μ*M* ATP.

2. 2 U p60c-src (Upstate Biotechnology, Lake Placid, NY) in 25 m*M* (pH 7.4), 35 m*M* magnesium chloride, 7 m*M* manganese chloride, 0.5 m*M* EGTA, 100 μ*M* ATP.
3. 1% bovine serum albumin (BSA) solution.

2.1.4.2. Antibodies

1. Monoclonal antiphosphotyrosine, fluorescein isothiocyanate (FITC) conjugate (Sigma-Aldrich, St. Louis, MO).
2. Monoclonal antiphosphoserine, FITC conjugate (Sigma-Aldrich, St. Louis, MO).
3. Fluorescein-5-ex-succinimidyl ester (FITC-NHS) for antibody labeling (Molecular Probes, Eugene, OR).

All preparations are made in dH_2O unless otherwise stated.

2.2. Equipment and Supplies

1. Pioneer™ Automatic Peptide Synthesizer (Applied Biosystems, Foster City, CA).
2. 96-well reaction system for high-throughput peptide synthesis (Robbins FlexChem®, Hudson, NH).
3. Vacuum concentrator centrifuge.
4. Lyophilizer.
5. Waters™ 600 HPLC station with a C_{18} semipreparative column.
6. ESI SMA™ arrayer (Ontario, Canada).
7. ArrayWoRx™ scanner (Applied Precision, Issaquah, WA).
8. NAP-5 Sephadex G-50 columns (Amersham, UK).
9. Clean glass slides and cover slips (Fisher Scientific, Pittsburgh, PA).
10. Slide hybridization chamber (Telechem, Sunnyvale, CA).

3. Methods

The method described herein has been used successfully to determine substrate specificity for the kinases the authors have tested.

3.1. Design and Synthesis of Kinase Substrates

An N-terminal cysteine or biotin may be added to virtually any synthetic peptide using the strategy described as follows. This makes it possible to apply peptides synthesized from various combinatorial strategies, such as positional-scanning libraries, to an array format (*see* **Note 1**). Within reasonable lengths, under 10 amino acid residues, Fmoc synthesis yields sufficiently pure peptides that may be directly applied to an array without extensive purification (also dependent on the nature of the residues within peptide and quality of synthesis). This makes it possible to use high-throughput synthetic measures on a 96-well format to rapidly generate an assortment of peptides or libraries that may be immediately applied for screening on an array (*see* **Note 2**). Standard Fmoc chemistry is employed in synthesizing peptide substrates for the kinases ***(13)***. The peptides the authors synthesized individually included YI*Y*GSFK and ALRRA*S*LG that served as substrates for p60c-src and PKA kinases, respectively.

1. Place the desired amount of resin (depending on the scale of synthesis) into the reaction vessel/reaction block.
2. Use TBTU, HOBt, and DIEA coupling chemistry (in 1:1:2 molar ratios) with a fourfold excess of suitably protected Fmoc amino acids for each coupling step. For automated

continuous flow systems the coupling is performed for at least 1 h, whereas for reaction blocks, the coupling is done for at least 2–4 h. Wash resin with DMF.
3. Deprotect Fmoc using 20% piperidine in DMF for 30 min. At the end, wash the resin thoroughly with DMF to remove all traces of piperidine.
4. Repeat **steps 2** and **3** as necessary for the length and sequence of peptide desired.
5. Thereafter, introduce two glycine residues to the N-terminal segment of the peptide to act as a spacer.
6. React with either cysteine or biotin, as desired. It is recommended that double coupling be performed for this final coupling step.
7. When the synthesis is completed, cleave the peptides off the solid support using reagent R, in a step that simultaneously removes the orthogonal protecting groups. Add a twofold volume equivalent of the cleavage cocktail (with respect to the resin) and incubate with mixing for 2 h (*see* **Note 3**).
8. Filter the mixture through a sintered glass or a cotton plug to remove the spent resin.
9. Transfer the filtrate to a polypropylene screw-cap vial containing 50 mL of cold diethyl ether to precipitate the peptide.
10. The equivalent procedure may be done in a 96-well format in the following way: First collect the filtrates from all 96 wells by vacuum into a deep well block. Concentrate the peptide mixtures, if required, using a SpeedVac, and thereafter precipitate using cold ether.
11. Isolate the peptide by centrifugation at 4000 rpm for 15 min. Wash a few times with diethyl ether to remove impurities. Because only peptides conjugated with cysteine or biotin in the final reaction step will react on the slide, extensive purification is not required as contaminants may be easily washed off the slide surface after the immobilization step.
12. If preferred, conduct further purification of the peptides using reverse-phase HPLC.
13. Confirm the identity of the peptide by mass spectrometry.
14. Reconstitute the purified peptide in H_2O and lyophilize to give a stable solid that may be stored for extended durations at –200°C (*see* **Note 4**).

3.2. Slide Functionalization

In addition to the standard thioester slides, the authors also derivatized slides with two types of PEG to remove nonspecific binding and minimize the background noise. With the use of PEG, they found it unnecessary to block the slides with BSA before the application of the antibody. PEG also serves as a linker to extend the ligated peptides away from the slide surface, presenting them more suitably for interaction with complementary ligands or enzymes. All glass slides (75 mm × 25 mm) are first cleaned before silanization and stored in piranha solution until ready for use. Before use, the slides are cleansed of the acid solution by extensive washing with dH_2O and air-dried (*see* **Note 5a,b**).

3.2.1. Preparing Avidin Slides

1. Soak the slides in a 1% solution of 3-glycidopropyltrimethoxysilane in 95% ethanol containing 16 m*M* acetic acid. After 1 h, cure the slides at 150°C for at least 2 h. Subsequently, rinse them with ethanol, and either air-dry or dry under a stream of nitrogen.
2. Place the resulting epoxy-functionalized slides in a solution of 1 mg/mL avidin in 10 m*M* sodium bicarbonate buffer (pH 9). After 30 min, wash the slides with H_2O to remove any unreacted avidin, and dry. Quench the remaining epoxy groups with a solution of 2 m*M* aspartic acid in 0.5 *M* sodium bicarbonate buffer (pH 9.0).

3. Rinse the slides in dH_2O and dry. Slides may be stored at 40°C for extended periods but are best prepared fresh prior to use.

3.2.2. Preparing Thioester Slides

The general scheme of making thioester functionalized slides is shown in **Fig. 3**.

1. First, generate amine slides by silanization using a solution of 3% aminopropyltriethoxysilane in 2% H_2O and 95% ethanol. After 1–2 h of reaction, wash the slides in ethanol and cure at 150°C for at least 2 h (*see* **Note 6**).
2. Incubate the amine slides in a solution of 180 m*M* succinic anhydride in DMF for 30 min, and thereafter transfer the slides to a boiling water bath for 2 min. Wash the slides in ethanol and dry under a stream of nitrogen.
3. Activate the carboxylic acid moiety created on the slide surface with a solution of TBTU, DIEA, *N*-hydroxysuccinimide (in 1:2:1 molar equivalents) in DMF. After a 3-h incubation, incubate the slides overnight (or for at least 8 h) in a solution of 120 m*M* DIEA and 100 m*M* benzylmercaptan in DMF.
4. Rinse the slides in ethanol and dry. Store the slides in a desiccator. Because the thioester functionality is highly reactive, prolonged storage beyond 1 mo may reduce the efficacy of the slides.

3.2.3. Preparing PEGylated Thioester Slides (see reaction scheme in ***Fig. 4***)

3.2.3.1. Starting With Epoxy Surfaces

1. First, generate epoxide slides as described in **Subheading 3.2.2., step 2**.
2. Apply a 100 m*M* solution of diamine PEG (Shearwater, Birmingham, AL) in sodium bicarbonate (pH 9) for 30 min.
3. After rinsing in ethanol and drying, follow **steps 3–5** in **Subheading 3.2.2.** to yield thioester moieties on PEGylated slides.

3.2.3.2. Starting With Amine Surfaces

1. First, generate amine slides as described in **Subheading 3.2.1., step 1**.
2. Apply a 100 m*M* SPA-PEG solution (Shearwater, Birmingham, AL) in sodium bicarbonate (pH 9) for 30 min.
3. Incubate overnight with a solution of 120 m*M* DIEA and 100 m*M* benzylmercaptan in DMF.
4. Rinse in ethanol and dry.

All thioester slides are stored as described in **Subheading 3.2.2., step 5**.

3.3. Printing the Peptides onto the Slides

The cysteine-functionalized peptides are immobilized on thioester slides, and the N-terminal biotinylated peptides are spotted on avidin slides, respectively.

1. Prepare the peptides to 3–5 mg/mL solutions in PBS (pH 7.4).
2. Spot the peptides using an ESI SMA™ Arrayer (Ontario, Canada), with a spot spacing of 220 μm. Incubate for 3–5 h after spotting to allow the peptides to stably attach onto the slide surface.
3. Rinse slides with PBS and H_2O to remove unbound reagents and dry.
4. Block slides with a solution of 1% BSA for a few hours. Rinse with H_2O and dry.

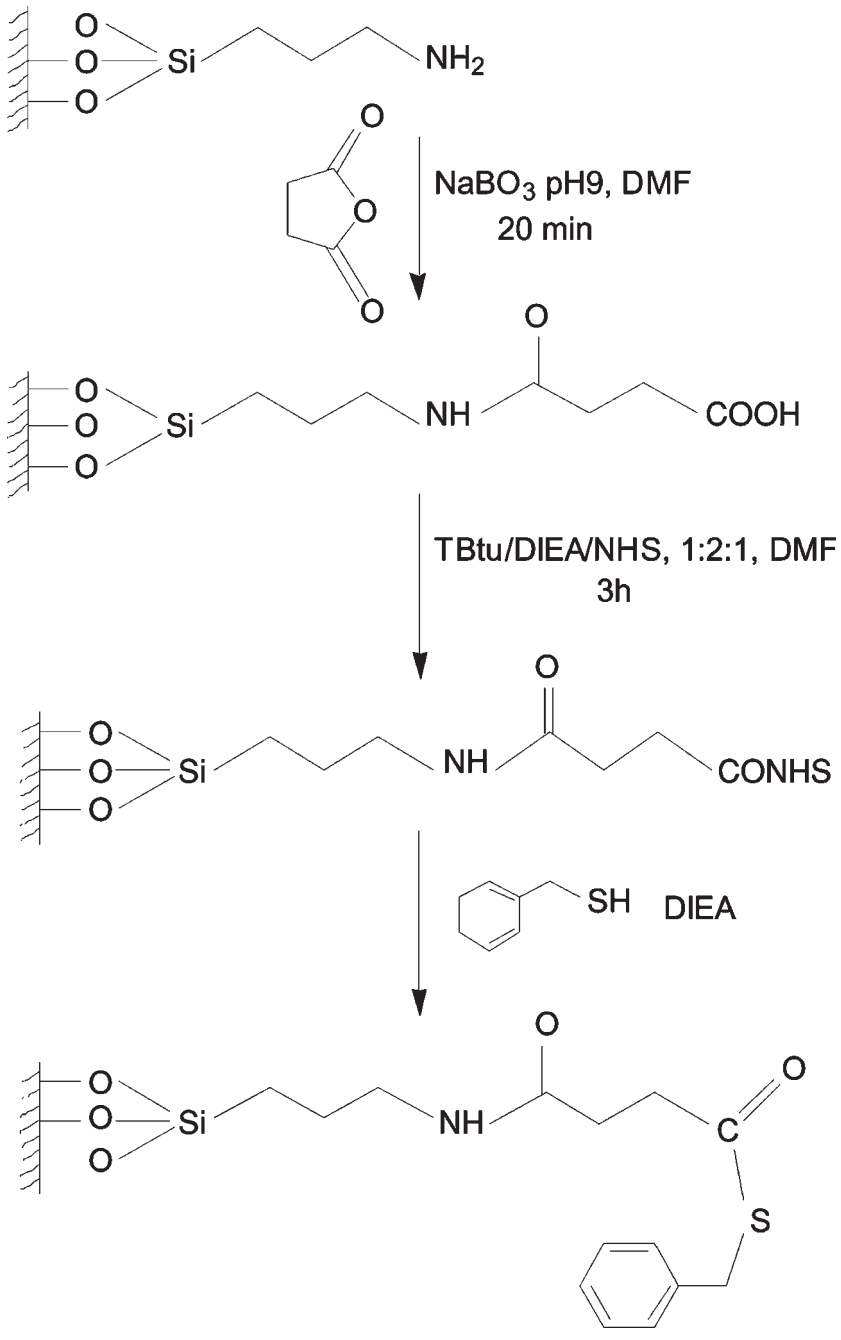

Fig. 3. Reaction schematic for generating thioester-functionalized slides.

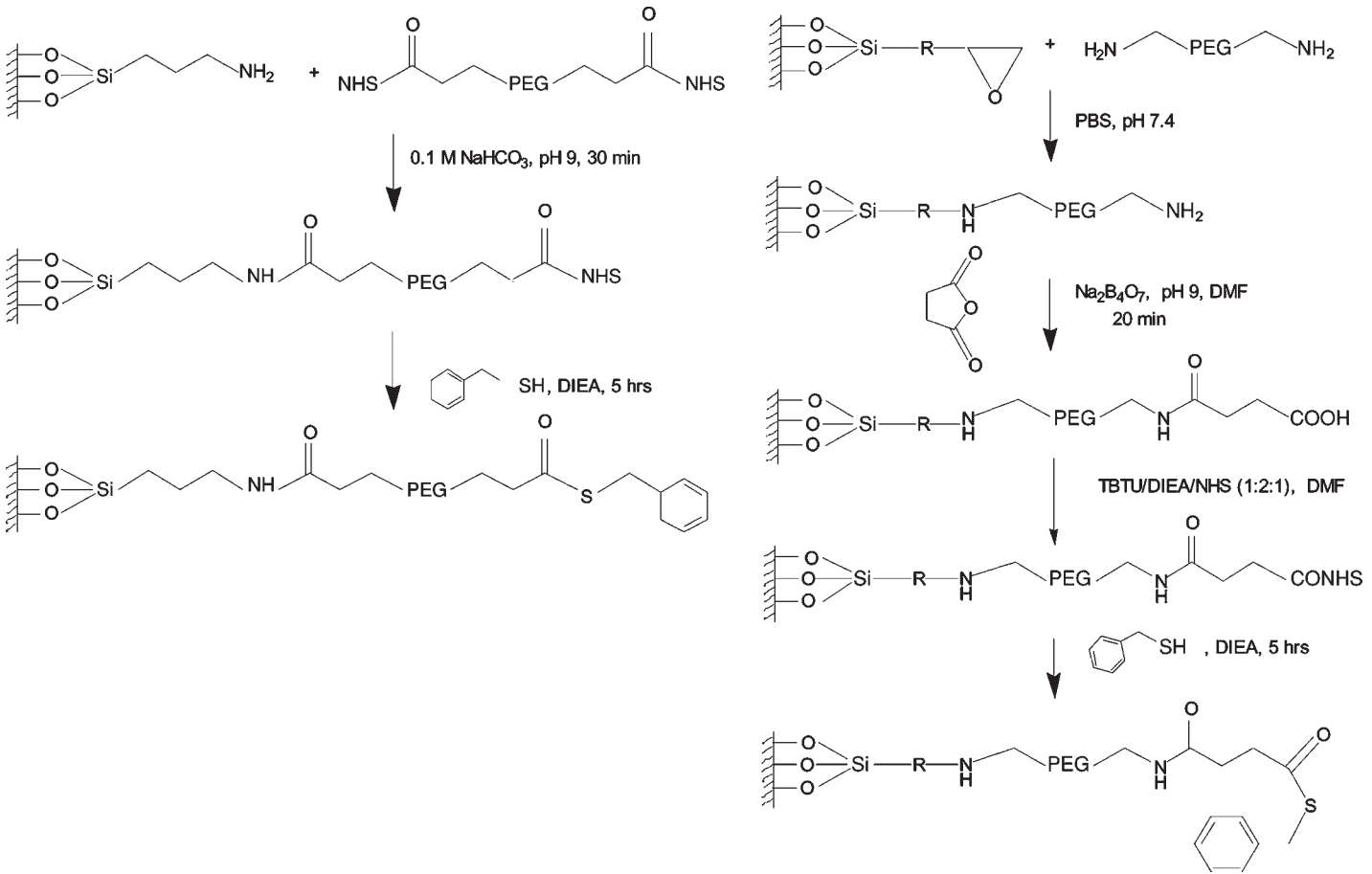

Fig. 4. Synthesis of PEGylated thioester slides.

3.4. Application of Kinase

1. Depending on kinase activity, the duration of incubation may be adjusted to optimize the reaction time required. For p60c-src and PKA, the authors applied the kinases to the slides for 5–6 h to obtain good fluorescence before detecting with the antibodies.
2. Conditions that have been optimized for PKA kinase reaction mix are the following: 2 U PKA (Upstate Biotechnology, Lake Placid, NY) in 25 m*M* Tris-HCl (pH 7.4), 15 m*M* magnesium chloride, 1 m*M* dithiothreitol, 2 m*M* EGTA, 100 μ*M* ATP.
3. Conditions that have been optimized for p60c-src activity on slides are the following: 2 U p60c-src (Upstate Biotechnology, Lake Placid, NY) in 25 m*M* pH 7.4, 35 m*M* magnesium chloride, 7 m*M* manganese chloride, 0.5 m*M* EGTA, 100 μ*M* ATP.
4. Apply 50 μL of the above kinase preparations using the cover-slip method (*see* **Note 5b**).

3.5. Application of Antibodies

Antiphosphoamino acid antibodies are commercially available and may be applied at concentrations as low as 1 μg/mL.

1. If necessary, label the antibody with fluorescent dye according to manufacturer's protocols. The dyes typically used for labeling include Cyanine 3 (Cy3), Cyanine 5 (Cy5) and fluorescein. Purify the labeled antibody with a NAP-5 column (Amersham, UK), and elute the antibody in PBS with 0.1% BSA.
2. Prepare the antibody in 50 μL PBS containing 1% BSA. Apply the solution to the slides using the cover-slip method as detailed in **Note 5b**. The hybridization step may be performed for 1–2 h, as necessary.
3. Wash away the unbound antibody with PBS and H_2O.
4. The slide may need to be washed with PBS (with 1% Tween-20) to remove nonspecifically bound antibodies in multiple washes, with each wash lasting 15 min.
5. Rinse slides with dH_2O and dry before scanning.

3.6. Scanning of the Slides

The slides are scanned on an ArrayWoRx™ scanner (Applied Precision, Issaquah, WA), under the designated fluorescence filters. An example of the typical results obtained is displayed in **Fig. 5**. The two nonphosphorylated CGG-containing substrates of p60 and PKA were spotted on thioester-functionalized slides, incubated with their respective kinases, and detected successfully with their corresponding fluorescently labeled antibodies. It is also possible to obtain quantitative data from microarray experiments (*see* **Note 7**).

4. Notes

1. The authors have already synthesized various Ala-Scan libraries and deletion libraries of peptides activated with N-terminal cysteines and have found them particularly useful in assessing kinase activity in parallel on an array for an assortment of kinase substrates (unpublished data).
2. The synthesis may be performed using a Pioneer™ Automatic Peptide Synthesizer (Applied Biosystems, Foster City, CA), or should multiple peptide sequences or libraries be required, fritted 96-well reaction systems (FlexChem® Organic Synthesis System) may be employed. For the latter, use rotating shakers for all incubation steps to allow for even mixing and efficient coupling of the amino acids onto the resin. Apply vacuum to remove

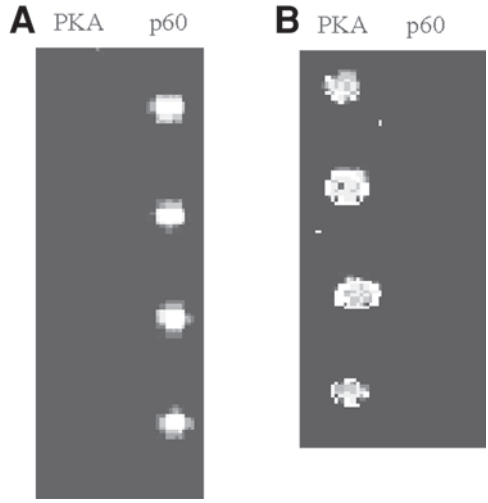

Fig. 5. Detection of kinase activity with FITC-labeled antibodies. Peptide substrates for PKA (*left column*) and p60 (*right column*) were spotted and phosphorylated with the corresponding kinases—p60 kinase in slide (**A**) and PKA kinase in slide (**B**) and probed with (**A**) FITC-labeled antiphosphotyrosine, and (**B**) FITC-labeled antiphosphoserine.

expended solvents and chemicals.

3. It is important to note that the choice of cleavage cocktail and duration of cleavage is dependent on both the type of resin used and the orthogonal protecting groups employed. For example, arginine residues protected with Mtr require 2 h of additional incubation with reagent R for every such residue there is within the peptide sequence—up to a maximum incubation time of 8 h. On the other hand, tryptophan residues are sensitive to reagent R and may be alkylated if exposed to scavengers for extended durations, and may result in peptide reattachment to the resin ***(11)***.
4. Some peptides are not readily soluble in H_2O, depending on the peptide length and degree of hydrophobicity. It then becomes necessary to first dissolve the peptide in small quantities of volatile solvents (methanol is an excellent candidate), and then dilute at least 10-fold in H_2O before the entire mixture is frozen. If a precipitate forms during the process of dissolving the peptide, repeat the process with an increased proportion of the organic solvent, if the peptide cannot be completely dissolved, or make a more dilute preparation using lower concentrations of peptides.

5a. When handling the slides, care must be taken to ensure that the slides are kept clean at all times, and that nothing comes into contact with the spotting surface. Dust especially may result in extraneous fluorescence and may affect the fluorescence readout when the slide is scanned. It is thus best to blow clean the slide under a stream of nitrogen before scanning. Also, gloves, if used, should be of the powder-free variety to ensure that the slides remain uncontaminated even after handling.

5b. If there is sufficient reagent, it may be convenient to react both surfaces of the slides by placing them on slide racks in deep well dishes. However, for expensive reagents, where it is preferable to use a conservative volume of the chemical, cover slips may be used. For

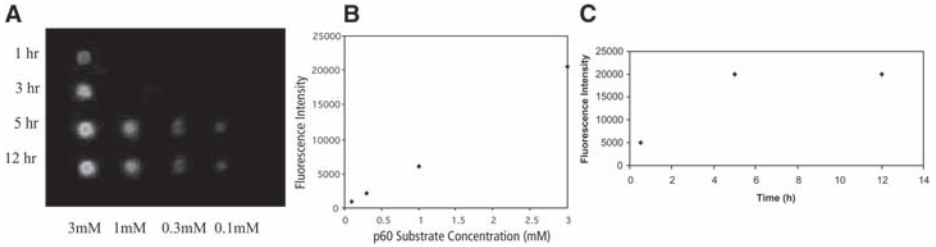

Fig. 6. Antibody-based fluorescence measurement of kinase activity. (**A**) Decreasing concentrations (3 m*M*, 1 m*M*, 0.3 m*M*, and 0.1 m*M*) of GGC p60 substrate in PBS, pH 7.4, were arrayed on thioester slides and incubated with the p60 kinase for increasing periods (1, 5, and 12 h). The slides were probed with FITC-labeled antiphosphotyrosine. (**B**) The fluorescence intensity was directly proportional to the phosphorylated substrate concentration, and the concentration-dependent kinase activity can be determined rapidly using FITC-labeled antibody. (**C**) The time-dependent kinase activity can also be determined by direct measurement of the fluorescence intensity.

a 22 × 60-mm cover slip, a 50 µL preparation is sufficient to allow for confluent coverage. Two methods may be used to apply the reagent on the surface. Either the reaction mix is first applied to the slide, and the cover slip is applied, or it could be applied to the cover slip, and the slide may be inverted on it. Both methods work equally well, but one ought to use the method that allows one to produce a uniform spread of the reagent across the slide surface, without introducing any bubbles or voids between the cover slip and the slide (where the reagent does not come in contact with the slide surface). Place the slides in a humid enclosed environment or use slide hybridization chambers for incubation. Cover slips may be slid off the slides after the reaction is complete, or be removed by vigorously shaking the slide within a water (or solvent) bath, until the cover slip slowly comes off.

6. APTES may stain glass surfaces white under prolonged exposure. It is thus advisable to wash away the APTES solution in the glass dish immediately after the functionalization step.
7. To facilitate real time detection of kinase activity, peptides immobilized on the array may be incubated with kinases for variable durations. Decreasing concentrations of p60 substrate (ranging from 0.1–3 m*M*) were arrayed on thioester glass slides and incubated with p60 kinase for increasing periods of time (1, 5, 12 h). The slides were incubated with the labeled antiphosphotyrosine antibody and scanned, with the fluorescence intensity of each spot quantitated and plotted graphically. The authors' results show a linear correlation of intensity of fluorescence with concentration of peptide immobilized (*see* **Fig. 6A**,**B**). This verifies the concentration-dependent kinase activity may be achieved on arrays. Furthermore, as expected, the increased incubation time quantifiably increased the fluorescence intensity detected. A kinetic plot was obtained that correlated well to solution phase-based assays, demonstrating the utility of microarrays in providing measurements of enzymatic activities (*see* **Fig. 6C**).

Acknowledgments

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18

Simultaneous Monitoring of Multiple Kinase Activities by SELDI-TOF Mass Spectrometry

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Summary

Cellular response to the external environment is often controlled by one or more protein kinases. We report a methodology for simultaneously monitoring multiple kinase activities across multiple signal-transduction pathways using ProteinChip® Array technology. Based on the addition of specific peptide reporters, kinase activity is detected by the presence of a mass shift of 80 Da (or multiple thereof) corresponding to the addition of one or more phosphate groups. These phosphorylated peptide substrates are then enriched using an immobilized metal affinity capture (IMAC)-Ga array and detected directly by surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF MS). SELDI-TOF MS is sensitive, tagless (nonradioactive, nonfluorescent), can be easily multiplexed for the analysis of several different kinases in a single reaction mixture (limited only by the specificity of the kinase for its substrate peptides), and is directly scalable through the use of robotic sample processing. By multiplexing kinase assays, one can dramatically increase the amount of information obtained from rare or volume-limited samples. More important, results reflect closely the complex interrelationships between kinases and show high correlation with in vivo assays.

Key Words:

SELDI-TOF MS; ProteinChip® Arrays; IMAC-Ga Array; phosphorylation; multiplexed kinase assay.

1. Introduction

Protein phosphorylation represents one of the most ubiquitous forms of posttranslational modification and is reflected in as many as one-third of eukaryotic gene products ***(1)***. Such modification can change the stability and/or function of a protein in numerous ways—for example, by increasing or decreasing its biological activity, by stabilizing or marking the protein for destruction, by facilitating or inhibiting move-

*These authors contributed equally to this manuscript.

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ment between subcellular compartments, or by initiating or disrupting protein–protein/DNA interactions. Common methods used to assess kinase activity employ radioactive or colorimetric/fluorescence tags that are incorporated into the phosphorylation site via the specific kinase activity (e.g., ^{32}P adenosine triphosphate [ATP]), or the use of antibody reagents raised against specific phosphothreonine, serine, or tyrosine residues/motifs. Although the use of these methods has been widely demonstrated in the literature, they either require specialized reagents (e.g, specific antiphospho antibodies) or are not suitable for detection of multiple kinase activities simultaneously.

A mass spectrometry method has been developed that allows for the determination of multiple kinase activities through the use of reporter peptide substrates. Following phosphorylation of the peptide substrate by its specific kinase, the substrates are captured and enriched from the sample using an IMAC-Ga protein biochip ***(2,3)***. Then, these biochips can be analyzed directly by SELDI-TOF MS. By using peptide substrates with different mass signatures, multiple kinase assays can be carried out simultaneously from a single sample.

2. Materials

2.1. Peptide Substrate Mix

1. Peptide KRP**pS**QRHGSKY, MW 1422.5 Da, (1 pmol/5 μL; Anaspec, San Jose, CA).
2. Peptide KRP**S**QRHGSKY, MW 1342.5 Da, (10 pmol/5 μL; Anaspec).
3. Peptide TRDIYETDY**pY**RK, MW 1702.8 Da, (1 pmol/5 μL; Anaspec).
4. Peptide TRDIYETDY**Y**RK, MW 1622.8 Da, (10 pmol/5 μL; Anaspec).
5. Peptide KRELVEPL**pT**PSGEAPNQALLR, MW 2398.7 Da, (3 pmol/5 μL; American Peptide, Sunnyvale, CA).
6. Peptide KRELVEPL**T**PSGEAPNQALLR, MW 2318.7 Da, (30 pmol/5 μL; American Peptide).

2.2. Enzymes and Their Specific Substrates (Upstate Biotechnology, Lake Placid, NY)

1. Protein kinase C (PKC)α kinase and its peptide substrate (QKRPSQRSKYL, MW 1390.6 Da, 500 μ*M*).
2. PKCα peptide inhibitor (RFARKGALRQKNV).
3. Akt1 and its substrate Crosstide (GRPRTSSFAEG, MW 1163.6 Da, 2 mg/mL).
4. Mitogen-activated protein kinase (MAPK) and its peptide substrate (APRTPGGRR, MW 967.2 Da, 2 mg/mL).
5. Protein kinase A (PKA) and its peptide substrate (GRTGRRNSI, MW 1016 Da, 2 mg/mL).
6. GSK3β kinase and its peptide substrate (YRRAAVPPSPSLSRHSSPHQS[p]EDEEE, MW 3029.1 Da, 0.76 mg/mL).
7. Lipid activator (*see* **Note 1**).

2.3. Buffers and Lysates

1. Kinase assay buffer A (5X): 100 m*M* major oocyst proteins, (pH 7.2), 125 m*M* β-glycerolphosphate, 5 m*M* sodium orthovanadate, 5 m*M* dithiothreitol, 5 m*M* $CaCl_2$.
2. Kinase reaction buffer B (25X ATP solution): 5 m*M* ATP and 1 *M* $MgCl_2$ in H_2O.
3. Chelating buffer C: 40 m*M* ethylene glycol *bis* (2-aminoethyl ether)-*N*,*N*,*N*'*N*'-tetraacetic acid (EGTA).

4. IMAC buffer D: 50 m*M* sodium acetate (pH 5.3), 1 *M* NaCl or 1 *M* ammonium acetate (as specified in results), 0.1% *n*-octyl β-D-glucopyranoside (OGP) and 2.5 m*M* sodium malate.
5. Lysis buffer E: 20 m*M* Tris-HCl (pH 7.5), 150 m*M* NaCl, 1 m*M* ethylenediaminetetraacetic acid (EDTA), 1 m*M* EGTA, 1% NP40, 2.5 m*M* sodium pyrophosphate, 50 m*M* β-glycerolphosphate, 1 m*M* sodium vanadate, 1 μ*M* microcystin LR, 0.1% β-mercaptoethanol, EDTA-free protease inhibitor cocktail (Roche).
6. Gallium nitrate: 50 m*M* solution.
7. Primary cortical neuronal (PCN) lysates: After culturing, cells are washed once with ice-cold phosphate-buffered saline. The washing buffer is then completely aspirated and the cells are lysed for 30 min with the addition of ice-cold lysis buffer E. After lysis, cells are scraped, transferred to microfuge tubes, and centrifuged at 23,000*g* for 20 min at 40°C. The supernatant is collected and used for multiplex kinase assays.

2.4. SELDI-TOF MS and Reagents

1. ProteinChip® Biomarker system (Ciphergen Biosystems, Fremont, CA) with integrated Biomek 2000 robotic workstation (Beckman Coulter).
2. IMAC-3, IMAC-30, and IMAC-40 and NP20 (normal phase) ProteinChip Arrays (Ciphergen Biosystems).
3. Bioprocessor (96-spot and 192-spot formats; Ciphergen Biosystems).
4. α-cyano-4-hydroxycinnamic acid (CHCA) prepared as a 20% saturated solution in 50% acetonitrile, 0.1% trifluoroacetic acid (Ciphergen Biosystems).

3. Methods

3.1. Enrichment of Phosphopeptide Substrates Using IMAC-Ga ProteinChip® Arrays

3.1.1. Preparation of NP20 Arrays

1. Add 1 μL of the peptide mixture to a NP20 ProteinChip Array.
2. Air-dry the sample (*see* **Note 2**), and then wash the spot once with 5 μL of deionized H_2O to remove all buffer salts. Air-dry again.
3. Add 0.8 μL of 20% CHCA to each spot and allow solvent to evaporate.
4. Read in the ProteinChip Reader.

3.1.2. Preparation of IMAC Arrays and Sample Binding

1. Add 5 μL of 50 m*M* gallium nitrate to spots on the IMAC array.
2. Place the array in a humidity chamber (*see* **Note 3**) and incubate for 5 min on a horizontal shaker with a low setting.
3. Remove array from the humidity box and aspirate the liquid from each spot, then repeat **steps 1–3** once more.
4. Add 5 μL IMAC buffer D to each spot and incubate 5 min on a horizontal shaker and in the humidity box. After incubation, aspirate the liquid from each spot.
5. Add 5 μL IMAC buffer D to each spot, and incubate 1 h on a horizontal shaker in the humidity chamber, then aspirate the liquid from each spot.
6. Add up to 10 μL of the peptide mixture containing phosphorylated and unphosphorylated peptides directly on the array spot, or add 25 μL of peptide mixture on each spot when using a bioprocessor (e.g., in **Subheadings 3.2.1.** and **3.2.2.**).

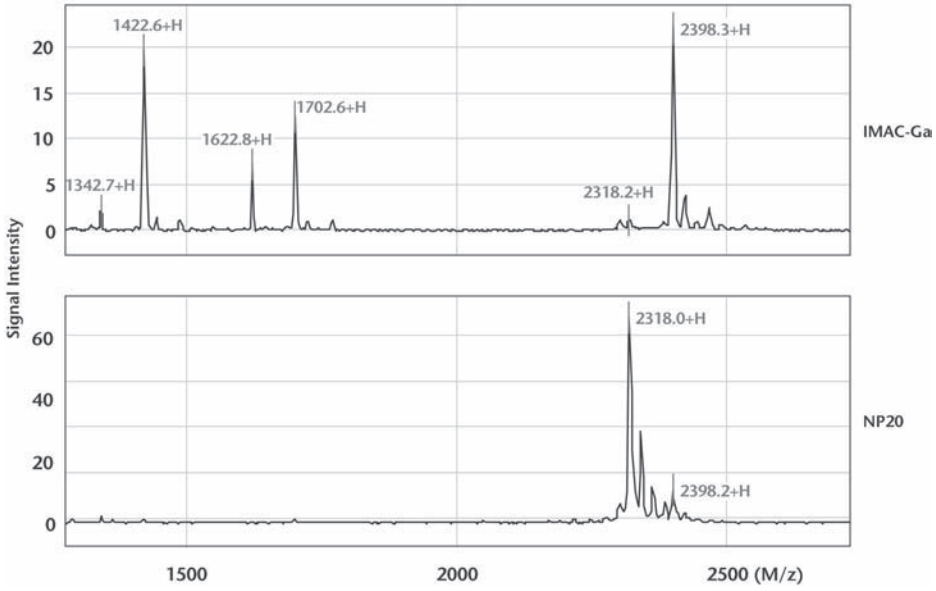

Fig. 1. Comparison of the enrichment of phosphopeptides using either NP20 (normal phase) or IMAC-Ga ProteinChip Arrays.

7. Place the arrays in a humid chamber or in the bioprocessor on a horizontal shaker and incubate for 30 min. After 30 min incubation, aspirate the liquid from each spot.
8. Wash each spot with 5 μL of IMAC buffer D, or each well in the bioprocessor with 50 μL of IMAC buffer D. Wash by adding the appropriate volume of buffer, then placing the array in a humid chamber or in the bioprocessor on a horizontal shaker and wash for 5 min.
9. Aspirate the liquid from each spot.
10. Repeat **steps 8** and **9** twice for a total of three washes.
11. Remove the arrays from the bioprocessor, rinse with deionized H_2O, and then allow spot to dry.
12. Add 0.8 μL of 20% CHCA to each spot and allow solvent to evaporate.
13. Read on the mass spectrometer. A typical result is depicted in **Fig. 1**.

3.2. Generation of Kinase Activity Standard Curve

A standard curve for protein kinase Cα (PKCα) activity is generated to assess reproducibility and quantitation. Kinase reactions are processed in a 96-well format using the Biomek 2000 robotic platform. Each concentration of enzyme is tested in quadruplets.

3.2.1. Generation of PKCα Activity Standard Curve

1. In a microfuge tube, mix 100 μL of PKC substrate, 40 μL of kinase reaction buffer B, 200 μL of lipid activator (*see* **Note 1**), 200 μL of kinase assay buffer A, and 260 μL of deionized H_2O to make kinase reaction mix. Place on ice (*see* **Note 4**).

2. Dispense 20 µL of the kinase reaction mix to each well of the first four columns of a 96-well V-bottom plate (A1-H4). Keep the plate at room temperature (*see* **Note 4**).
3. Prepare a serial dilution of PKCα in eight different tubes (or in one column of a 96-well plate) so that the final concentration of the kinase in each tube is (in sequence) 20, 6, 2, 0.6, 0.2, 0.06, 0.02, and 0 ng/µL. Dilute the kinase in 1X kinase assay buffer A. Make sure total volume in each tube exceeds 25 µL. Keep the kinase dilution series on ice (*see* **Note 4**).
4. The reaction is done at ambient temperature. Set a timer for 6 min. Start the reactions by adding 5 µL of diluted kinase to each well at 5-s intervals between each well. For example, add 5 µL of 0 ng/µL PKCα to wells A1, A2, A3, and A4, 5 µL of 0.02 ng/µL to wells B1, B2, B3, and B4, and so forth.
5. At the end of 6 min, place the 96-well microfuge plate on ice, and stop the reaction by adding 100 µL of IMAC buffer D to each well, again at 5-s intervals, starting at well A1 to H4.
6. Transfer 25 µL of the above reaction mix to IMAC-40 arrays and process according to **Subheading 3.1.2.** A typical result is depicted in **Fig. 2**.

3.2.2. Generation of PKCα Inhibition Curve

1. In a microfuge tube, mix 100 µL of PKC substrate, 200 µL of lipid activator (*see* **Note 1**), 200 µL of kinase buffer A, 120 ng of PKCα (3 ng/assay point) and 100 µL of deionized H_2O to make kinase reaction mix. In a separate tube, dilute 40 µL of reaction buffer B (ATP/$MgCl_2$) 1:5 with 160 µL of deionized H_2O. Keep on ice (*see* **Note 4**).
2. Dispense 15 µL of the kinase reaction mix to each well of the first four columns of a 96-well V-bottom plate (A1-G4). Keep the plate at ambient temperature.
3. Prepare serial dilutions of PKCα inhibitor in eight different tubes (or in one column of a 96-well plate) so that the final concentration of the inhibitor in each tube is (in sequence) 100,10, 1, 0.1, 0.01, 0.001 and 0 µ*M*. Dilute the inhibitor in 1X kinase assay buffer A. Make sure the total volume in each tube exceeds 25 µL.
4. Add 5 µL of the previously diluted PKC inhibitor to each well in the plate. For example, add 0 µ*M* to wells A1, A2, A3, and A4, 5 µL of 0.001 µ*M* to wells B1, B2, B3, and B4, and so forth.
5. The reaction is done at ambient temperature. Set a timer for 6 min. Start the reaction by adding 5 µL of reaction buffer B (diluted 1:5 in **Subheading 3.2.2, step 1**) to each well at 5-s intervals between each well.
6. At the end of 6 min, place the 96-well microfuge plate on ice, and stop the reaction by adding 100 µL of IMAC buffer D to each well, again at 5-s intervals, starting at wells A1 to H4.
7. Transfer 25 µL of the above reaction mix to IMAC-40 arrays and process according to **Subheading 3.1.2.** A typical result is depicted in **Fig. 2**.

3.3. Multiplexed Kinase Assays

Multiplexed kinase assays can be performed by mixing multiple kinases and their corresponding substrates in a single reaction tube. After a suitable incubation time, phosphorylated products can be transferred to IMAC-Ga arrays for subsequent capture and detection. When this approach is taken, it is essential that substrate cross-phosphorylation studies be carried out across numerous kinases (*see* **Fig. 3**). Multiplex kinase assays using SELDI can be performed by two different methods: (a) Tradi-

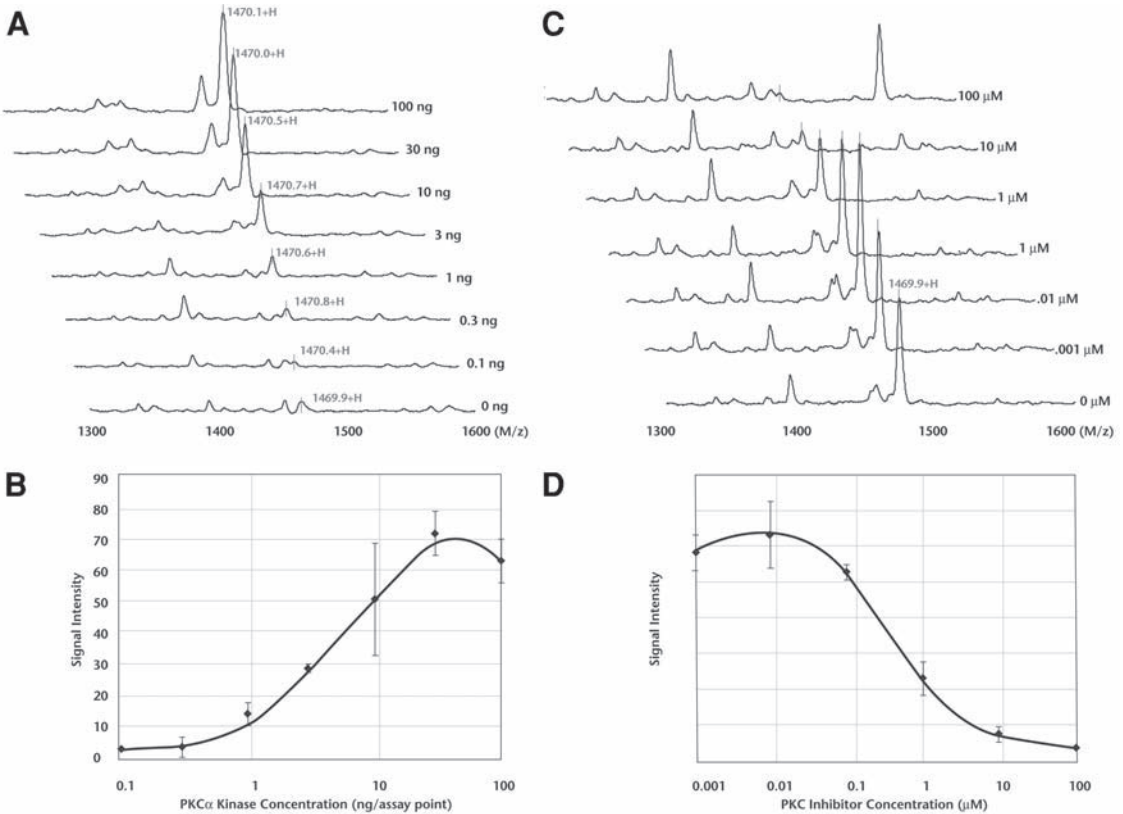

Fig. 2. Generation of standard curves: **A** shows representative spectra of phosphorylated product (1469.5 Da) in a dose-dependent manner. **B** summarizes the data by plotting PKCα concentration (in log scale) against phosphorylated peptide peak intensity (normalized against total ion current). **C** shows representative spectra of decreasing amount of phosphorylated product with increasing amount of the inhibitor. **D** shows concentration-dependent inhibition of PKCα activity by this inhibitor. Half-maximal inhibitory dose (IC_{50}) was calculated as 0.3 µ*M*, in agreement with published data of 0.2. µ*M* ***(4)***.

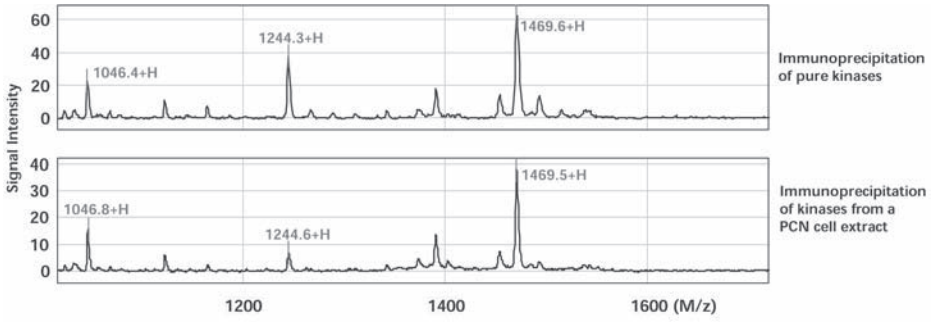

Fig. 3. Validation of substrate specificity of AKT, MAPK, and PKC kinase activity with pure kinases. Individual kinases were incubated with substrates for all three kinases and ATP. AKT, MAPK, and PKC phosphorylated only their specific substrates and did not cross-phosphorylate substrates of the other two kinases. But when the three kinases were incubated with the three substrates in a single reaction, then all the substrates were phosphorylated.

tional immunocapture of kinases by antibodies followed by kinase activity (immunoprecipitation followed by kinase activity [IPK]); (b) directly assay kinase activity in the lysate by adding specific substrate to kinases (in-solution kinase assay) and monitoring the product formation using IMAC-Ga arrays. Both methods are described in this section.

3.3.1. Protocol for Immunoprecipitation Followed by Kinase Activity of Multiple Kinases in a Single Assay (IPK)

To maximize recovery of kinase activity, perform all steps at 40°C, or on ice, as indicated in the following protocol.

1. To 300 µL cell/tissue extracts (approx 1 mg) or a mixture of purified kinases (e.g., 2 µL MAPK, 0.5 µL PKC, and 2 µL AKT), add a volume of antikinase antibodies, such as 5 µL of anti-MAPK, 2 µL of anti-PKC, and 2 µL of anti-AKT.
2. Incubate the cell/tissue extract and antibodies overnight at 4°C with gentle rotation.
3. To the cell/tissue extract-antibody preparation add 10 µL of packed protein-A and 20 µL of packed protein-G beads.
4. Incubate for 1 h at 4°C on a rotator mixer to thoroughly mix the components during the incubation.
5. Pellet the agarose beads at 8600*g* for 45 s.
6. Remove the supernatant fraction. Wash the beads twice with lysis buffer E and once with 1X kinase assay buffer A.
7. Prepare a solution containing 3 µL of MAPK substrate, 1.5 µL of AKT substrate, 5 µL PKC substrate, 5 µL of lipid activator (*see* **Note 1**), 1 µL of kinase reaction buffer B, 5 µL of kinase assay buffer A (5X). Add this to the beads prepared in **step 6**.
8. Mix well, then immediately remove a 5-µL aliquot, and add this aliquot to 20 µL IMAC buffer D. This will stop the reaction and the sample will act as a 0-min time-point.
9. Incubate the remaining solution from **step 7** for 20 min at 30°C with constant shaking (*see* **Note 5**).

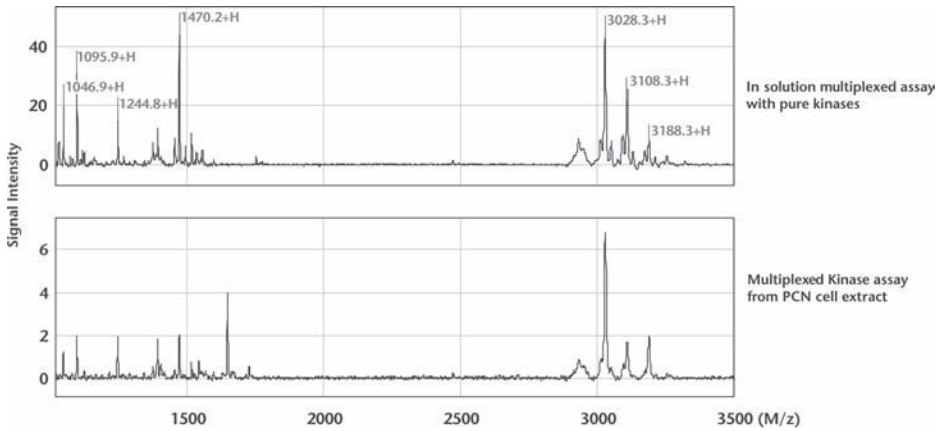

Fig. 4. Multiplex kinase assay with immunoprecipitated pure kinases or kinases immunoprecipitated from PCN cells.

10. After 20 min take a 5-μL aliquot, and add this aliquot to 20 μL IMAC buffer D. This will stop the reaction, and the sample will act as a 20-min time-point for Ca^{2+}- and Mg^{2+}-dependent kinase activity.
11. To the remaining reaction mix from **step 10** add 1.5 μL of chelating buffer C. Continue the reaction for another 30 min.
12. After 30 min, take a final 5-μL aliquot, and add this aliquot to 20 μL IMAC buffer D. This will stop the reaction, and the sample will act as a 30-min time-point for Ca^{2+}- and Mg^{2+}-sensitive kinase activity.
13. For each of the three 5-μL samples taken in **steps 8**, **10**, and **12**, process the sample on an IMAC-3 array using the method described in **Subheading 3.1.2.** A typical result is depicted in **Fig. 4**.

3.3.2. Protocol for In-Solution Multiplex Kinase Assays

To maximize recovery of kinase activity, perform all steps at 40°C or on ice, as indicated in the following protocol.

1. To 7 μL cell/tissue extracts (approx 14 μg) or a mixture of purified kinases (e.g., 2 μL MAPK, 0.1 μL PKCα, 0.5 μL PKA, 0.1 μL GSK3β, and 1 μL AKT), add a mixture of 3 μL of MAPK substrate, 1.5 μL of AKT substrate, 1 μL of PKA substrate, 1 μL of GSK3β substrate, 5 μL of PKC substrate, 5 μL of lipid activator (*see* **Note 1**), 1 μL of buffer B, 5 μL of 5X buffer A.
2. Mix well. Take 5 μL for IMAC-3 array, and add it to 20 μL buffer D (addition of buffer D terminates the reaction; good for five repeats).
3. Incubate for 20 min in a 30°C shaking incubator.
4. Take 5 μL for IMAC-3 array and add it to 20 μL buffer D.
5. To the remaining reaction mix add 1.5 μL of buffer C. Continue the reaction for another 30 min.
6. Take a 5-μL aliquot for IMAC-3 array at 1 hr, and add it to 20 μL buffer D.

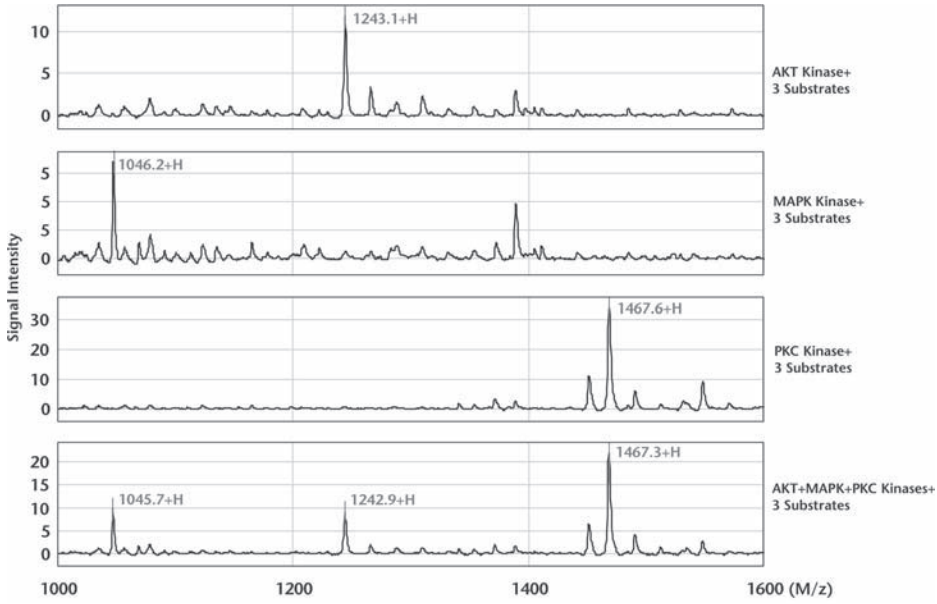

Fig. 5. Designing in-solution multiplexed kinase assays. Assay optimization of multiplexed assays using pure kinases in solution and demonstration of in-solution multiplexed assays using PCN cell extracts.

7. Process sample on IMAC-3 array similar to the method in **Subheading 3.1.2.** A typical result is depicted in **Fig. 5**.

4. Notes

1. Sonicate lipid activator on ice for at least 1 min before use.
2. Arrays can be air-dried or placed on a 37°C warm plate to accelerate drying.
3. A humidity box is used to prevent excess evaporation from the liquid droplet placed on the array spots. A humidity box can be made from an empty pipet tip box. Remove the plastic insert used to hold the tips. Fill the bottom of the box with dH_2O. Replace the plastic insert used to hold the tips. Place a wet Kimwipe on top of the insert. This plastic insert is then used as the platform to hold the arrays.
4. Keep reagents, kinases, and reaction mixes on ice all the time unless otherwise noted.
5. Assay mixture must be thoroughly mixed throughout the reaction time to ensure that the substrate and the enzyme immunocomplex interact maximally.

Acknowledgments

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19

Cytokine Protein Arrays

Ruo-Pan Huang

Summary

Cytokines play important roles in many aspects of cell physiology and pathology. The simultaneous determination of multiple cytokine-expression levels is receiving much attention in the research community. Multiple cytokine-expression levels can be simultaneously determined using enzyme-linked immunosorbent assay (ELISA)-based protein array technology. In this approach, target proteins are captured by the arrayed capture antibody and then detected in a sandwich ELISA format using a cocktail of biotinylated detection antibodies. The signals are visualized by either horseradish peroxidase (HRP)-conjugated streptavidin and enhanced chemiluminescence, or cy3-conjugated streptavidin and laser scanner. Several key factors and steps are described, including selection of solid supports, selection of suitable antibodies, determination of specificity and sensitivity of cytokine protein arrays, array design, sample preparation, and detailed experimental procedures for macroarray and microarray formats. An account of the successful development and application of cytokine protein arrays is presented.

Key Words:

Protein array; protein chip; antibody array; cytokine; chemokine; growth factor; secreted protein; ELISA.

1. Introduction

Cytokines are broadly defined as a group of proteins that act as mediators of cell communication. They play important roles in a wide range of physiological processes, such as cell growth, differentiation, apoptosis, wound healing, and homeostasis ***(1–3)***. Frequently, cytokine expression is coordinately regulated because multiple cytokines often share the same upstream signal pathway, and one cytokine can regulate other cytokine-expression levels. Deregulation of cytokine expression is often associated with disease status, particularly cancer, cardiac diseases, and arthritis ***(4–7)***. Therefore, simultaneous detection of multiple cytokines will undoubtedly provide a powerful tool in biomedical discovery. For instance, by profiling cytokine expression in certain types of diseases, potential molecular targets involved in those disease pro-

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cesses may be identified. By profiling cytokine expression in response to drugs, the efficacy of medicines can be monitored. New biomarkers may be discovered as a result of profiling cytokine-expression levels in patient specimens. Finally, by profiling cytokine-expression levels in cells treated with certain stimulators or transfected with a given gene, the molecular mechanisms involved in a particular stimulator or gene can be determined.

One approach to measuring multiple cytokine-expression levels is to use cytokine protein arrays. Several cytokine array technologies have been developed ***(8–12)***. The development and application of human cytokine arrays in membrane-based and HydroGel-based formats is discussed to illustrate a method used for cytokine protein array establishment.

2. Materials

1. Pairs of antibodies against cytokines.
2. HRP-conjugated streptavidin (BD PharMingen, San Diego, CA).
3. Substrates, including Hybond enhanced chemiluminescence (ECL) membrane (Amersham Corp., UK), other types of membranes and HydroGel slides (PerkinElmer Life Science, Meriden, CT).
4. Blocking buffer: 5% bovine serum albumin (BSA)/1X tris-buffered saline (TBS) (BSA: Roche Molecular Biochemistry).
5. TBS/0.1% Tween-20: 0.01 *M* Tris-HCl, pH 7.6, 0.15 *M* NaCl, 0.1%Tween-20.
6. TBS: 0.01 *M* Tris-HCl, pH 7.6, 0.15 *M* NaCl.
7. ECL reagents A and B (Amersham Corp., Amersham, UK).
8. Radioactive immunoprecipitation assay (RIPA) buffer: 10 m*M* Tris-HCl, pH 7.5, 0.15 *M* NaCl, 1% sodium deoxycholate, 1% TX-100.
9. HRP-IgG.
10. Cy3-conjugated streptavidin (Rockland, Gilbertsville, PA).
11. Arrayer.
12. Laser scanner.
13. Kodak X-OMAT film and film processor or Chemiluminescence imaging system.
14. Orbital shaker.
15. Light box.
16. Small plastic boxes or containers or chamber for washing steps.
17. Frame and cover slip.
18. Other general laboratory equipment, such as a centrifuge.

3. Methods

3.1. Principle

The principle of all cytokine protein arrays so far developed is based on the sandwich ELISA as shown in **Fig. 1**. Pairs of antibodies, which recognize two different epitopes of the same antigen, are used. A group of antibodies are spotted onto membranes or slides, serving as a capture. Samples are then incubated with array membranes or array slides. Unbound materials are washed away. The array membranes or array slides are then incubated with a cocktail of biotinylated antibodies serving as a detector. To visualize the signals, HRP-conjugated streptavidin or cy3-or cy5-conjugated streptavidin is used. By comparing signal intensities between samples, the rela-

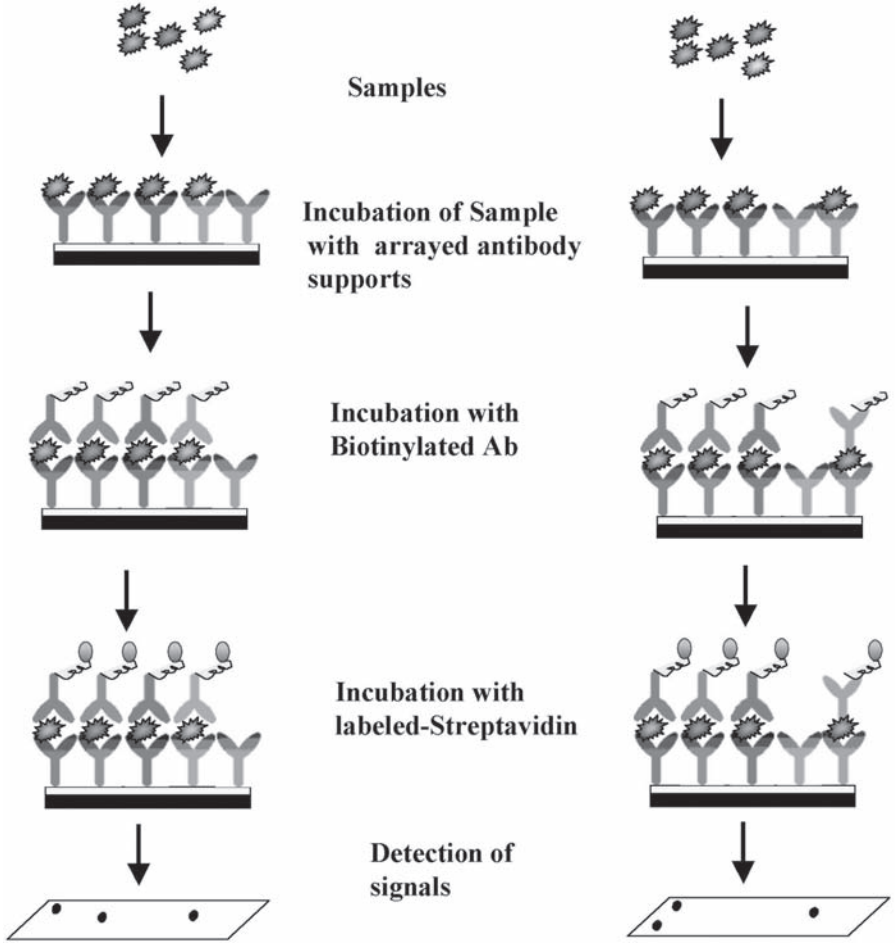

Fig. 1. Schematic representation of ELISA-based protein cytokine arrays. Capture antibodies against cytokines are spotted onto solid support. Arrayed supports are incubated with samples. After removing unspecific binding, a cocktail of biotin-conjugated antibodies is applied to detect bound cytokines. Signals are visualized with HRP-conjugated streptavidin in combination with ECL or fluorescence-labeled streptavidin coupled with a laser scanner.

tive expression levels of cytokines can be determined ***(10–12)***. By comparing signal intensities generated from standard cytokines, the exact expression levels of cytokines can be quantitatively measured ***(10,13,14)***.

3.2. Basic Steps

The prerequisites for cytokine protein arrays are array membranes or array slides and experimental samples. The following steps are fairly straightforward and relatively easy.

1. Block the membranes or slides with BSA for 30 min at room temperature.
2. Incubate array membranes or slides with samples at room temperature for 1–2 h.
3. Wash array membranes or slides with TBS/Tween-20 and TBS.
4. Incubate array membranes or slides with a cocktail of biotin-labeled second antibodies at room temperature for 1–2 h.
5. Wash array membranes or slides with TBS/Tween-20 and TBS.
6. Incubate array membranes with HRP-streptavidin or slides with cy3-streptavidin or cy5-streptavidin at room temperature for 30 min to 1 h.
7. Wash array membranes or slides with TBS/Tween-20 and TBS.
8. Visualize the signals.

3.3. Construction of Array Membranes or Array Chips

3.3.1. Selection of Suitable Solid Supports

Glass slides, membranes, and plates are three major supports currently being used for cytokine protein arrays. Glass slides are suitable for high throughput and have low background for fluorescence detection. The major problem with glass slides is their poor protein-binding capacity. Several glass slides, including HydroGel, aldehyde, aminosilane, S&S nitrocellulose, and salicylhydroxamic modified-surface slides (Versalinx™ Technology, Prolinx, Bothell, WA), are available ***(15)***. Among them, HydroGel slides are particularly suitable for protein arrays with high-binding capacity and low background ***(10,13)***. Such HydroGel slides can be used for a variety of sample sources, such as tissue-culture supernatant, cell lysate, tissue lysate, and body fluids ***(10)***. Different sophisticated surface chemistries for protein array applications are being developed in many companies and academic laboratories. It is still too early to conclude which substrates are best, but only the best one will survive in future practice. Membranes, on the other hand, have been widely used in Western blotting assays and have a high-binding capacity. Proteins can bind to membranes through noncovalent interactions with hydrophobic (nitrocellulose and polystyrene) or positively charged (poly-L-lysine and aminosilane) surfaces. Cost effectiveness is the major advantage of membranes. The entire procedure can be done in any laboratory without sophisticated equipment when membranes are used as the macroarray format. The author routinely uses membranes as the macroarray format for testing new concepts at the proof-of-principle stage and for developing new assays ***(10–12,16)***. Another support is tissue-culture plates, usually 96-well plates ***(17,18)***. The advantage of this format is that experiments can be performed using the existing automatic liquid handle system. However, it is difficult to create a high-density array system using 96-well plates.

To select the suitable membranes for cytokine arrays, several membranes can be used. The array membranes are generated as described in **Subheading 3.3.** Cytokine MCP-1 is used to test the procedure as described in **Subheading 3.5.** A typical result is shown in **Fig. 2**. Hybond ECL membrane from Amersham Corp A was selected for cytokine arrays because of low background, high sensitivity, and good absorption.

3.3.2. Array Design

Antibodies can be selected based on the following criteria: (a) ability to be used in ELISA assay or to specifically detect a single band in Western blot analysis; (b) avail-

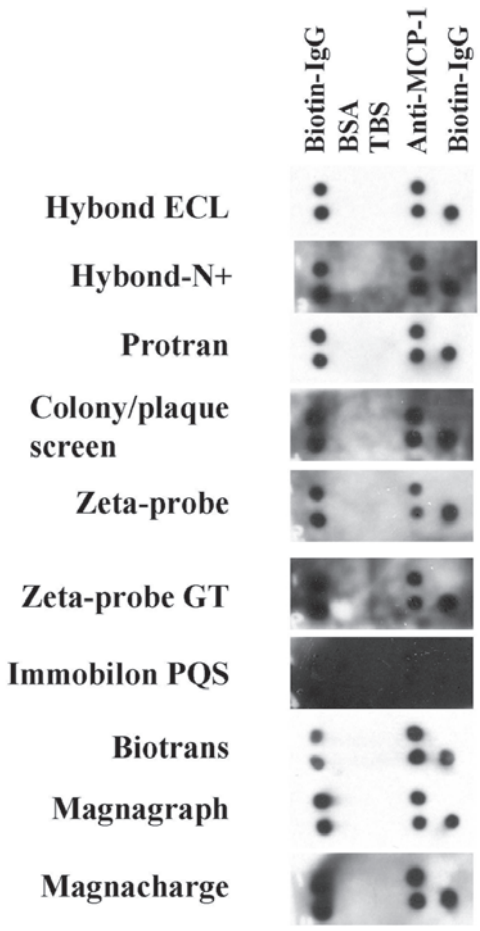

Fig. 2. Selection of suitable membranes for protein arrays. Different membranes were spotted with anti-MCP-1 antibody and other controls as indicated. The membranes were then incubated with MCP-1. After washing, membranes were incubated with biotin-labeled anti-MCP-1. HRP-conjugated streptavidin was used to indicate the presence of biotin-conjugated antibody. The signals were visualized with ECL.

ability of monoclonal antibodies to assure future supply; (c) availability of at least more than two antibodies against different epitopes, allowing one to select a pair of antibodies; and (d) availability of corresponding antigens so that the specificity and sensitivity of the arrays can be evaluated.

Positive controls can include one or all of the following: (a) HRP-conjugated bovine IgG for macroarrays and Cy3- and Cy5-conjugated bovine IgG for microarrays; (b) biotin-conjugated bovine IgG (BIgG), which serves to normalize streptavidin incubation efficiency; and (c) anti-β-galactosidase (a constant amount of β-galactosidase is included in sample), which serves to normalize the whole process.

Negative controls include BSA, TBS, and antiluciferase or other proteins whose sequences are not coded in mammalian cells.

To assure quality, each antibody should be spotted at least in duplicate.

3.3.3. Selection of Specificity of Antibody

To select the specificity of antibodies for cytokine arrays, array membranes spotted with all capture antibodies are incubated with a single recombinant cytokine (usually, 100 ng/mL) and a single biotin antibody against corresponding cytokine (usually, 10 ng/mL) as described in **Subheading 3.5.** At this stage, large amounts of cytokines are used to confirm the specificity. Based on the results, antibodies with high specificity are selected for generation of cytokine array membranes. **Figure 3** illustrates how to determine the specificity of arrays. If the data show crossreactivity, the particular pair of antibodies must be removed from the array.

3.3.4. Determination of Sensitivity of Arrays

Standard curves are generated using recombinant cytokines. This serves two major purposes: One is to determine the sensitivity of a particular cytokine and the other is to determine the detection dynamic range. The detection sensitivity is different for every cytokine. As a rule of thumb, 100 ng/mL to 1 pg/mL can be used for the test in 10-fold increments. The experiments are performed as described in **Subheadings 3.4.** and **3.5.** The intensities of signals are plotted against known amounts of cytokine, as shown in **Fig. 4**.

3.3.5. Array Membranes or Array Chips

Array membranes can be generated manually or using an arrayer. The arrayer from GeneMachine (San Carlos, CA) can be adjusted to spot antibodies onto membranes easily. For other arrayers, membranes must be adhered to glass slides. Manufacturers provide detailed instructions that explain how to print antibodies onto glass slides.

To manually spot antibodies onto membranes, a template is placed on top of the white light box. A membrane is then put on top of the template. This template is used as a guide to spot solution onto membranes. Through the light, dark spots in the template are clearly visible on the membranes. Solution in the amount of 0.20–0.25 μL is manually loaded onto a single spot by a 2 μL pipetman. Typically, it takes about 30–50 min to spot 500 spots. If an array membrane contains 40 antibodies, it takes only 15 min to produce one array membrane. A trained technician should be able to produce up to 40 array membranes to detect 40 cytokines per day for research demand. This production is more than adequate for most research laboratories. Templates with dark spots are computer generated. The distance between spots is 3 mm.

Captured antibodies are diluted into 200 μg/mL with 5% BSA. Antibodies and control solutions are spotted onto membranes in duplicate.

For array chips (glass slides), antibodies must be printed using an arrayer. Two major types of arrayers are commercially available: contact and noncontact arrayers. To avoid evaporation, the print must be done under high humidity and, if possible, at 4°C.

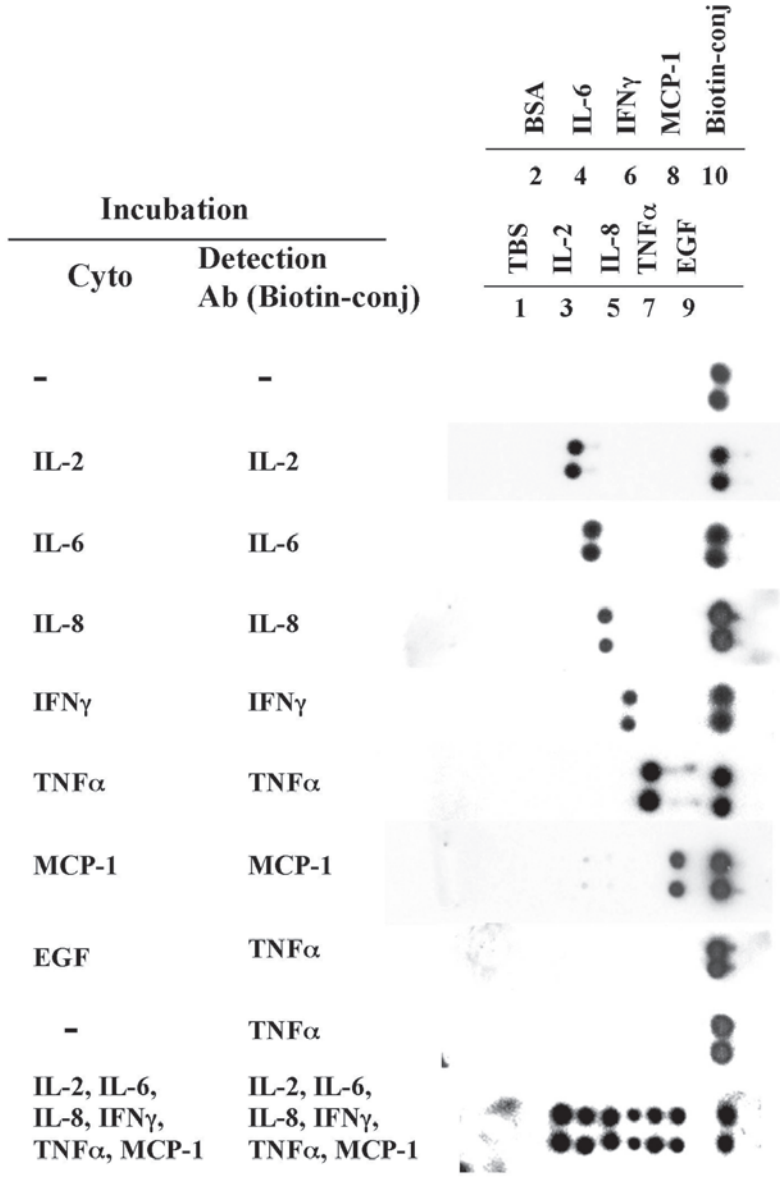

Fig. 3. Selection of specificity of human cytokine arrays. Hybond ECL membranes spotted with different capture antibodies against different cytokines were incubated subsequently with a single cytokine or all six cytokines or a control as indicated in the figure; they were then incubated with corresponding biotin-conjugated anticytokines or a control as showed in the figure. The signals were visualized by ECL.

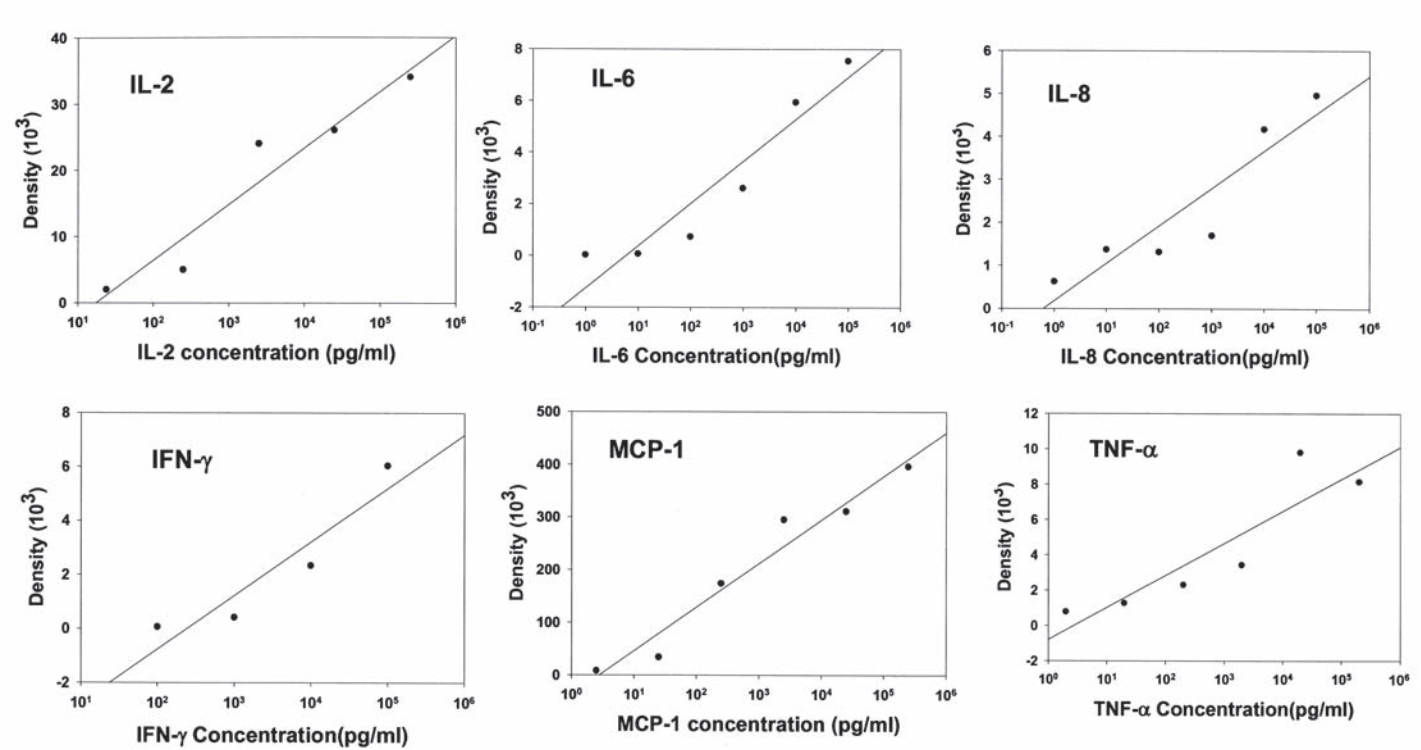

Fig. 4. Determination of detection sensitivity of cytokine protein arrays. Cytokine array membranes were incubated with different concentrations of cytokines as indicated. The intensities of signals were detected by densitometry and plotted against the concentrations of cytokines.

3.4. Sample Preparation

For conditioned medium, it is better to prepare serum-free or low-serum medium because some sera also contain cytokines. If serum is absolutely necessary for cell growth, prepare the control medium at the same condition.

1. At day 0, seed 1×10^6 cells in 100 mm tissue-culture plate with complete medium.
2. At day 3, medium is removed and replaced with low-serum medium (e.g., medium containing 0.2% calf serum).
3. At day 5, medium is collected into a 15-mL tube. Spin at 1000*g* in desktop centrifuge at 4°C for 10 min. Save the supernatant. Aliquot the supernatant into a 1.5-mL Eppendorf tube. Store supernatant at –80°C until experiment. The sample can be stored this way for at least 1 yr.
4. Harvest cells and determine the protein concentration as a routine procedure or count cell numbers. Protein concentrations or cell numbers are used to normalize the amount of samples for cytokine arrays.

For cell lysate or tissue lysate, the author routinely uses RIPA buffer to extract proteins.

1. Harvest cells into Eppendorf tube.
2. Add RIPA buffer to sample (e.g., add 500 μL to 1×10^6 cells); vortex for about 1 min.
3. Spin down tube in Eppendorf centrifuge at maximal speed for 5 min at 4°C.
4. Save supernatant. Aliquot and store at –80°C until experiment.
5. Determine protein concentration and use 100–500 μg of sample for cytokine protein arrays.

For tissue lysate, the author also uses RIPA to extract protein.

1. Obtain tissues from patient tissues or animal tissues. Cut tissues into small pieces. Add RIPA to tissue (e.g., for 10 mg tissue, add 500 μL of RIPA).
2. Homogenize samples until tissue is dissolved well.
3. Spin down sample in Eppendorf centrifuge at maximal speed for 5 min at 4°C.
4. Save supernatant. Aliquot and store at –80°C until experiment.
5. Determine protein concentration and use 100–500 μg of sample for cytokine protein arrays.

3.5. Cytokine Protein Macroarrays

This protocol describes membrane-based cytokine protein macroarrays. After construction of array membranes and preparation of samples as described, the experiment can be relatively easily performed.

1. Put membranes in suitable containers such as 8-well tissue-culture plates or other small containers or plastic bags to save samples and reagents.
2. Block nonspecific binding by incubation of membranes with 5% BSA/TBS at room temperature for 30 min.
3. Incubate membranes with 1 mL of sample at room temperature for 1–2 h. More of the sample can be used if signals are too weak. If signals are too strong, the sample can be diluted further. Dilute sample using 5% BSA/TBS if necessary.
4. Remove samples.

5. Wash membranes three times with TBS/0.1% Tween-20 at room temperature, 5 min per wash.
6. Wash membranes two times with TBS at room temperature, 5 min per wash.
7. Add 1 mL of cocktail of biotin-conjugated antibodies. Dilute biotin-conjugated antibodies into 10–100 ng/mL with 5% BSA/TBS. Incubate at room temperature.
8. Wash as directed in **steps 5** and **6**.
9. Add 2 mL of 2.5 ng/mL of HRP-conjugated streptavidin. Dilute HRP-conjugated streptavidin with 5% BSA.
10. Incubate at room temperature for 30–60 min.
11. Wash as directed in **steps 5** and **6**.
12. Visualize signals using ECL according to manufacturer's instructions coupled with film development, or using a chemiluminescent imaging system. Expose membranes several times for 30 s to 10 min.

3.6. Cytokine Protein Microarrays

This protocol describes glass-slide-based cytokine protein microarrays. After construction of array slides and preparation of samples as described, the experiment can be done as follows. The author is using HydroGel slides as shown in **Fig. 5**, but the same experimental procedures can be used for other types of glass slides.

1. Print antibodies onto HydroGel slides according to the manufacturer's instructions.
2. Block slides with 5% BSA in TBS for 30 min at room temperature.
3. Rinse slides with 1X TBS/0.1% Tween-20 three times for 5 min at room temperature.
4. Rinse slides with 1X TBS twice for 5 min at room temperature.
5. Spin slides in desktop centrifuge for 2 min at 500–1000*g*.
6. Place frame around arrays. Add 50 μL of sample. Seal frames with cover slips.
7. Incubate at room temperature for 2 h with gentle rotation.
8. Remove hybrid frames and cover slips. Wash as in **step 4**.
9. Spin slides as in **step 5**.
10. Place frame around arrays. Add 50 μL of cocktail of biotin-labeled antibodies. Seal frames with cover slips.
11. Incubate at room temperature for 1 h with gentle rotation.
12. Remove frames and coverslips. Wash as in **step 4**.
13. Spin slides as in **step 5**.
14. Place frame around arrays. Add 50 μL of 1 μg/mL of Cy3- or Cy5-labeled streptavidin. Dilute streptavidin with 5% BSA. Seal the frames with cover slips.
15. Incubate at room temperature for 30 min with gentle rotation.
16. Remove frames and cover slips. Wash as in **step 4**.
17. Spin slides as in **step 5**.
18. Scan slides with laser scanner according to manufacturer's instructions.

3.7. Quantitative Cytokine Arrays

Both cytokine protein microarrays and macroarrays can be used to quantitatively measure cytokine levels. The experiments are performed as described above with additional standard curves.

For generation of standard curves, a mixture of recombinant cytokines from 10 pg/mL to 100 ng/mL is used to probe cytokine array membranes or array slides. Signals are determined by fluorescent intensities or density intensities. The signal intensities

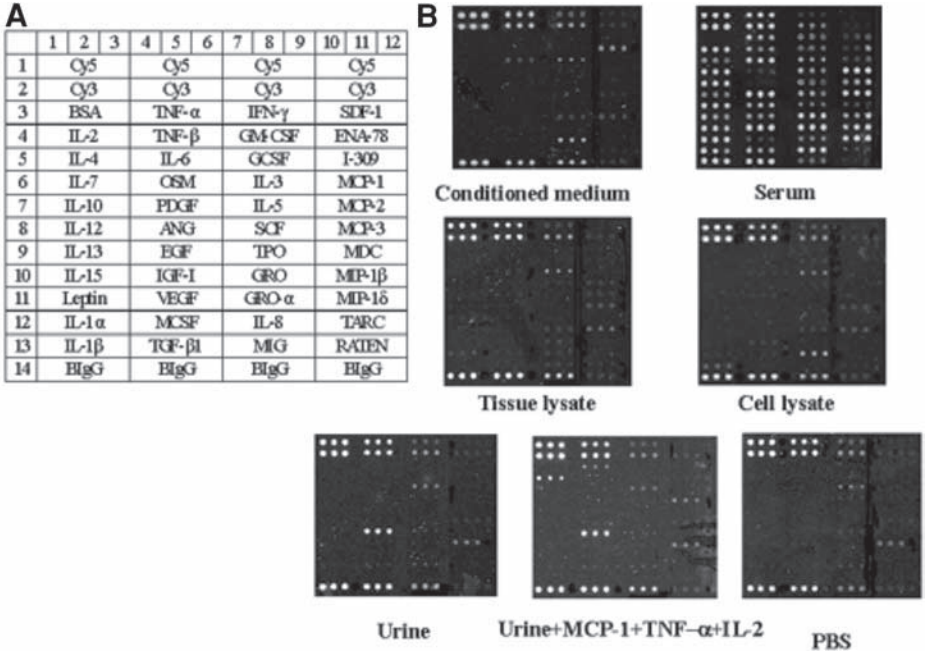

Fig. 5. Detection of cytokines in microarray format. (**A**) A map for the location of capture antibodies spotted onto protein arrays. (**B**) Protein arrays printed with 43 cytokine capture antibodies were incubated with samples from different sources: conditioned medium (human breast cancer cells BT549), serum (from an individual enrolled in the author's cardiac and antioxidant study), cell lysate (human MDA-MB-231), tissue lysate (human endometriosis tissue), urine (from a normal individual), urine (from a normal individual plus purified recombinant cytokines as indicated in the figure), and a control (phosphate-buffered saline).

are plotted against known amounts of cytokines. From the standard curves, the levels of cytokines in samples can be determined.

3.8. Examples

The primary application of cytokine protein arrays is to simultaneously detect multiple cytokine-expression levels semiquantitatively and quantitatively. In addition, cytokine protein arrays can be used to detect protein–protein interactions and protein modifications. Following is an example of the application of cytokine arrays to analyze the molecular mechanisms of tumor suppression by gap junction protein, connexin 43 (Cx43) ***(10)***.

Step 1. Collect conditioned media from Cx43-transfected cells and control-transfected cells.

1. Seed 1×10^6 cells at d 0 with complete medium. Incubate cells at 37°C and 5% CO_2 for 48 h.
2. Replace medium with lower serum medium containing 0.2% fetal calf serum at d 3. Incubate cells at 37°C and 5% CO_2 for 48 h.

	a	b	c	d	e	f	g	h	I	j	k	l
1	Pos	Neg	Neg	ENA-78	GCSF	GM-CSF	GRO	GRO-_	I-309	IL-1_	IL-1β	L-2
2	Pos	Neg	Neg	ENA-78	GCSF	GM-CSF	GRO	GRO-_	I-309	IL-1_	IL-1β	IL-2
3	IL-3	IL-4	IL-5	IL-6	IL-7	IL-8	IL-10	IL-12	IL-13	IL-15	IFN-γ	MCP-1
4	IL-3	IL-4	IL-5	IL-6	IL-7	IL-8	IL-10	IL-12	IL-13	IL-15	IFN-γ	MCP-1
5	MCP-2	MCP-3	MCSF	MDC	MIG	MIP-1β	MIP-1δ	Rantes	SCF	SDF-1	TARC	TGF-β
6	MCP-2	MCP-3	MCSF	MDC	MIG	MIP-1β	MIP-1δ	Rantes	SCF	SDF-1	TARC	TGF-β
7	TNF-_	TNF-β	EGF	IGF-I	Ang	OSM	Tpo	VEGF	PDGFβ	Leptin		
8	TNF-_	TNF-β	EGF	IGF-I	Ang	OSM	Tpo	VEGF	PDGFβ	Leptin		

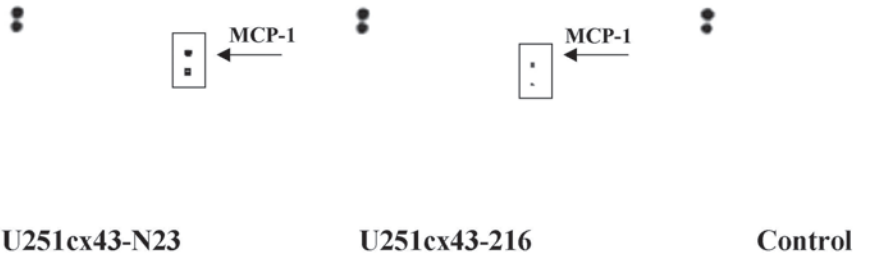

Fig. 6. Analysis of proteins secreted into conditioned media from cx43-transfected and control-transfected cells by the human cytokine array system. (**A**) A template of human cytokine arrays. (**B**) Human cytokine arrays from cx43-transfected cells and control-transfected cells. 1 mL of 10-fold diluted conditioned media and control media was incubated with human cytokine array membranes. After removing unbound materials, the membranes were incubated with a cocktail of biotin-labeled antibodies. Signals were detected with HRP-conjugated streptavidin and ECL.

3. Collect supernatant at d 5 into 15-mL centrifuge tubes. Spin down supernatant at 1000*g* in Beckman top-deck centrifuge for 10 min. Save the supernatant.
4. Wash dishes and collect cells.
5. Dissolve cell pellet in 400 µL of 10 m*M* $NaHCO_2$.
6. Determine the protein concentrations of cell lysates.

Step 2. Cytokine protein arrays.

1. Use the protein concentrations to normalize equal amounts of conditioned media for cytokine arrays.
2. Add 1 mL of conditioned media for the sample containing high protein concentrations to 8-well plate containing array membranes. Add appropriate volume of other sample with equal protein concentrations to another 8-well plate.
3. Proceed with incubation, and wash as described in **Subheading 3.6.**

Step 3. Detection of cytokine-expression.

1. Following washing steps, membranes are incubated in ECL solution. After development, signals are visualized on X-ray film as shown in **Fig. 6**.

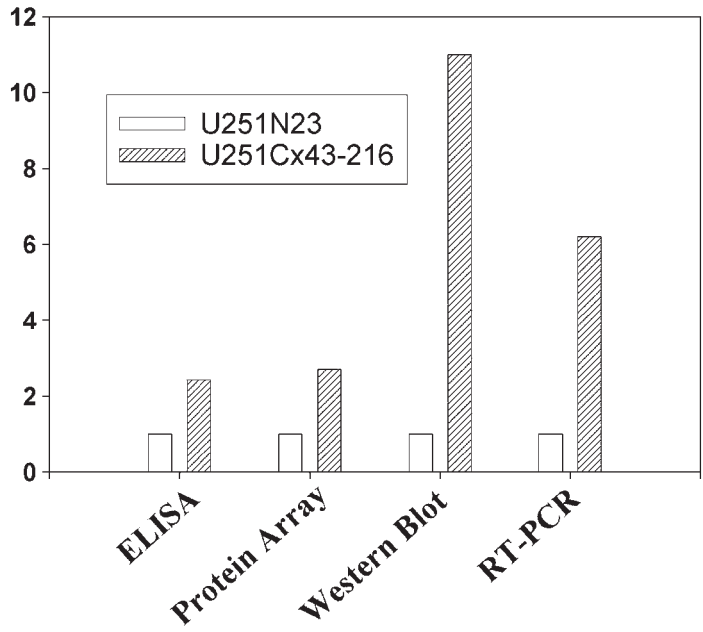

Fig. 7. Confirmation of MCP-1 expression using ELISA, Western blotting, and RT–PCR. The relative expression levels of cytokines were determined and plotted against Cx43-transfected (U251cx43-216) and control-transfected (U251N23) cells.

2. Scan the film using Bio-Rad densitometry. Normalize the signals with positive control. Compare the signals between Cx43-transfected cells and control-transfected cells.

The results indicated that cx43-transfected U251 cells significantly reduce MCP-1 expression.

Step 4. Confirmation of expression data.

1. The cytokine protein array data is further confirmed using ELISA, Western blotting analysis, and reverse transcription polymerase chain reaction (RT–PCR), as shown in **Fig. 7**.
2. A series of tumor biology experiments using recombinant MCP-1 and neutralization antibody against MCP-1 demonstrate that MCP-1 is a target of cx43 in cx43-transfected human glioblastoma cells (*see* **Fig. 8**).

4. Notes

1. Good antibodies with the highest affinity and specificity to cytokines are the prerequisite for successful construction of cytokine protein arrays. Therefore, effort must be made to select good antibodies at the initial stage of development.
2. Because conditioned media, patient sera, or any biological fluid can be directly used in protein arrays without any purification step, cytokine protein arrays provide a much more efficient way of detecting their presence than cDNA microarrays. Cytokines are traditionally detected by ELISA; however, cytokine protein arrays have several advantages

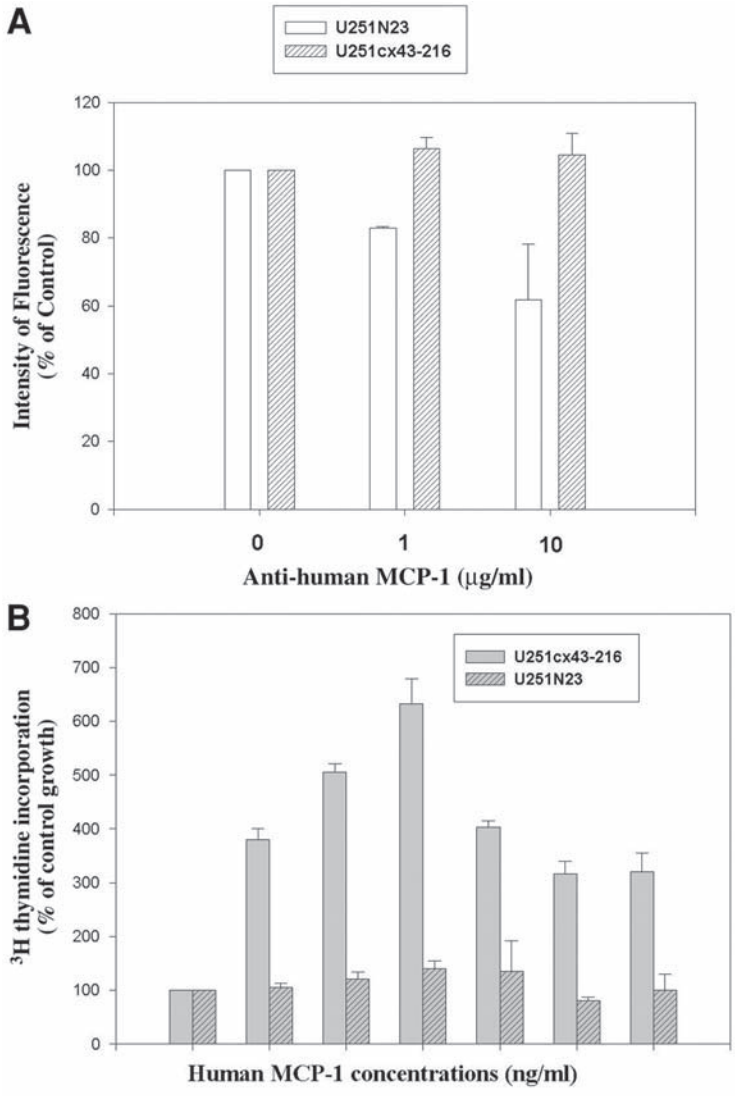

Fig. 8. Functional analysis of MCP-1 expression in cx43-transfected cells. (**A**) Inhibition of MCP-1-mediated proliferation of human glioblastoma cells using neutralizing antibody to MCP-1. Cx43-transfected cells and control-transfected cells were cultured with medium alone or medium containing different concentrations of anti-MCP-1, and inhibition of cell proliferation was determined by CyQUANT cell proliferation assay according to manufacturer's instruction. The values represent the percentage of inhibition of cell proliferation from triplicate experiments. (**B**) Effect of exogenous MCP-1 on cx43-transfected and control-transfected cells. Cx43-transfected and control-transfected cells were incubated with medium alone or medium containing different concentrations of recombinant human MCP-1. After 48 h of incubation, cell growth stimulation by MCP-1 was determined by ^{3}H thymidine incorporation assay.

over ELISA. First and more important is that cytokine protein arrays can simultaneously detect many cytokines. Second, the microarray format substantially reduces the cost and labor associated with such experiments because very small amounts of antibodies are required. Furthermore, the detection range is usually greater than ELISA.

3. The detection sensitivity is different from cytokine to cytokine. In general, if pairs of antibodies have a high sensitivity in an ELISA assay, they also have a high sensitivity in an array assay. However, because different substrates have different properties, in some cases, array systems may have a higher sensitivity than ELISA assays and conversely, ELISA assays may have a higher sensitivity than array systems in some cases.
4. The signals can be detected by different means. The most sensitive approach to detecting proteins is chemiluminescence, because the signal is amplified by turnover of the enzyme. Chemiluminescence detection is used to detect proteins on filter arrays ***(10–12,16,19,20)***, glass arrays ***(21)*** and tissue-culture plates ***(17)***. Initial study suggests that chemiluminescence detection can be quite quantitative and have dynamic ranges ***(11,12,17)***. The signals can be recorded on X-ray films; therefore, no major equipment is required. The major limitations of chemiluminescence are the short window to detect a signal and the availability of only one signal channel. The most common approach to detecting proteins is fluorescence. Fluorescence can be directly labeled into proteins ***(22)*** or conjugated into other detection molecules such as streptavidin ***(9,10)***. Fluorescence detection offers multiple channel detection capacity and long-lasting signals. The sensitivity of fluorescence detection is usually lower than chemiluminescence. Because there is low autofluorescence in glass slides, protein arrays are normally constructed on glass when using fluorescence detection. Low autofluorescence membranes have been produced by Schleicher & Schuell (Schleicher & Schuell Dassel, Germany; www.s-und-s.de). The fluorescent signals can then be detected by laser scanner or charge-coupled device (CCD) cameras.
5. To date, almost all cytokine protein array systems reported in the literature and on the market are based on ELISA. ELISA-based array systems require detection antibodies, which greatly limits the development of a higher density protein array system because it is very difficult, and possibly even impossible, to mix hundreds if not thousands of antibodies together. Therefore, the goal is to eliminate or reduce the use of detection antibodies. Several different approaches can be used to accomplish this goal. One method is to develop an aptamer system in which the bound cytokines can be detected by the indication of a protein. A second approach is to develop detection antibodies that can recognize a common domain; therefore, only several detection antibodies are required for the array system. Another method is to label proteins with fluorescent dye, gold particles, or biotin.
6. The primary application of cytokine protein arrays is to detect cytokine-expression levels semiquantitatively and quantitatively. In addition, cytokine protein arrays can be used to study cytokine-interacting proteins such as cytokine receptors ***(23)***. Briefly, cell lysate containing cytokines and cytokine-interacting proteins is incubated with cytokine protein arrays. Cytokine-interacting proteins are captured to corresponding cytokine antibodies through interacting with cytokine. Through the identification of cytokine-interacting proteins (e.g., using antibodies against a certain receptor) and the position where the cytokine antibodies are deposited, the cytokines interacting with cytokine-interacting proteins can be identified. Another potential application is to determine protein modifications. In this regard, after incubation of samples with cytokine arrays, the arrays are incubated with modification-specific antibodies, for example, antibodies against tyrosine phosphorylation, serine phosphorylation, or acetylation. The signals reveal the particular modification of certain cytokines.

Acknowledgments

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Fabrication and Application of G Protein-Coupled Receptor Microarrays

Ye Fang, Brian Webb, Yulong Hong, Ann Ferrie, Fang Lai, Anthony G. Frutos, and Joydeep Lahiri

Summary

The increased number of drug targets and compounds demands novel high-throughput screening technologies that could be used for parallel analysis of many genes and proteins. Protein microarrays are evolving promising technologies for the parallel analysis of many proteins with respect to their abundance, location, modifications, and interactions with other biological and chemical molecules. This chapter specifically describes the fabrication of G protein-coupled receptor (GPCR) microarrays, a unique subset of protein microarrays, using contact-pin printing technology. The bioassays and potential applications of GPCR microarrays for the determination of compound affinities and potencies are also included.

Key Words:

G protein-coupled receptor microarrays; protein microarrays; G protein-coupled receptor; contact-pin printing; saturation assay; competitive binding assay.

1. Introduction

With the completion of the human genome sequence, the number of available biological targets has significantly increased. Because proteins are the ultimate products of genes and, typically, are the targets against which drugs are designed, understanding proteins will aid in deciphering the biochemical mechanisms of disease and help create drugs with reduced side effects and greater probability of success in clinical trials. Proteomics, the large-scale study of proteins, is the key to translating the knowledge of genomics into new therapies for human disease ***(1)***. Protein microarray technologies ***(2,3)*** have been used to profile protein abundance ***(4,5)*** and modification ***(6)*** and to obtain information about protein–protein and protein–small molecule interactions ***(7–9)***. Target selectivity of a compound plays a critical role in determining its potential as a drug; the greater the selectivity, the lower the probability of harmful side

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effects. Traditionally, compound selectivity is characterized further downstream in the drug discovery process, generally in lead optimization. Protein microarrays hold great promise in bridging primary and secondary (i.e., selectivity) screening efforts by offering high-throughput capacity simultaneously with compound profiling.

Membrane-bound proteins represent the single most important class of drug targets ***(10)***. Of the current drug targets, 50% are membrane bound; 20% of the top 200 best selling drugs target GPCRs. GPCRs share common structural motifs, including seven transmembrane helices, a glycosylated extracellular N-terminus and an intracellular C-terminus. GPCRs mediate some important cellular signaling pathways through ligand-mediated activation of the receptor-associated G proteins, which ultimately leads to activation of downstream signaling pathways. There are an estimated 400–700 GPCRs, 190 of which have known ligands. GPCRs for which ligands are unidentified are termed orphan GPCRs, many of which are expected to be important drug targets based on the historical performance of known GPCRs.

This chapter describes the fabrication of GPCR microarrays on γ-aminopropylsilane (GAPS)-modified surfaces using robotic pin printing technology and their use for pharmacological profiling (binding affinity, potency, etc.) of compounds.

2. Materials

2.1. Chemicals

1. Human β-adrenergic receptor subtype 1 (β1) membrane preparation (Biosignal, Montreal, Canada).
2. Human β-adrenergic receptor subtype 2 (β2) membrane preparation (Biosignal).
3. Human α-adrenergic receptor subtype 2A (α2A) membrane preparation (Biosignal).
4. Human neurotensin receptor (NTR) subtype 1 membrane preparation (NEN Life Sciences, Boston, MA).
5. Bodipy-TMR-CGP 12177 (BT-CGP) (Molecular Probes, Eugene, OR).
6. Bodipy-TMR-neurotensin (BT-NT) (NEN Life Sciences, Boston, MA).
7. CGP 12177 hydrochloride (Tocris, Ballwin, MO).
8. ICI 118551 hydrochloride (Tocris, Ballwin, MO).
9. Neurotensin (Sigma, St. Louis, MO).
10. (-)- Propranolol (Tocris, Ballwin, MO).
11. Xamoterol hemifumarate (Tocris, Ballwin, MO).
12. NaCl.
13. $MgCl_2$.
14. Tris-HCl.
15. Dimethyl sulfoxide (DMSO).
16. Ethylenediaminetetraacetic acid (EDTA).
17. Bovine serum albumin (BSA) (Sigma).
18. Deionized H_2O (>18 *M*Ω; MilliQ-UV, Millipore, Bedford, MA).

2.2. Instruments and Consumables

1. Genepix 4000B scanner (Axon Instruments, Union City, CA).
2. Quill-pin printer (Cartesian Technologies Model PS 5000, Irvine, CA).
3. Quill-pin CMP3 (Telechem, Atlanta, GA).

4. Pipettors and pipet tips.
5. Vortexer.
6. White low-volume 384-well microplate (cat. no. 3674, Corning, Acton, MA).
7. GAPS slide (made in house; unavailable commercially for use in GPCR arrays).
8. FlexiPERM micro 12 chambers (Sigma).

3. Methods

3.1. General Considerations for the Fabrication of GPCR Microarrays

Fabricating GPCR microarrays is challenging (*see* **Note 1**) because GPCRs require association with lipid membranes to retain their correctly folded conformation. The authors have demonstrated the fabrication of GPCR microarrays using cell membrane preparations containing GPCRs of interest from a cell line overexpressing a GPCR (*see* **Note 2**) in combination with contact-pin printing (*see* **Note 3**) ***(7–9)***. Several parameters to determine printing quality include spot size, spot uniformity, printing reproducibility, and array performance. The main factors that affect these parameters are listed as follows:

1. Surface chemistry. Surface chemistry plays a major role in determining the quality and assay feasibility of a GPCR array (*see* **Note 4**). The authors have found that surfaces modified with GAPS are suitable for the fabrication of GPCR microarrays ***(7)***. The optimal type of GAPS slide for GPCR microarrays is not commercially available. Corning, Inc. has filed patent applications for the use of GAPS surfaces for GPCR arrays ***(11–12)***. Parties interested in obtaining this proprietary GAPS surface for GPCR arrays should contact Corning, Inc.
2. Pin size. The size and type of quill pin used for printing determines the shape and diameter of the microspots, as well as the amount of material transferred onto the surface during printing. Under the experimental conditions described here, the authors use CMP3 model pins (Telechem), which produce GPCR microspots with a spot diameter of approx 130 μM.
3. Printing conditions. Printing GPCR membrane preparations is tricky because biological membranes are extremely susceptible to environmental changes, and biological membranes can significantly alter the wetting and dewetting properties of pins during the printing. Optimal printing conditions are trade secrets and cannot be fully disclosed.
4. Receptor membrane quality. Membrane preparations made from cell lines overexpressing a receptor of interest are used for fabricating GPCR microarrays. Receptor membrane quality is determined by several key parameters. They are (a) homogeneity, (b) active receptor concentration (so-called B_{max}), (c) concentration of total membrane proteins, and (d) buffer composition. The greater the homogeneity, the higher the active receptor and total membrane protein concentration, the better array performance one can achieve. The buffer composition in which the receptor membranes are suspended also plays a role in printing quality and array performance. Typically, a buffer containing 10% sucrose/10% glycerol, or 5% sucrose/5% glycerol is used.
5. Postprinting treatment of the arrays. Immobilization of biological membranes to achieve stable association with the surface occurs relatively slowly. Therefore, an incubation period (typically 1 h) under high humidity is required following printing.

3.2. General Considerations for Bioassays Using GPCR Microarrays

Generally, multiple microarrays of GPCRs are fabricated onto a single GAPS slide (1 × 3 in.). To perform binding assays, each array is incubated for 1 h with 10 μL of a buffered solution containing fluorescently labeled ligands, or mixtures of fluorescently labeled ligands and compounds, for competitive binding assays. After incubation, the solution is carefully removed using a pipet tip attached to a vacuum pump. The slides are rinsed briefly with H_2O, dried under a stream of nitrogen, and imaged in a fluorescence scanner. Alternatively, a FlexiPERM micro 12 chamber is attached to a slide so as to position the printed arrays within the wells. An assay solution (30 μL) is added to each well. Following incubation, similar washing and drying protocols are used after the FlexiPERM is removed. Factors that affect assay performance are listed as follows:

1. Probe concentration. The authors use fluorescence imaging to monitor the binding of ligands to the receptors in the arrays. For this purpose, a fluorescently labeled ligand is used as a probe. The probe concentration determines the total binding signal and specificity. Generally, a concentration close to the kd of the probe to the receptor is used to achieve optimal binding signal and high specificity. Because the nonspecific binding of the probe to the surface (so-called background) is a function of the probe concentration, the choice of the probe concentration is critical to achieve a high signal-to-background ratio. Generally probes with an affinity of subnanomolar or less are used.
2. Assay buffer composition. For better results, the assay buffer composition must be optimized for each receptor. BSA is included in the buffer to reduce nonspecific binding of the probe to the surface, and to increase the stability and the confinement of microspots ***(13)***.
3. Incubation time and volume. A range of incubation times between 30 and 90 min and solution volume between 10 and 30 μL give acceptable array performance. Typically a 1-h incubation is sufficient for ligand binding.
4. DMSO concentration. DMSO concentrations below 5% have no obvious effect on array performance; higher concentrations of DMSO cause spreading of the biological membranes within the microspots.

3.3. Fabrication and Storage of GPCR Microarrays

In a typical print run, 5–10 μL of a "probe" GPCR membrane preparation is added to each well of a 384-well microplate. A single insertion of the pin into the solution prints up to 400 identical spots, with printing coefficients of variance (CVs) less than 7% across the spots (*see* **Note 5**). The amount of membrane solution printed per spot corresponds to about 0.5 nL, demonstrating the huge reagent savings afforded by microarray technology. After printing, the arrays are incubated in a humid chamber at room temperature for 1 h and then used for ligand-binding experiments. An example is shown in **Fig. 1**. For longer-term storage, the arrays are stored at 4°C in a desiccator filled with nitrogen.

1. Load 5 μL of each solution containing a GPCR of interest into each well of a 384-well microplate.
2. Clean the quill pin in an ultrasonic water bath.
3. Insert the pin into the probe receptor solution and load the solution.
4. Preprint some microspots on a spare slide.
5. Continuously print a given number of microspots on GAPS slides in a given format.

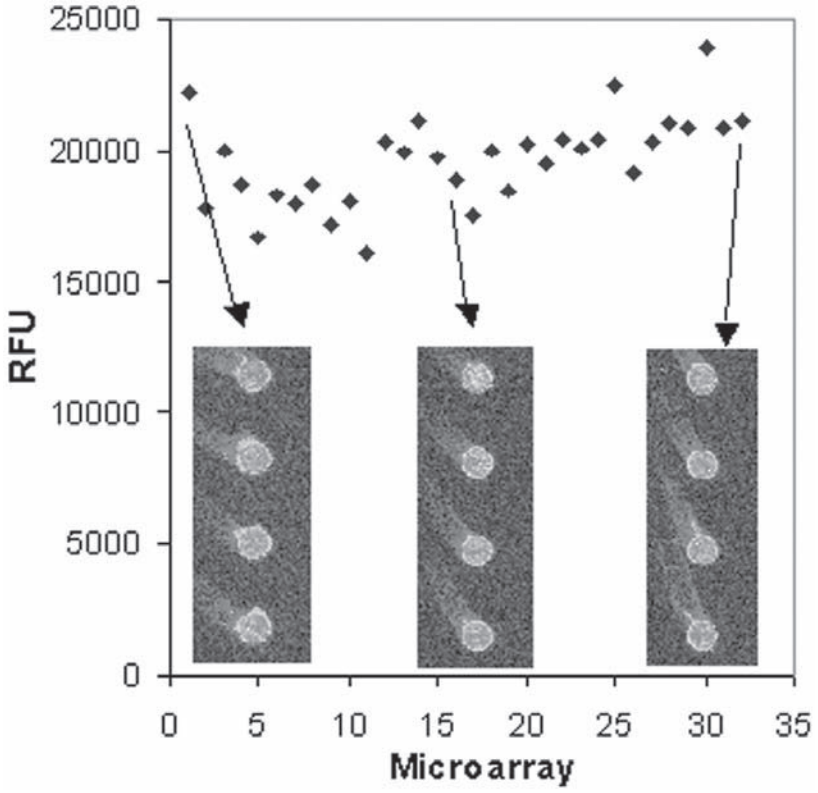

Fig. 1. Demonstration of robust printing of GPCR microarrays on GAPS. Multiple arrays of the β1 adrenergic receptor (each array consisting of four replicates) were printed on GAPS slides. A single sample pickup was used to print 256 microspots on four GAPS slides. Half of these microarrays were incubated with 2 nM BT-CGP in binding buffer, and half were incubated with 2 nM BT-CGP in the presence of 10 μ*M* CGP 12177. The inset shows the fluorescence images of the 1st, 16th, and 32nd arrays after the binding of 2 nM BT-CGP12177. The graph shows the fluorescence intensity of each microarray. Each data point represents the average fluorescence signal (RFU) of four replicates in a single array. The intraslide and interslide CVs after assays are approx 9% and 12%, respectively.

6. Repeat **steps 2–5** to fabricate microarrays of multiple GPCRs.
7. Transfer slides with the printed arrays to a humidity chamber.
8. Incubate for 1 h.
9. Store the slides in a desiccator filled with nitrogen at 4°C until use.

3.4. Preparation and Storage of Binding Buffer, Probes, and Compounds

For this chapter, the authors use four different receptors to demonstrate the potential pharmacological applications of GPCR microarrays. The four receptors are human β-adrenergic receptor subtype 1 (β1), human β-adrenergic receptor subtype 2 (β2),

human α-adrenergic receptor subtype 2A (α2A), and human NTR subtype 1. BT-CGP, a fluorescently labeled CGP 12177 analog, is used as probe for β1 and β2. CGP 12177 is a β-adrenergic receptor-selective partial agonist that binds to β1 and β2 with similar affinity. BT-NT, a fluorescently labeled neurotensin, is used as a probe for NTR subtype I. Neurotensin is a natural agonist for NTR1. The binding buffer for all four receptor contains 50 m*M* Tris-HCl, pH 7.4, 10 m*M* $MgCl_2$, 1 m*M* EDTA, and 0.1% BSA.

1. BT-CGP is dissolved in DMSO to a final concentration of 2 μ*M*, aliquotted, and stored at –80°C (*see* **Note 6**).
2. BT-NT is dissolved in DMSO to a final concentration of 2 μ*M*, aliquotted, and stored at –80°C.
3. Propranolol, CGP 12177, xamoterol, ICI 118551, and neurotensin are individually dissolved in DMSO to a final concentration of 1 m*M*, and stored at –20°C. Repeated freeze/thawing cycles should be minimized. The recommended maximum number of freeze/thawing cycles is 15.
4. Prepare buffer containing 50 m*M* Tris-HCl, pH 7.4, 10 m*M* $MgCl_2$, 1 m*M* EDTA. The buffer is stored at room temperature.
5. Prepare a 2% BSA solution that should be used within the same day.
6. Prepare a binding buffer containing 50 m*M* Tris-HCl, pH 7.4, 10 m*M* $MgCl_2$, 1 m*M* EDTA, and 0.1% BSA. This should be used within the same day.

3.5. Saturation Assays Using GPCR Microarrays

To examine the activity of the receptors in the microarrays and to determine the binding constant of fluorescently labeled ligands to the receptors in the arrays, saturation assays are employed. To determine this, the binding of BT-NT to the NTR1 arrays is examined. In the assay, two sets of NTR1 arrays are used. The arrays in the first set are incubated with BT-NT at different concentrations to obtain the total binding signals. The arrays in the second set are incubated with BT-NT at the same concentration in the presence of excess unlabeled neurotensin to obtain the nonspecific binding signal. The amount of specific binding of BT-NT at different concentrations is obtained by subtracting the nonspecific signal from the total binding signal. A standard Scatchard analysis is used to determine the binding constant of BT-NT to the NTR1 in the arrays (*see* **Fig. 2**).

1. Prepare a series of solutions of BT-NT in binding buffer at different concentrations: 0, 0.125, 0.25., 0.5, 1, 2, and 5 nM.
2. Prepare a second series of solutions of BT-NT in binding buffer in the presence of 1 μ*M* neurotensin at different concentrations: 0, 0.125, 0.25, 0.5, 1, 2, and 5 nM.
3. Apply 15 μL of each of the above solutions to each NTR1 microarray on a slide.
4. Incubate for 1 h at room temperature.
5. Remove the solution, briefly rinse the slides with H_2O, and dry immediately.
6. Scan the slide using a Genepix scanner.
7. Analyze the image to examine the total binding signal and nonspecific binding signal.

3.6. Competitive Binding Assays Using GPCR Microarrays

Competitive binding assays using GPCR microarrays can be used to screen compounds by monitoring the competitive binding of fluorescently labeled probe ligands

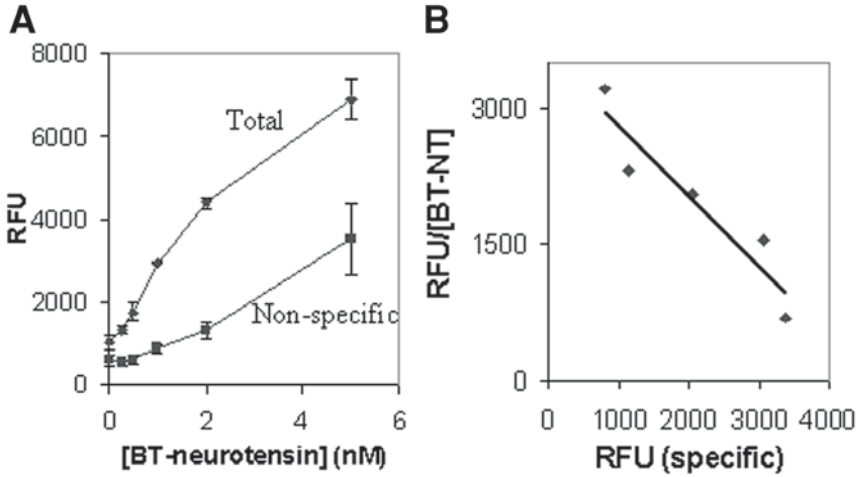

Fig. 2. Demonstration of the use of GPCR microarrays for determining the binding constant of labeled ligands to the receptors using a saturation assay. The binding of fluorescently labeled neurotensin (Bodipy-TMR-neurotensin, BT-NT) to arrays of the NTR (human subtype I, NTR1) was examined ***(7,8)***. (**A**) Plot of the fluorescence intensity of NTR1 arrays as a function of the concentration of BT-NT in the absence (total signal) and presence (nonspecific signal) of excess unlabeled NT. (**B**) Scatchard plot for estimation of the binding constant for BT-NT. The binding constant of BT-NT to NTR1 in the arrays was 1.3 nM, consistent with that reported in the literature for solution-based assays ***(14)***.

in the presence of compounds of interest. Compound screening using GPCR microarrays can be carried out in many different formats and with different types of GPCR probes. For example, compounds can be tested against an array consisting of one member of each GPCR family, or against an array consisting of all of the GPCRs within a family (e.g., the adrenergic receptors), or against a full-index GPCR array that contains receptors from different families. Furthermore, one can also obtain relative potencies of different compounds to a given receptor using competitive binding assays (*see* **Fig. 3**). The authors use three members of the adrenergic receptor family (β1, β2, and α2A) as a model system to evaluate the potential of GPCR microarrays for compound selectivity screening. The assays described in the following list are carried out on slides containing arrays of multiple GPCRs printed on the surface, with each array being treated with different compounds or compounds at different concentrations (*see* **Fig. 4**).

1. Prepare a series of solutions of BT-CGP 12177 at a fixed concentration (2 nM) in the presence of different compounds at different concentrations. The binding buffer mentioned previously is used.
2. Apply 15 μL each of the above solutions to an array of adrenergic receptors on a slide.
3. Incubate for 1 h at room temperature.
4. Remove the excess solution, briefly rinse the slides with H_2O, and dry immediately.

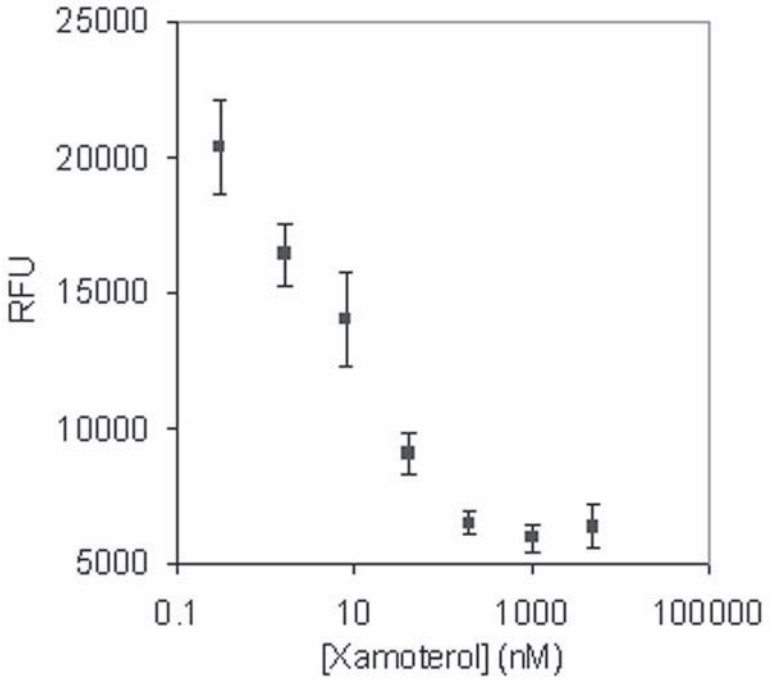

Fig. 3. Demonstration of the use of GPCR arrays for relative potency studies using a competitive binding assay. The binding of BT-CGP 12177 to microarrays of the β1 receptor as a function of xamoterol concentration was examined. The IC_{50} was found to be 32 nM for xamoterol, close to that reported in the literature for solution-based assays ***(15)***. The error bars represent the standard deviation of the four replicates in a single array.

5. Scan the slides using a Genepix scanner.
6. Analyze the image to determine the total binding signal.

4. Notes

1. There are several unique issues for fabricating GPCR microarrays that are fundamentally different from fabricating conventional deoxyribonucleic acid and protein microarrays ***(7–9)***. First, for the receptor to retain its correctly folded conformation and its biological function coimmobilization of the GPCR *and* the lipid membrane in which it is embedded is required. Second, lipid membranes need to be offset from the surface to avoid misfolding of the extramembrane domains of the receptor. Third, the receptor lipid membranes need to be stably associated with the surface after being deposited onto the surface. However, covalent immobilization of the entire membrane is not desirable because lateral mobility is an intrinsic and physiologically important property of biological membranes. Fourth, the GPCR-G protein complex should be preserved after being arrayed onto a surface because the correct complexation of the receptor and G protein is a prerequisite for the binding of agonists to the receptor with physiological binding affinity. Fifth, the lipid membranes should remain in predetermined locations throughout the bioassays.

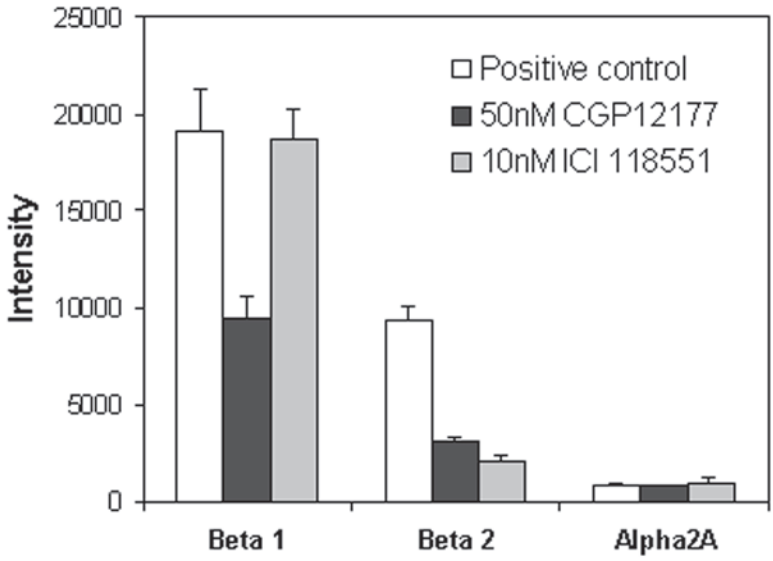

Fig. 4. Demonstration of selectivity screening using GPCR arrays. Three separate arrays of the β1, β2, and α2A receptors were printed on a single GAPS-coated gold slide. The arrays were incubated with a solution containing 2 nM BT-CGP in the absence (positive control) and presence of either CGP 12177 (50 nM), or ICI 118551 (10 nM). Analysis of the histogram shows that the presence of 50 nM CGP 12177 significantly inhibits the binding of BT-CGP to microspots corresponding to β1 and β2 receptors. These are consistent with the fact that CGP 12177 binds to β1 and β2 with similar affinity (Ki value is approx 0.6 nM for both receptors) ***(16)***. However, the presence of 10 nM ICI 118551 significantly inhibits the binding of BT-CGP only to microspots corresponding to the β2 receptor. This is consistent with the fact that ICI 118551 binds to β2 with much higher affinity (Ki is 1.2 nM) and binds to β1 with much lower affinity (Ki is 120 nM) ***(17)***. The α2A receptor was included in the array as a negative control ***(7–9)***.

Sixth, special printing protocols and handling are needed for fabricating GPCR microarrays because it is well known that biological membranes are susceptible to environmental changes.

2. Membrane preparations were purchased from either Biosignal Packard (Montreal, Canada) or PerkinElmer Life Sciences (Boston, MA) and were received as membrane-associated suspensions in a buffer. Detailed information is available from the suppliers' Web sites. In most cases, these membrane preparations were used for printing as received without further purification or dilution. In some cases, these preparations are resuspended in a desired buffer or are rehomogenized using a homogenizer.
3. The fabrication of microarrays was carried out using a quill-pin printer (Cartesian Technologies). The authors have not investigated other printing techniques, such as those involving solid pins, ink jet printers, and so forth. They believe that other printing techniques should be feasible. There may be, however, issues with particular printing methods; for example, thermal ink jet printing may denature proteins in the membrane or lead to phase transitions of the membrane itself.

4. Ideally, a surface to be used as a substrate for GPCR arrays should have the following properties. First, the size of the microspots should be controllable to enable fabrication of an array with a desired spot density. Second, the printed microspots should be confined in predetermined or designed locations throughout a bioassay. Third, GPCRs in each microspot should retain their biological function and demonstrate specificity and affinities similar to those exhibited in homogenous assays. Fourth, the printed microspots of biological membranes should be physically stable and should resist desorption from the surface over the course of an assay. Fifth, nonspecific binding of labeled target molecules should be minimal. Sixth, lipids in the printed membrane should retain their lateral fluidity, an intrinsic and physiologically important property of biological membranes. Seventh, lipid membranes printed onto the surface should not spread beyond predetermined tolerances during deposition and immobilization processes. The authors have found that surfaces modified with GAPS provide an optimal balance of all these characteristics. Specifically, supported membranes on GAPS exhibit long-range lateral fluidity yet are resistant to desorption, and the size and uniformity of the microspots as well as printing reproducibility are consistent with high-quality arrays.
5. The calculation of printing CV is based on the "autofluorescence signal" of the microarrays using fluorescence imaging with high power and high photomultiplier tube voltage in the Cy3 channel. The autofluorescence signal arises from two different sources: (a) scattering of light by salt particles contained in the spots after drying, and (b) intrinsic autofluorescence of the cell membranes. Incubation with buffer during the assay greatly reduces the autofluorescence because of salt particles. The intrinsic autofluorescence of the membranes is much lower than the signal from the binding of fluorescently labeled ligand probe and can be subtracted out of the total signal.
6. Fluorescently labeled ligands should be protected from light to minimize photobleaching of the dye molecules. All ligands and compounds can be stored at low temperature and should be stable over 6 mo. Freeze/thaw cycles should be minimized.

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ProteinChip® Array-Based Amyloid β Assays

Lisa E. Bradbury, James F. LeBlanc, and Diane B. McCarthy

Summary

Amyloid-β (Aβ) fragments are found in plaques of patients with Alzheimers. Three secretases cleave the amyloid precursor protein, producing multiple Aβ fragments that accumulate in the brain and fluids of patients with Alzheimers. Aβ peptides are difficult to detect using standard methods because of their small size and multiple isoforms. However, multiple peptide fragments can be detected using a single ProteinChip Array-Based assay. Specific antibodies recognizing various amyloid epitopes are immobilized on a ProteinChip Array. Crude samples, such as tissue lysates, serum, cerebral spinal fluid (CSF), or cell culture media, are applied to the antibody-coated arrays. Aβ peptides are specifically retained by the antibody, whereas other sample components are removed by washing. The multiple peptide fragments are detected by surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF MS), which can easily resolve the different fragments because of the corresponding changes in peptide mass.

Key Words:

Amyloid-β; protein arrays; Alzheimer's disease; amyloid precursor protein; α-secretase; β-secretase; γ-secretase.

1. Introduction

Accumulation of amyloid-β (Aβ) fragments in different areas of the brain, especially the cerebral cortex and hippocampus, is associated with the progression of Alzheimer's disease. These fragments result from cleavage of the amyloid precursor protein (APP) by three endoproteases: the α, β, and γ secretases. α-Secretase cleaves at position 17, β-secretase cleaves at positions 1 and 11, and γ-secretase cleaves less specifically at the C-terminal end, primarily at positions 40 and 42 (*see* **Fig. 1**). Aβ 1–42 is especially hydrophobic and tends to accumulate in senile plaques. The two most commonly studied forms of Aβ, the 1–40 and 1–42 fragments, can be measured using antibodies to the C-terminus in an enzyme-linked immunosorbent assay (ELISA)

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Fig. 1. Sequence and cleavage sites of Aβ 1–42 fragment in human and mouse. The amino acid sequence of human and mouse Aβ differ at three positions near the N-terminus (amino acids 5, 10, and 13). These differences are responsible for the species specificity of the 6E10 antibody, which recognizes the human but not the mouse Aβ. Note also the presence of a methionine residue at position 35. This residue sometimes becomes oxidized, resulting in a mass shift of 16 Dalton.

format. However, there is growing interest in the presence and relative abundance of other cleavage products when evaluating the effects of in vivo or in vitro treatments or gene knockouts. The small size, overlapping amino acid sequence, and multiple cleavage sites of these fragments make it quite difficult to resolve the various forms by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and standard antibody-based methods. ProteinChip® Technology facilitates the analysis of multiple forms of Aβ by capturing the peptides on antibody-coated ProteinChip Arrays, removing contaminating molecules, and detecting bound Aβ fragments by SELDI-TOF MS. SELDI-TOF MS provides sufficient resolution to readily identify cleavage-site usage based on peptide molecular weight. Selection of antibodies with different epitopes can allow the detection of cleavage products resulting from all three secretases. The ProteinChip Aβ assay has been used to detect Aβ fragments in cell culture media ***(1–6)***, serum ***(7)***, brain lysates ***(7–11)*** and CSF.

2. Materials

1. Preactivated ProteinChip® Arrays—PS10 or PS20 (Ciphergen Biosystems, Fremont, CA).
2. Antibody against desired species and epitope (6E10 works well).
3. Aβ standard peptides.
4. Phosphate-buffered saline (PBS).
5. 0.5 *M* ethanolamine in PBS, pH 8.0 (or 0.1 *M* Tris-HCl, pH 8.0).
6. 2% fetal bovine serum in PBS.
7. 0.5% Triton X-100 in PBS.
8. α-cyano-4-hydroxycinnamic acid (CHCA).
9. 10 m*M* HEPES buffer, pH 7.0.
10. High-performance liquid chromatography (HPLC)-grade acetonitrile.
11. HPLC-grade trifluoroacetic acid (TFA).
12. 2% sodium dodecyl sulfate (SDS) in H_2O, with added protease inhibitors (for tissue extraction).
13. Soluble fraction buffer: 1 L of ddH_2O, 50 mL of 0.1 *M* NaH_2PO_4, 150 mL of 0.1 *M* Na_2HPO_4, 0.5 g NaN_3, 2 m*M* ethylenediaminetetraacetic acid, 0.4 *M* NaCl, pH to 7.0, add 10 g bovine serum albumin, 0.5g 3-[(3-cholamidopropyl) dimethylammonio]-1-propane sulfonate (CHAPS) and 0.05% SDS.

14. 70% formic acid (for tissue extraction).
15. Insoluble fraction buffer: 1 *M* Tris-HCl base, 0.5 *M* NaH_2PO_4 (dibasic) and 0.5% Triton X-100.
16. Humidity chamber.
17. Ciphergen ProteinChip Reader (PBS-II or PBS-IIc).

Items 1–9 can be purchased as a kit from Ciphergen Biosystems. This kit includes the 6E10 antibody, which recognizes amino acids 4–12 of the human Aβ peptide.

3. Methods

Key experimental parameters for successful ProteinChip β-amyloid peptide assays are described in **Subheadings 3.1.–3.4.**: basic ProteinChip Immunoassay protocol, sample preparation, quantitation, and alternative bead-based methods. There are numerous sample types in both clinical and basic research containing amyloid-β peptides, including serum, CSF, conditioned cell media, and tissue extracts. Serum, CSF, and conditioned media from cells that overexpress the APP can often be directly applied to antibody-linked ProteinChip Arrays (*see* **Fig. 2**). Tissue extraction (*see* **Subheading 3.2.1.**) yields at least two fractions, both of which may be analyzed for Aβ peptides (*see* **Fig. 3**). Signals from samples having very low concentrations of Aβ peptides can be amplified by doing a traditional solution immunoprecipitation followed by SELDI-TOF MS analysis (*see* **Subheading 3.4.**).

3.1. General Protocol for Antibody Capture of Aβ Peptides on ProteinChip Arrays

ProteinChip Array antibody-capture experiments rely on direct detection of the bound peptides without an amplification step. As a result, these assays can be less sensitive than other antibody-based methods. Attention to detail will greatly improve experimental success—for example, ensuring that all binding steps reach equilibrium. Incubations on a shaker capable of frequency and/or direction changes will facilitate binding and washing. Both PS10 and PS20 ProteinChip Arrays should be tested initially to determine which provides the most sensitive and selective capture (*see* **Note 1**).

3.1.1. Coupling the Antibody to the ProteinChip Array

Antibodies that are free of contaminating proteins and other sources of free amine groups (such as Tris-HCl or azide concentrations above 0.01%) can be coupled directly to the ProteinChip Array. Other antibodies must be purified before use or can be applied to ProteinChip Arrays coated with protein G or protein A. Additional tips on antibody selection can be found in **Notes 2** and **3**.

1. To couple the antibody covalently to the ProteinChip Array, apply 2 µL of purified antibody (0.25 µg/µL in PBS) to each spot of a PS10 or PS20 ProteinChip array. Include a negative control antibody to identify nonspecific binding.
2. Incubate in a humid chamber for 2 h at room temperature or overnight at 4°C. Do not allow the spots to dry.
3. Remove the antibody and block the remaining active sites by placing the array into a 15-mL conical tube with approx 10 mL of 0.1 *M* Tris-HCl, pH 8.0, and 1% Triton X-100

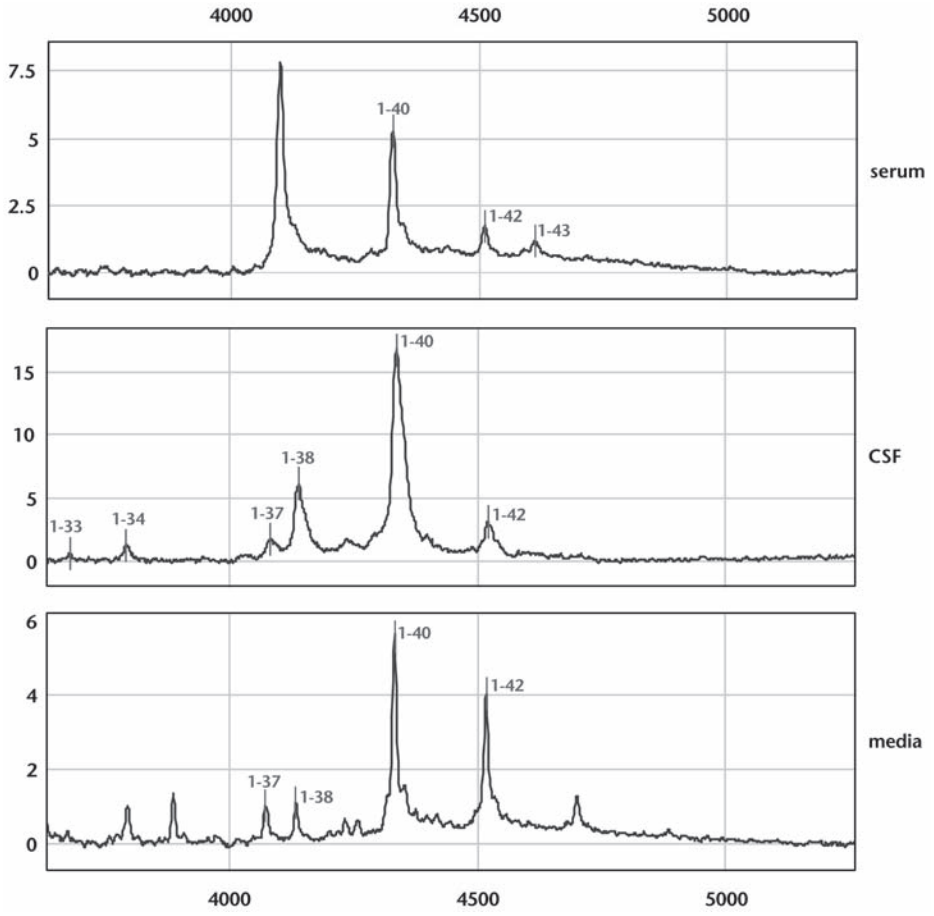

Fig. 2. Aβ fragments in serum, CSF, and conditioned media. Aβ fragments were captured from serum, CSF, and cell culture media using the 6E10 antibody coupled to a PS10 or PS20 ProteinChip Array. In addition to the 1–40 and 1–42 fragments, which are observed in all three samples, several sample-specific fragments are also observed, including the 1–43 fragment in serum and the 1–33 and 1–34 fragments in CSF.

or 0.5 *M* ethanolamine in PBS, pH 8.0 for two vigorous washes of 15 min each. Two arrays can be placed back to back and washed together.

4. Wash the array two to three times with 0.5% Triton X-100 in PBS.
5. Rinse the array twice with PBS to remove excess detergent (this can be done in a 15-mL conical tube). Do not allow the array to dry prior to adding the sample. If samples are not yet ready to be applied, the array may be stored briefly in PBS.

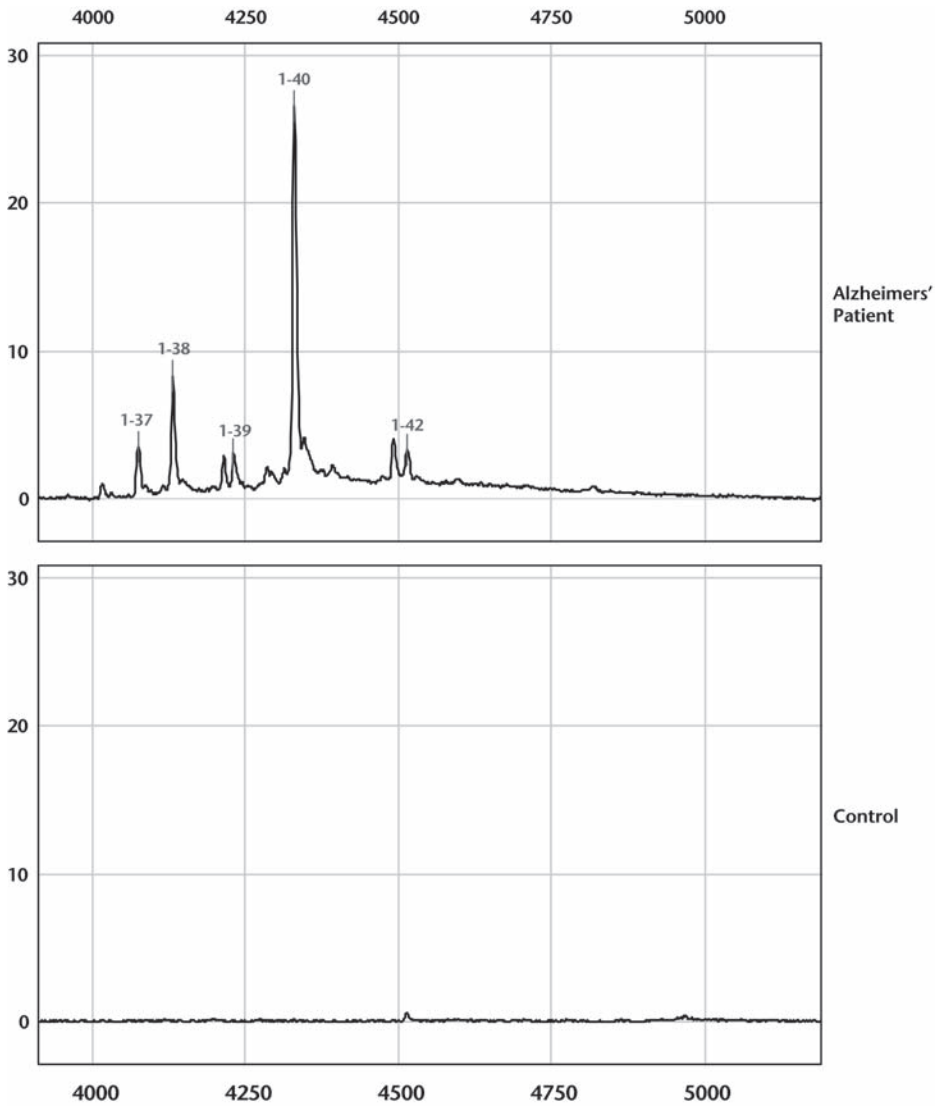

Fig. 3. Aβ fragments detected in soluble and insoluble fractions of human brain tissue. Aβ was captured from soluble and insoluble human brain fractions using the 6E10 antibody coupled to a PS10 preactivated ProteinChip Array. The soluble fraction was prepared by extracting brain tissue with 6 *M* guanidinium and 2% CHAPS. The insoluble fraction was prepared by incubating the pellet from the initial preparation overnight with formic acid.

3.1.2. Capture of Aβ Peptides From Sample and of Energy Absorbing Molecule (EAM) Addition

Small sample volumes (less than 5 μL) can be added directly to the array; however, high concentrations of detergents can cause the sample to spread beyond the active surface. More frequently, arrays are assembled into a ProteinChip Bioprocessor. This allows larger sample volumes to be added and facilitates mixing during the binding and washing steps. Samples are typically applied diluted in PBS and 0.5% Triton X-100. The minimum and maximum volumes that can be applied are 25 and 300 μL, respectively. For samples with very low concentrations of Aβ peptides, several aliquots can be applied. In general, tissue-culture supernatants have less total protein and, therefore, less nonspecific binding. As a result, less stringent binding and washing conditions can sometimes be used for these samples. This protocol recommends 0.5% Triton X-100, but other concentrations and/or nonionic detergents can be used. Additional tips on reproducibility and reducing nonspecific binding can be found in **Notes 4** and **5**.

1. Centrifuge the sample at 14,000*g* to precipitate any particulates before diluting into PBS and 0.5% Triton X-100. Dilution factors will vary depending on sample type and concentration of Aβ fragments.
2. Remove the antibody-coupled ProteinChip Array from PBS. Very gently blot remaining liquid off the array with a KimWipe.
3. Immediately add 1–2 μL of PBS to each spot to keep them wet until sample addition. Check that the PBS beads up on the spot, indicating reestablishment of the hydrophobic barrier. Do not let the spots dry out.
4. Place the arrays into the bioprocessor for use with larger volumes.
5. If using the bioprocessor, add 25–200 μL of appropriately diluted sample to each well. For small volumes (<5 μL), samples can be applied directly to the array. Purified protein G can be used as a positive control for antibody coupling (molecular weight varies with vendor; check prior to use).
6. Incubate, with strong shaking, at least 1 h at room temperature (30 min for 25 μL). Samples can be incubated overnight at 4°C. Optimal binding time will depend on antigen concentration, sample volume, and mixing.
7. Prepare the CHCA for peptide detection prior to washing the array. Dilute the saturated CHCA two- to fivefold in solvent (50% acetonitrile/0.1% TFA) to reduce noise in the mass spectrum.
8. After binding, aspirate the sample from the bioprocessor. For small volumes, remove sample with a KimWipe or aspirate. Be careful to avoid touching the active surface.
9. Wash two to four times with 200–400 μL/well of 0.01–0.5% of NP40 or Triton X-100 (or more) in PBS, up to 5–10 min. Additional or longer washes are recommended if nonspecific binding is a problem.
10. Wash the array twice for 2 min with PBS.
11. Quickly rinse the array once with 1–5 m*M* HEPES, pH 7–7.5. (Because the pH of laboratory H_2O can vary significantly, it is important to control the pH.) Remove the array from the bioprocessor and shake off the excess liquid. Air-dry.
12. Add 1.0 μL diluted CHCA to the spots within 5 min of final wash.
13. Allow the ProteinChip Array to dry completely prior to data collection.

3.1.3. Data Collection

Automatic data collection is highly recommended for these experiments. This results in data that are collected using the same protocol on each spot and, thus, gives the best relative quantitation. The optimal instrument settings will depend on instrument model (PBS I, PBS II, PBS IIc), amount of peptide captured, and other experimental parameters. The optimization range (for setting the time-lag focus) is typically 3–5 kDa, with a 4 kDa center point, but this will depend on which peptides are of greatest interest. Optimization of data collection parameters should focus on generating spectra where the most intense Aβ peaks remain on scale and the least intense Aβ peaks are still visible. If this is not possible, data can be collected at two settings. Laser energy and detector sensitivity should be adjusted to achieve the best result. Appropriate external mass calibration should be applied, with peptide standards collected using the same optimization center or lag time as the experimental samples. If peptide signals are very weak or absent, consider adding slightly more CHCA to the spots. Data should be collected up to at least 20 kDa for optimal use of total ion current normalization within the ProteinChip Software. This normalization is highly recommended as it compensates for slight variations on each spot and will improve overall reproducibility.

3.2. Sample Preparation

Aβ fragments can be detected in cell media and bodily fluids such as serum, plasma, and CSF as well as in lysates from brain tissue. Because Aβ peptides are hydrophobic and tend to aggregate, extra care must be taken during sample handling to prevent peptide loss. Extended storage and repeated freeze–thaw cycles can cause modification and cleavage of Aβ peptides (*see* **Notes 6** and **7**). Amyloid peptides are also susceptible to oxidation, resulting in 16 Dalton mass shifts. Low concentrations of dithiothreitol (DTT) or β-mercaptoethanol (1–2 m*M*) can be added to reduce oxidation during sample preparation and immunoprecipitations. High concentrations of reducing agents will reduce the antibody and therefore should be avoided.

3.2.1. Tissue Extraction: Soluble and Insoluble Aβ

Aβ peptides can be extracted from brain tissue using several different extraction methods. The soluble Aβ is typically extracted with buffer containing high concentrations of detergent with or without guanidine isothiocyanate or urea. Insoluble Aβ (from senile plaques) can be extracted with formic acid. One recommended extraction protocol is described as follows:

1. Weigh frozen brain tissue.
2. Add sufficient volume of 2% SDS in H_2O with protease inhibitor tablets or liquid (complete) to give a final ratio of 150 mg tissue/1 mL SDS.
3. Using the appropriate volume/brain, sonicate three times for 10 s each time. This can be done in Beckman 5-mL Ultraclear centrifuge tubes (for the floor model Ultra), Beckman Eppendorf-size 1.5-mL tubes, or 100 µL in 1.5-mL tubes.
4. Centrifuge at 100,000*g* (32,000 rpm for floor model; 43,000 rpm for tabletop) for 1 h at 4°C.

5. Save the supernatant, which is the SDS or soluble fraction.
6. Sonicate the pellet in 70% formic acid in H_2O using the same volume as used previously. Avoid using extended sonication times that will heat the sample; three or four pulses of 15–30 s is generally sufficient. (This step can be extended by mixing overnight at 4°C.)
7. Centrifuge at 100,000*g* (32,000 rpm for floor model, 43,000 rpm for tabletop) for 1 h at 4°C.
8. Save the supernatant, which is the formic acid or insoluble fraction.
9. Dilute the SDS/soluble fraction (typical dilution = 1:40) into soluble fraction buffer.
10. Dilute the formic acid/insoluble fraction (typical dilution = 1:20) into insoluble fraction buffer.
11. Apply 5–200 µL of diluted fraction further diluted to a total volume of 250 µL with PBS and 0.5% Triton X-100 to antibody-coated ProteinChip Arrays.

3.2.2. Preparation of Biological Fluids (Serum, Plasma, Cerebral Spinal Fluid)

Serum, CSF, and conditioned media from cells that overexpress APP can often be directly applied to antibody-coated ProteinChip Arrays. Centrifuge the sample at 14,000*g* to remove any particulates and dilute into PBS and 0.5% Triton X-100 immediately before array binding. Typical dilutions are 1:5 to 1:20 but may vary depending on the concentration of Aβ peptides. Samples should be aliquoted and stored frozen for optimal results. As with all protein samples, repeated freeze–thaw cycles should be avoided.

Aβ peptides have been detected in the CSF of mice, rats, dogs, and humans. CSF is typically diluted in PBS and 0.5% Triton X-100. CSF can contain very high levels of Aβ peptides, thus, a dilution series is recommended for any semiquantitative assay.

Serum and plasma of transgenic animals expressing high levels of Aβ peptides can be analyzed by diluting the sample into PBS and 0.5% Triton X-100. Aβ fragments 1–40, 1–42, and 1–43 have been observed in the serum of APP/PS1 mice ***(6)***. Detection is more challenging in human samples and nontransgenic animal models, which have much lower Aβ peptide concentrations (*see* **Note 8**). ELISA-based studies have shown that Aβ binds with high affinity to proteins in human plasma, preventing the peptide epitopes from binding to antibodies ***(12)***. Denaturation and chromatographic separation, which have been used to increase the accessibility of Aβ epitopes in ELISAs ***(12)***, should also prove useful for SELDI-based assays.

Tissue-culture supernatants collected from in vitro systems can be analyzed for multiple Aβ peptides. Supernatants are collected, centrifuged at low speed to remove any suspended cells, then centrifuged at high speed (100,000*g*) to remove particulates.

3.3. Quantitation of Aβ Peptides: Standard Curves and Relative Changes

Antibody-based peptide capture on the ProteinChip arrays can generate quantitative information if extra care is taken with experimental design and execution. There is a great deal of interest in measuring the relative change of the various Aβ peptides in disease and in vitro model systems. Changes in the amount of one peptide can be measured by comparison to peptides that do not change significantly. Concentration determination can be done with a standard curve but assumes that concentration of only one of the captured peptides changes, whereas the others remain constant. This may not occur in experimental samples. Typical antibody-capture experiments on

ProteinChip arrays have a dynamic range of two to three orders of magnitude. This is influenced by antibody affinity, sample type, peptide concentration, and other experimental details. Because multiple Aβ cleavage products contain the same epitope, competition among these will affect the capture of individual peptide species. This effect will be increased as the total amount of reactive peptides increases, as predicted by equilibrium equations. This section describes methods to generate a standard curve and determine relative amounts of peptide (*see* **Notes 4–8**).

3.3.1. Generation of Aβ Peptide Standard Curves

Standard curves are easily created using common methods transferred to the ProteinChip Technology. The pure peptide (one of the Aβ peptides) is spiked into a solution biochemically similar to the unknown samples. This similarity must extend beyond buffer components to include total protein concentration and complexity as each of these will impact the efficiency of peptide capture. Thus, if peptides are to be captured from an unknown serum sample diluted fivefold in PBS and Triton X-100, then peptides for the standard curve must be spiked into similarly diluted serum. If working with tissue-culture supernatants, there are significant differences between fresh media and conditioned media, so standard curves should be made in conditioned media for the most accurate results. Each peptide species requires its own standard curve because detection efficiency is peptide specific. A peptide dilution series of two- to fourfold will generally give a fairly linear standard curve. A useful standard curve with high affinity 6E10 monoclonal antibody to capture the 1–42 peptide diluted into 50 μL of PBS/Triton X-100 can be generated with peptide concentrations of 0.1–10.0 fmol/μL (*see* **Fig. 4A**). Larger sample volumes (50–200 μL) require vigorous mixing and longer incubations, but they usually result in better reproducibility and sensitivity. Duplicates are recommended, and average peak intensities can be plotted against concentration, as shown in **Fig. 4B**.

3.3.2. Relative Quantitation (Semiquantitation) of Aβ Peptides

Relative changes in the amyloid peptides are easier to determine because no standard curve is required. Relative changes in one form (i.e., Aβ 1–42 or 1–38) can be measured by comparing the peak intensity of this fragment to that of another Aβ fragment (i.e., Aβ 1–40). Large changes in the total amount of Aβ may adversely affect this ratio because of competition for binding sites. Therefore it is advisable to evaluate the broader range of Aβ peptides in addition to focusing on changes associated with specific fragments.

3.4. Alternative Bead-Based Methods

Efficient capture of Aβ peptides directly on antibody-coated ProteinChip Arrays requires high-affinity antibodies of high purity. In cases where the antibody required to recognize a specific epitope does not have sufficient affinity for efficient capture on ProteinChip Arrays, or where Aβ peptides are present at very low concentrations, a standard immunoprecipitation can be used to enrich for Aβ peptides prior to analysis. Most immunoprecipitation protocols are compatible with analysis on ProteinChip Arrays. Peptides are often captured in PBS containing detergent to reduce nonspecific

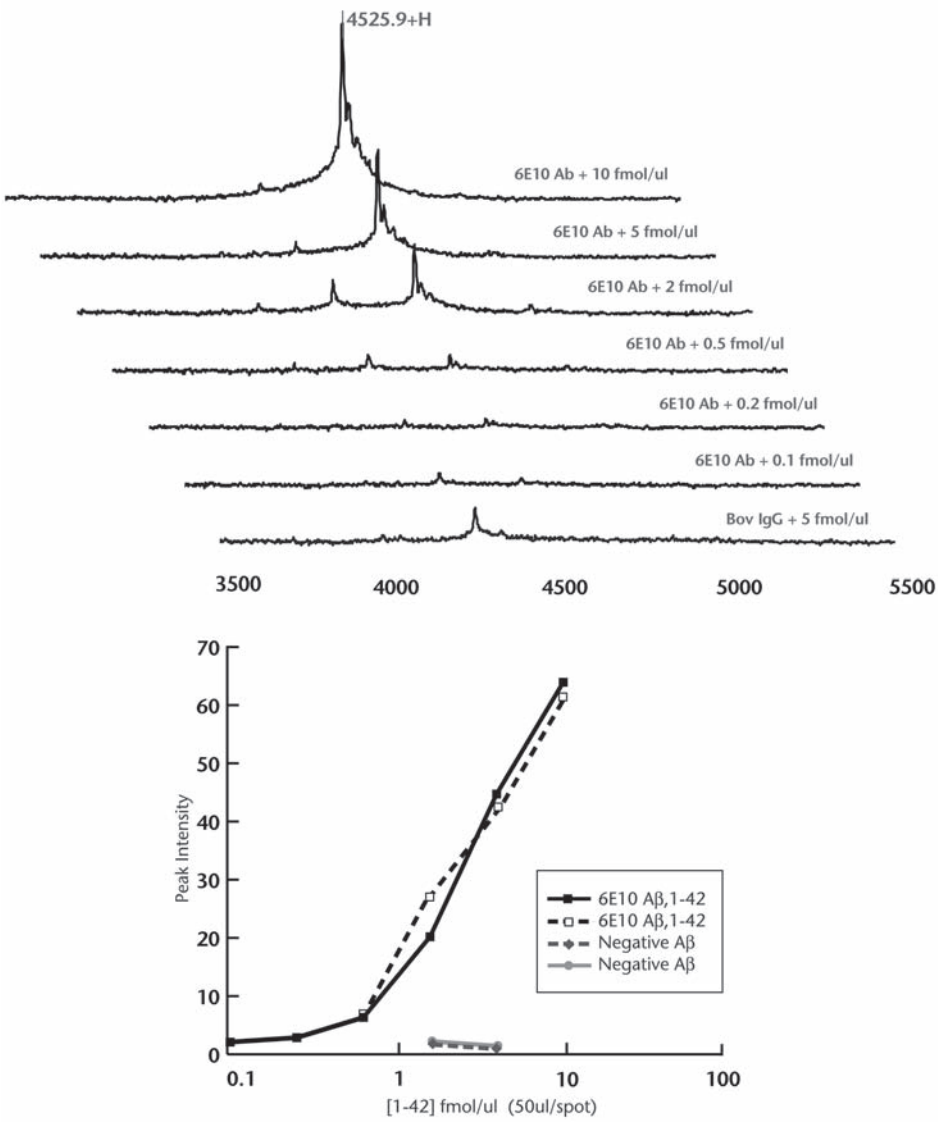

Fig. 4. Quantitation of Aβ 1–42 fragment. Known concentrations of pure Aβ 1–42 peptide were spiked into a PBS and 0.5% Triton X-100 binding buffer. 50 μL of diluted peptide was added to each spot of the ProteinChip Array with 6E10 or negative control antibody. The peak height of the Aβ 1–42 peptide (at 4526 Dalton) was imported into Excel to plot the standard curve.

binding to the beads. If SDS is used as a detergent during immunoprecipitation, it should be followed by several washes containing Triton X-100 or another nonionic detergent. The final washes should contain no detergent. If high salt buffers are used (i.e., PBS), the salt concentration should be reduced in the final washes. Because pH is an important factor in antibody–antigen interactions, dilute buffer at the same pH is the ideal wash solution (i.e., 5–10 m*M* HEPES, pH 7.4 for immunoprecipitations performed in PBS). The Aβ peptides can be extracted from the antibody-linked beads with small volumes of 50% acetonitrile/0.5% TFA (15–30 μL) and applied to a reversed-phase (H4) ProteinChip Array. The solution is quite volatile and therefore should be applied to the array soon after vortexing the beads. In most cases, dilute CHCA (5–25%) can then be directly applied to the spots after the sample dries. However, if there is very little signal, it may indicate carryover of salts or other contaminants from the immunoprecipitation. If this occurs, the spots can also be washed with 0.1% TFA or H_2O after the sample dries but before adding CHCA.

4. Notes

1. Choice of ProteinChip Array type: Antibodies can be covalently coupled to either PS10 or PS20 ProteinChip Arrays. The different chemistries of these two types of arrays result in different nonspecific-binding characteristics. When optimizing an assay, both array surfaces should be tested, along with different binding and wash stringencies.
2. Antibody selection: The 6E10 antibody commonly used for ProteinChip Aβ peptide assays recognizes an epitope at the N-terminus. Consequently this antibody can detect peptides generated by cleavage at position 1. However, 6E10 cannot detect fragments beginning at positions 11 or 17. The 4G8 antibody, recognizing the 17–24 region, can be used to detect fragments beginning at 11 or 17 but has a lower overall affinity for Aβ peptides. Other antibodies may also be used. Immunoprecipitation followed by profiling on chromatographic ProteinChip Arrays can often be used to improve the signal with lower affinity antibodies.
3. Antibody quality: All antibody preparations should be free of other proteins and other sources of free amines (such as Tris-HCl, azide). Antibody preparations should always be tested for quality by applying approx 0.5–1 μg to an H50 array. Allow the sample to air-dry on the array. Adding 2–3 μL of 1% TFA on each spot with the antibody may improve the signal. If the antibody preparation contains salts, rinse the spots quickly with H_2O. Add 2 applications of saturated sinapiniz acid (SPA) (0.5–1 μL) and analyze, focusing on the high mass range. Intact antibodies should have a molecular weight of 145–152 kDa. The presence of large amounts of heavy (45–50 kDa) and/or light (20–25 kDa) chains indicates reduction and possible separation of these chains. Small molecular weight contaminants such as Tris-HCl and azide can be removed by dialysis, spin columns, or filters. Purification of the antibody on protein A or protein G beads can be used to remove albumin and other protein contaminants.
4. Reproducibility tips: Some suggestions for good reproducibility:
   - Measure and add CHCA carefully as this can be a major source of variability.
   - Be consistent in the drying time immediately prior to CHCA addition. Less than 5 min is optimal.
   - Be consistent with postcapture wash times once the assay protocol has been optimized. Very little peptide is captured on the array surface and the antibody off-rate will result in peptide loss during extended wash times.

5. Nonspecific binding: Nonspecific binding can be a problem, especially when the peptides are in serum or tissue extracts. There are several protocol modifications that can help:
   - Perform binding and washing steps in the cold.
   - Increase wash times or number of washes. Although there is risk of some peptide loss, with high-affinity antibodies this should be tolerable. The reduction in noise from the nonspecific binding often results in better sensitivity overall, but this must be empirically determined.
   - Increase binding and or washing buffer stringency. Optimal buffers will depend on the antibody as well as the sample. In some cases, even 0.5 *M* urea will not interfere with peptide capture.
   - Dilute the sample more, and compensate if necessary by using a larger volume for the binding step.
6. Oxidation of amyloid peptides during storage or in samples: Aβ peptides contain a single methionine residue at position 35. Methionines are frequently oxidized, resulting in a mass shift of 16 Dalton. Although the antibody capture will not be adversely affected, the peptide peak will be split into two species, and this will reduce the overall sensitivity of the assay. For qualitative experiments this is not a major problem as long as the peaks are still detected. For relative quantitation this is a problem if only one of the compared peptides is oxidized. If both are oxidized to approximately the same extent, the ratio (i.e., 1–40:1–42) should not be altered substantially. Oxidation will diminish opportunities for comparison to standard curves for concentration values. For best results, store all samples in a nonoxidating environment (freezing helps), and consider adding 1 m*M* DTT or β-mercaptoethanol to sample during storage or prior to binding if necessary. Avoid multiple freeze–thaw cycles.
7. Cleavage of 1–16 peptide to 1–15: The C terminal lysine residue of the 1–16 peptide (included in the Ciphergen Aβ kit as a standard) is sometimes cleaved in serum and cell culture media containing serum, converting it to the 1–15 fragment.
8. Peptide complex with serum proteins reduces availability for antibody capture: As in any binding experiment, the availability of the analyte for antibody capture dramatically affects the result. If Aβ peptides are aggregating or complexed with other proteins in the sample, the antibody capture efficiency will decrease. Detergent or denaturants can be added directly to the sample to disrupt protein–protein interaction. If necessary, these can be diluted prior to antibody capture step.

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Serum Protein-Expression Profiling Using the ProteinChip® Biomarker System

Kate Gilbert, Sharel Figueredo, Xiao-Ying Meng, Christine Yip, and Eric T. Fung

Summary

Protein-expression profiling of serum is a common approach to the discovery of potential diagnostic and therapeutic markers of disease. Like any other proteome, the serum proteome is characterized by protein expression across a large dynamic range. This single facet requires the employment of fractionation procedures prior to detection of protein. The authors use a combination of conventional column chromatography with array-based chromatography to simplify the serum proteome into subproteomes, thus providing a greater representation of the serum proteome. Robotics is employed to increase the throughput of sample processing. These procedures result in large amounts of data that are analyzed through a series of preprocessing and postprocessing steps. A well-designed serum profiling project can therefore result in the discovery of statistically sound, clinically meaningful protein biomarkers.

Key Words:

SELDI; ProteinChip Array; protein-expression profiling; serum.

1. Introduction

Because serum is one of the most easily procured patient specimens, and because it is perceived to contain many of the molecules that might indicate systemic function, serum is the sample source that is most often profiled in the hopes of identifying sets of biomarkers for clinical use. The reference value of protein content in human serum is 6–8 g/dL; albumin constitutes about 50% of that content and immunoglobulin another 20%. The most common causes of diminished total protein content are malnutrition, liver disease, and certain types of renal disease. Hyperproteinemic states are unusual but can be seen in cases of hyperlipidemia and other metabolic conditions. Because of the large amount of albumin in serum and because total protein content itself can be an indication of patient well-being, the authors feel that it is unwise to

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attempt to normalize for protein content in serum and instead use a fixed volume in the authors' experiments.

The protocol begins with an anion-exchange fractionation step that uses stepwise pH elutions; six fractions are collected and each fraction is bound to a variety of array surfaces. Each of the six fractions is collected twice and the two collections pooled. This helps to ensure that the pH changes appropriately and also gives greater reproducibility in the fractionation as well as better partitioning of proteins into their respective fractions. For human serum the authors recommend 180 μL of resin; for rat serum they recommend 240 μL of resin. There has been some concern regarding whether the fractionation should be done at room temperature or at 4°C. Although it is true that, in general, proteins should be handled in the cold as much as possible, serum appears to be a unique case where this is not necessary. This is, in part, because serum naturally contains a preponderance of proteases (e.g., clotting factors) as well as protease inhibitors and, therefore, is less susceptible to artificial degradation once it reaches the researcher's hands.

The authors list in the following protocol the recommended array-binding conditions; however, in a particular application one may of course experiment with other binding conditions. The authors always profile the same fraction on the various array types at one time so that they do not subject the fractions to multiple freeze–thaw cycles. The protocol provided here describes the processing of serum; however, the same principles can be applied to virtually any other type of sample, including cerebrospinal fluid and tissue extracts. High throughput is readily achieved using an automated liquid handler; the protocol provided here describes the use of the Biomek® 2000 laboratory automation workstation, as implemented in the Biomarker system.

2. Materials (*see* Notes 1–7 regarding materials and buffers)

1. 1 *M* Tris-HCl, pH 9.0.
2. 1 *M* Tris-HCl, pH 8.0.
3. 1 *M* HEPES, pH 7 (Sigma cat. no. H-0887).
4. 27.8 g/L 0.2 *M* sodium phosphate monobasic (use the monohydrate salt).
5. 53.65 g/L 0.2 *M* sodium phosphate dibasic (use the heptahydrate salt).
6. 1*M* acetic acid, 11.6 mL glacial acetic acid/L H_2O.
7. 27.2 g/L 0.2 *M* sodium acetate (use the trihydrate salt).
8. 42.02 g/L 0.2 *M* citric acid (Sigma, cat. no. C-1909).
9. 59.8 g/L 0.2 *M* sodium citrate dihydrate (Sigma, cat. no. S-4641). Do not use the pentahydrate salt.
10. Acetonitrile >99.93%, high-pressure (performance) liquid chromatography (HPLC)-grade (Sigma-Aldrich cat. no. 27, 071-7).
11. 2-propanol 99.5%, HPLC-grade (Sigma-Aldrich cat. no. 27, 049-0).
12. Octyl glucopyranoside: Make a 10% (w/v) stock solution (Sigma, cat. no. O-9882).
13. Trifluoroacetic acid (TFA) (Sigma-Aldrich cat. no. 29, 953-7).
14. Urea (Sigma cat. no. U-5128).
15. 3-[(3-cholamidopropyl) dimethylammonio]-1-propane sulfonate (CHAPS) (Sigma cat. no. C-9426).
16. U9: 9 *M* urea, 2% CHAPS, 50 m*M* Tris-HCl, pH 9.0.

17. U1 (U9 diluted in 50 m*M* Tris-HCl pH 9.0 to a final concentration of 1 *M* urea, 0.22% CHAPS, 50 m*M* Tris-HCl, pH 9.0).
18. Wash buffer 1: 50 m*M* Tris-HCl with 0.1% *n*-octyl β-D-glucopyranoside (OGP) pH 9.0.
19. Wash buffer 2: 50 m*M* HEPES with 0.1% OGP pH 7.0.
20. Wash buffer 3: 100 m*M* sodium acetate with 0.1% OGP pH 5.0.
21. Wash buffer 4: 100 m*M* sodium acetate with 0.1% OGP pH 4.0.
22. Wash buffer 5: 50 m*M* sodium citrate with 0.1% OGP pH 3.0.
23. Wash buffer 6: 33.3% isopropanol/16.7% acetonitrile/0.1% trifluoroacetyl acid.
24. Silent Screen 0.45 m*M* filtration plate (VWR Scientific cat. no. 13586-240).
25. Q Ceramic HyperD® F (Ciphergen cat. no. 20066).
26. V-well 96-well dishes—low protein binding. (VWR cat. no. 62409-112).
27. Immobilized metal affinity capture (IMAC)3 ProteinChip Array (Ciphergen cat. no. C553-0022).
28. IMAC binding buffer: 100 m*M* sodium phosphate, 0.5 *M* NaCl, pH 7.0.
29. IMAC charging solution: 100 m*M* $CuSO_4$.
30. CM binding buffer and IMAC neutralization solution: 100 m*M* sodium acetate pH 4.0.
31. CM10 ProteinChip Array (Ciphergen cat. no. C553-0075).
32. H50 ProteinChip Array: (Ciphergen cat. no. C553-0065).
33. 10% Acetonitrile (ACN), 0.1% TFA (H50 binding buffer).
34. 1% TFA *(TFA should be stored in a dark glass bottle).*
35. SAX2 ProteinChip Array (Ciphergen cat. no. C553-0027).
36. 96-well bioprocessor (Ciphergen cat. no. C503-0006).
37. Bioprocessor gasket and reservoir (Ciphergen cat. no. C503-0007).
38. Pap pen (Vector Laboratories cat. no. H-4000).
39. Sinapinic acid (Ciphergen cat. no. C300-0002)—reconstituted with 400 μL of 50% ACN, 0.5% TFA to tube and vortex 5 min at room temperature.
40. α-Cyano-4-hydroxycinnamic acid (CHCA) (Ciphergen cat. no. C300-0001)—reconstitute with 200 μL of 50% ACN, 0.25% TFA to tube and vortex 5 min at room temperature.
41. All-in-1 Protein Standard (Ciphergen cat. no. C100-0004).

3. Methods

The methods describe below outline (a) anion-exchange fractionation of serum, (b) binding of individual fractions to ProteinChip Arrays, and (c) the reading of arrays and preprocessing of data.

3.1. Anion-Exchange Fractionation

The steps required for anion-exchange fractionation of serum are described in **Subheading 3.1.** Because proteins bind to anion-exchange resins at high pH, serum is denatured in a high-pH buffer containing urea and CHAPS. Denaturation disrupts protein–protein interactions, thereby increasing the reproducibility of the fractionation procedure. A stepwise descending pH gradient is employed. Each of the six fractions is collected twice and the two collections pooled.

This protocol describes loading the Q Ceramic HyperD F sorbent into the filtration plate before fractionation. Alternatively, prefilled plates are available separately (Ciphergen cat. no. C540-0018) or as part of the serum fractionation kit (Ciphergen cat. no. K100-0007); these plates contain Q Ceramic HyperD F in a dehydrated form.

If using this plate, rehydrate and equilibrate the plate as described in the product insert and proceed to **Subheading 3.1.4.**

3.1.1. Equilibrate Sorbent

1. Transfer 14 mL of the Q Ceramic HyperD F slurry (approx 10 mL of beads) into a 50-mL Falcon tube.
2. Allow the beads to settle; aspirate the solution from the top of the beads.
3. Determine the volume of the beads and then add 3 bed volumes of 50 m*M* Tris-HCl, pH 9.0.
4. Invert the tube several times to mix the beads.
5. Repeat **steps 2–4** three more times.
6. Aspirate the solution from the top of the beads, then add 1 bed volume of 50 m*M* Tris-HCl, pH 9.0, to create a 50% suspension. This can be stored at 4°C.

3.1.2. Denature Serum Protein

1. Bring serum samples to ambient temperature. Spin at 20,000*g* for 10 min at 4°C.
2. Aliquot 20 µL of serum sample to each well of a standard V-bottom 96-well microplate. (*see* **Notes 8** and **9**).
3. Add 30 µL of U9 buffer to each well.
4. Cover the microplate with adhesive sealing film and mix on the MicroMix 5 (available from Diagnostic Product, Corp. Los Angeles, CA; also included in the Biomek 2000 package from Ciphergen). The MicroMix 5 should be set at 20, 5, 20; mix for 20 min at 4°C.

3.1.3. Loading and Equilibration of Sorbent into Filter Plate

1. Pipet 180 µL of the 50% sorbent slurry into each well of the 96-well filtration plate (*see* **Notes 10** and **11**).
2. Apply vacuum to remove the buffer (*see* **Note 12**).
3. Add 200 µL of U1 buffer to each well; apply the vacuum.
4. Repeat two more times.

3.1.4. Binding Sample With Sorbent

1. Pipet 50 µL of sample from each well of the sample microplate to the corresponding well in the 96-well filtration plate.
2. Add 50 µL of U1 to each well of the sample microplate. Mix five times.
3. Pipet 50 µL from each well of the sample microplate to the corresponding well in the 96-well filtration plate (*see* **Note 13**).
4. Cover the filtration plate with adhesive sealing film and mix on MicroMix 5 (set at 20, 7, 30) for 30 min at 4°C.

3.1.5. Fraction Collection (see ***Note 14***)

1. Label 6 V-well 96-well plates F1–F6.
2. Aliquot adequate volumes of elution buffers in a buffer dish (*see* **Note 15**).

   Fraction 1:

   a. Place the 96-well microplate labeled F1 underneath the filtration plate.
   b. Apply the vacuum and collect the flowthrough into the F1 plate.
   c. Add 100 µL of wash buffer 1 to each well of the filtration plate.
   d. Mix for 10 min on MicroMix 5 (set at 20, 7, 10) at room temperature.
   e. Apply the vacuum and collect the eluate into the F1 plate. Fraction 1 contains the flowthrough and the pH 9.0 eluate.

Fraction 2:
a. Add 100 µL of wash buffer 2 to each well of the filtration plate.
b. Mix for 10 min on MicroMix 5 (set at 20, 7, 10) at room temperature.
c. Place the 96-well microplate labeled F2 underneath the filtration plate.
d. Apply the vacuum and collect the eluate into the F2 plate.
e. Add 100 µL of wash buffer 2 to each well of the filtration plate.
f. Mix for 10 min on MicroMix 5 (set at 20, 7, 10) at room temperature.
g. Collect remainder of fraction 2 in the F2 plate. Fraction 2 contains the pH 7.0 eluate.

Fraction 3:
a. Add 100 µL of wash buffer 3 to each well of the filtration plate.
b. Mix for 10 min on MicroMix 5 (set at 20, 7, 10) at room temperature.
c. Place the 96-well microplate labeled F2 underneath the filtration plate.
d. Apply the vacuum and collect the eluate into the F2 plate.
e. Add 100 µL of wash buffer 3 to each well of the filtration plate.
f. Mix for 10 min on MicroMix 5 (set at 20, 7, 10) at room temperature.
g. Collect remainder of fraction 3 in the F3 plate. Fraction 3 contains the pH 5.0 eluate.

Fraction 4:
a. Add 100 µL of wash buffer 4 to each well of the filtration plate.
b. Mix for 10 min on MicroMix 5 (set at 20, 7, 10) at room temperature.
c. Place the 96-well microplate labeled F2 underneath the filtration plate.
d. Apply the vacuum and collect the eluate into the F2 plate.
e. Add 100 µL of wash buffer 4 to each well of the filtration plate.
f. Mix for 10 min on MicroMix 5 (set at 20, 7, 10) at room temperature.
g. Collect remainder of fraction 4 in the F4 plate. Fraction 4 contains the pH 4 eluate.

Fraction 5:
a. Add 100 µL of wash buffer 5 to each well of the filtration plate.
b. Mix for 10 min on MicroMix 5 (set at 20, 7, 10) at room temperature.
c. Place the 96-well microplate labeled F2 underneath the filtration plate.
d. Apply the vacuum and collect the eluate into the F2 plate.
e. Add 100 µL of wash buffer 5 to each well of the filtration plate.
f. Mix for 10 min on MicroMix 5 (set at 20, 7, 10) at room temperature.
g. Collect remainder of fraction 5 in the F5 plate. Fraction 5 contains the pH 3.0 eluate.

Fraction 6:
a. Add 100 mL of wash buffer 6 to each well of the filtration plate.
b. Mix for 10 min on MicroMix 5 (set at 20, 7, 10) at room temperature.
c. Place the 96-well microplate labeled F2 underneath the filtration plate.
d. Apply the vacuum and collect the eluate into the F2 plate.
e. Add 100 mL of wash buffer 6 to each well of the filtration plate.
f. Mix for 10 min on MicroMix 5 (set at 20, 7, 10) at room temperature.
g. Collect remainder of fraction 6 in the F6 plate. Fraction 6 contains the organic solvent eluate.

3. Seal the six collection microplates and store until proceeding with the ProteinChip Array binding protocol. If the samples are to be analyzed within 24 h store at 4°C; longer term storage should be at –20°C or lower.
4. Dispose of the sample plate as biohazardous waste if human serum sample is used.

3.2. Array Binding

To minimize freeze–thaw cycles, it is advisable to bind each fraction to all of the selected array conditions at one time. The choices of arrays and matrix are discretion-

ary. When resources are ample, it is advisable to bind each fraction to each of the array surfaces because each array surface binds an overlapping subset of the proteome. In addition, different matrices give different profiles. In situations where resources are limited, only specific array types might be used, or limiting to a single matrix may also be employed. In the authors' experience, fraction 2 usually gives the least amount of useful information and so may be profiled last. A final way of reducing the amount of resources used is to pool fractions 1 and 2 and to pool fractions 5 and 6. This effectively yields four fractions to bind to the various ProteinChip Array conditions. In any instance, all binding should be done at least in duplicate (preferably in triplicate). Only the number of arrays that can be read in a reasonable period of time after binding (i.e., 1 wk) should be prepared.

3.2.1. Array Preparation and Sample Binding: IMAC3 (see ***Note 16***)

1. Assemble the ProteinChip Arrays in the Bioprocessor.
2. Add 50 µL of IMAC charging solution (100 m*M* $CuSO_4$) to each well. Mix for 5 min at room temperature.
3. Remove the buffer from the wells. Rinse with H_2O.
4. Add 50 µL of IMAC neutralization buffer (100 m*M* sodium acetate pH 4.0) to each well. Mix for 5 min at room temperature.
5. Remove the buffer from the wells. Rinse with H_2O.
6. Add 150 µL of the IMAC binding buffer (100 m*M* sodium phosphate, 0.5 *M* NaCl, pH 7.0) to each well. Mix for 5 min at room temperature.
7. Remove the buffer from the wells.
8. Repeat **steps 6** and **7** for a total of two washes.
9. Add 90 µL of the IMAC binding buffer to each well.
10. Add 10 µL of the sample to each well. Mix for 30 min at room temperature.
11. Remove the samples from the wells.
12. Wash each well with 150 µL IMAC binding buffer for 5 min, with agitation. Repeat twice for a total of three buffer washes.
13. Remove the washing buffer from the wells and rinse each well with deionized H_2O.
14. Drain the wells and remove the arrays from the bioprocessor.
15. Allow the arrays to air-dry.
16. Using a Pap pen draw a circle around each spot.
17. Apply 1.0 µL EAM solution per spot. Two applications of energy absorbing molecule (EAM) solution can be used to increase the intensities of high-mass proteins.
18. Allow the arrays to air-dry.
19. Analyze the arrays using the ProteinChip Reader.

3.2.2. Array Preparation and Sample Binding: H50

1. (Optional) Bulk-wash the ProteinChip Arrays with 50% methanol or acetonitrile for 5 min. Repeat once. Dry the array for an hour after bulk wash to minimize any spot-to-spot cross-contamination.
2. Assemble the ProteinChip Arrays in the Bioprocessor.
3. Add 100 µL of the H50 Binding Buffer to each well. Mix for 5 min at room temperature.
4. Remove the buffer from the wells.
5. Add 45 µL of the H50 Binding Buffer to each well.
6. Add 5 µL of the sample (fraction) to each well. Mix for 30 min at room temperature.

7. Remove the samples from the wells.
8. Wash each well with 100 μL H50 Binding Buffer for 5 min, with agitation.
9. Repeat once for a total of two buffer washes.
10. Remove the washing buffer from the wells and rinse each well with deionized H_2O.
11. Drain the wells and remove the arrays from the Bioprocessor.
12. Allow the arrays to air-dry.
13. Apply 1.0 μL EAM solution per spot. Two applications of EAM solution can be used to increase the intensities of high-mass proteins.
14. Allow the arrays to air-dry.
15. Analyze the arrays using the ProteinChip Reader.

3.2.3. Array Preparation and Sample Binding: CM10

1. Assemble the ProteinChip Arrays in the Bioprocessor.
2. Add 150 μL of the CM binding buffer (100 m*M* sodium acetate pH 4.0) to each well. Mix for 5 min at room temperature.
3. The buffer from the wells.
4. Repeat **steps 2** and **3** for a total of two washes.
5. Add 90 μL of the CM binding buffer to each well.
6. Add 10 μL of the sample to each well. Mix for 30 min at room temperature.
7. Remove the samples from the wells.
8. Wash each well with 150 μL CM binding buffer for 5 min, with agitation.
9. Repeat twice for a total of three buffer washes.
10. Remove the washing buffer from the wells and rinse each well with deionized H_2O.
11. Drain the wells and remove the arrays from the bioprocessor.
12. Allow the arrays to air-dry.
13. Apply 1.0 μL EAM solution per spot. Two applications of EAM solution can be used to increase the intensities of high-mass proteins.
14. Allow the arrays to air-dry.
15. Analyze the arrays using the ProteinChip reader.

3.3. Array Reading and Data Preprocessing

When arrays have been bound with protein, it is recommended that they be stored dust-free in the dark at room temperature. Proteins are denatured so there is no need to refrigerate or freeze the arrays prior to reading in the ProteinChip Reader. However, over time the signal will decrement. It is often possible to "rescue" the signal by depositing additional matrices, although this is not recommended as routine practice because the reproducibility of this step is not predictable. The ProteinChip Reader should be monitored at least weekly for performance. This can be done by the employment of various reference standards. The IgG array is an array on which a fixed amount of immunoglobulin is deposited; this array is used to monitor instrument sensitivity. The insulin array is an array on which a fixed amount of insulin is deposited; this array is used to monitor instrument resolution. The results of such readings should be recorded in a log book. A mass calibration array should be run periodically and always at the beginning of a project. Mass calibration should be performed by using five calibrants in the mass range of interest. Different calibrants should be used for the low-mass range vs the high-mass range. Spot protocols should be determined prior to

the start of the project. In general, at least 160 shots should be acquired and averaged. Spots should be read at both low and high energies. In general, if the laser energy needs to be set too high, an increase in detector voltage may be warranted. For data analysis, a general rule of thumb for the baseline fitting width should be eight times the fitting width rather than the default settings. Representative spectra are shown in **Figs. 1** and **2**.

It is often desirable to standardize data from multiple runs. If this is the case, the use of a standard sample is advisable (*see* **Note 17**). This sample can be fractionated and a fraction spotted on multiple spots. The spots are read at a variety of instrument settings, and the resulting total ion current at each of the settings is recorded. Future assays can then be set to approximate previous assays by repeating the procedure of fractionating, array binding, and reading at multiple settings. The setting that should be used is the one that generates total ion currents approximating those of the previous runs. Further normalization can be accomplished using the software feature of total ion current normalization. This normalization should be done over a mass range appropriate to the analysis, but always omitting the matrix region.

4. Notes

1. Check the pH of the house H_2O before making buffers to make sure the pH is not drifting, which may indicate malfunctioning of the filtration system.
2. Use HPLC-grade H_2O when making all solutions and buffers.
3. Make buffers for entire project at beginning. Estimate amount required and make at least twice as much. Record the date on which the buffer was made.
4. Organic solvents are light sensitive. Store in amber glass bottles, preferably in the dark. Organic solvents should be stored in dark bottles and/or in the dark.
5. Store buffers in large bottles. Pour each day's working aliquot into a smaller bottle or conical. *Never* pipet directly from the stock bottle.
6. Urea is heat and light sensitive. It is best to make a large quantity (e.g., 500 mL) of U9 and to immediately aliquot into 1-mL portions. These aliquots should be stored in the dark at –80°C. To make 100 mL of U9: 2 g of CHAPS, 54.054 g of urea, 60 mL H_2O, 5 mL 1 *M* Tris-HCl pH 9.0. When making U9, the solution will get cold (an endothermic reaction). Gentle heat may be applied but not so much that the solution goes above room temperature. Stir until dissolved. Add H_2O to 100 mL.
7. Matrices such as sinapinic acid and CHCA used for detection are light sensitive. These should be made fresh each day and stored in the dark when not in use.
8. Typically, it is recommended that serum be aliquoted into 25 µL portions at the time of initial preparation (i.e., in the clinical laboratory). If this has not been done, it should be done upon the first thaw.
9. The samples should be distributed randomly throughout the 96-well plate. Do not place all samples of one sample group (e.g., healthy) in one column or in one row.
10. Using pipet tips with the ends cut off can make the transfer of the beads easier.
11. The user should constantly shake the tube of 50% Q Ceramic HyperD F because the resin will sink to the bottom of the tube. If this is not done, less and less resin will be deposited from well to well in the 96-well filter plate.
12. The vacuum pressure should be set at 15 in Hg. Typical vacuum time is 1–2 min.
13. This step is included because there is a dead volume when pipetting with the robot; when

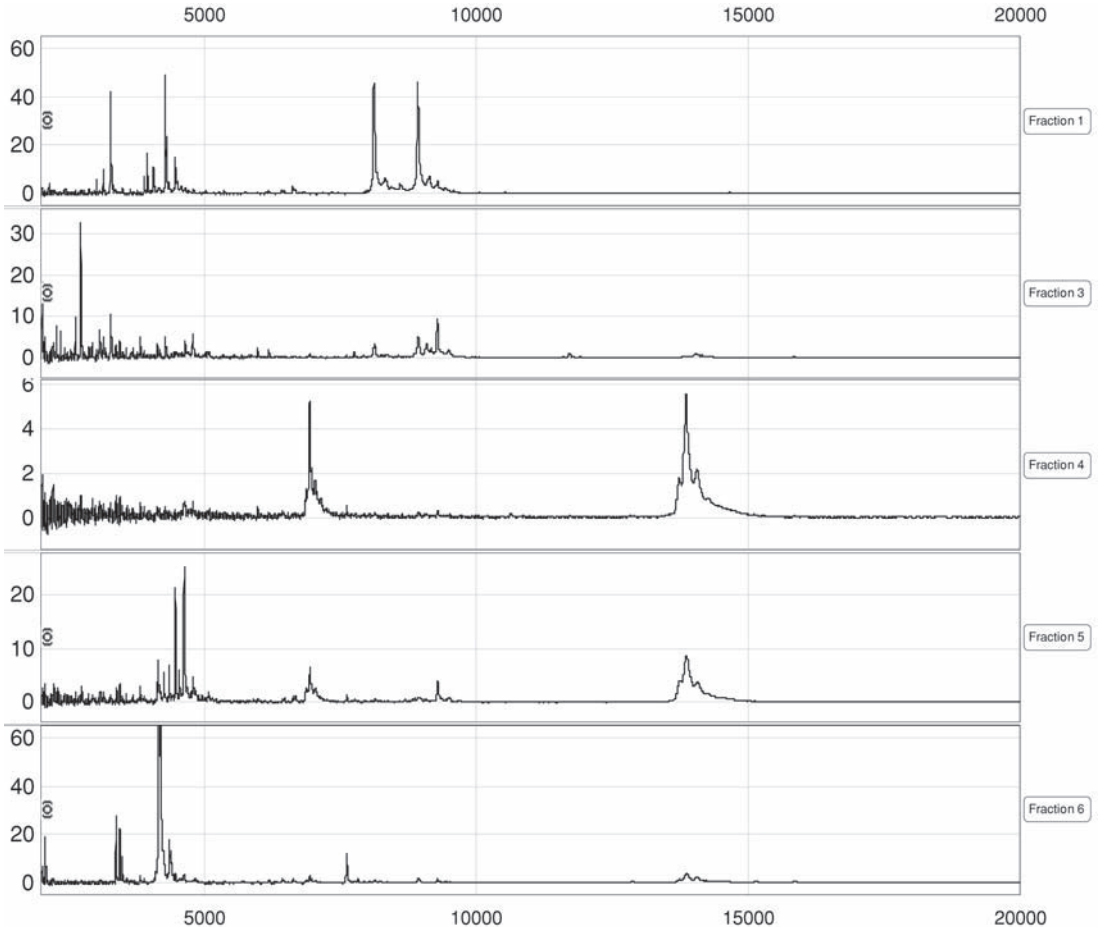

Fig. 1. Representative spectra of fractionated serum incubated on IMAC ProteinChip arrays, using sinapinic acid as the matrix.

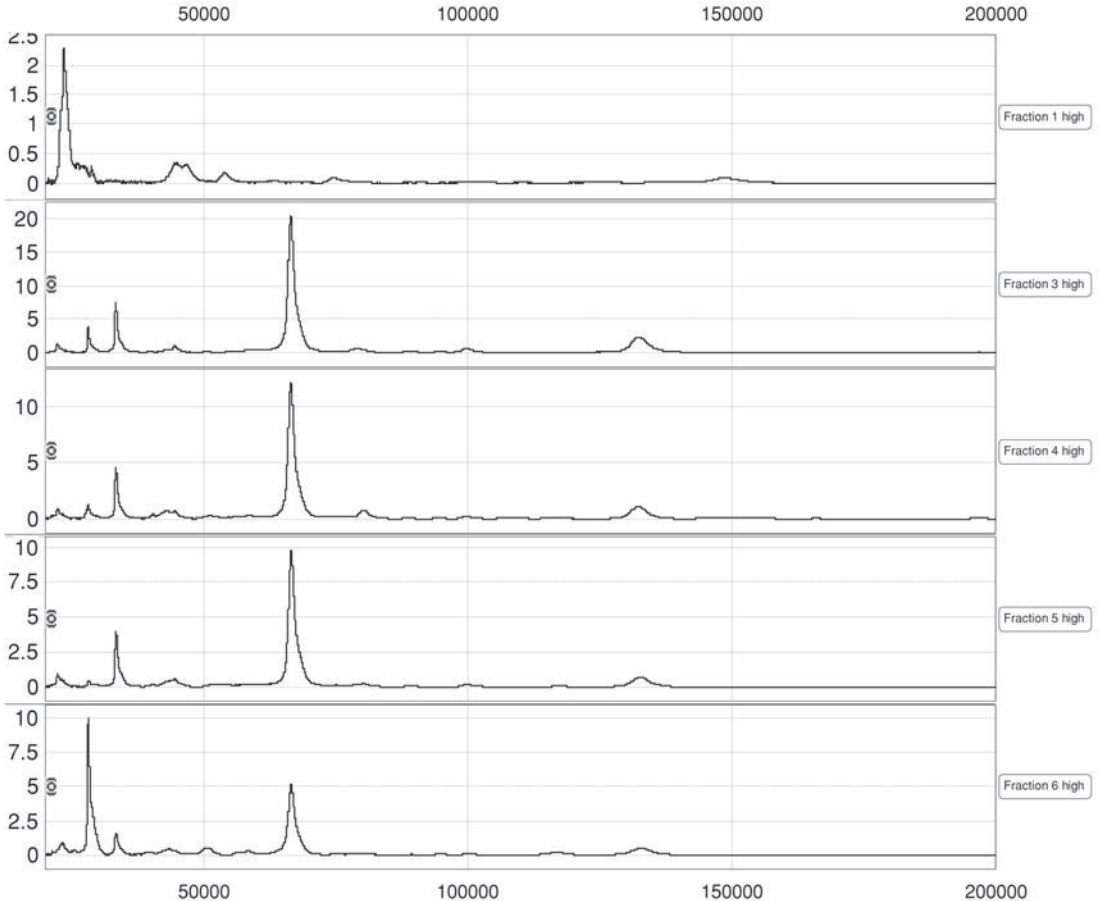

Fig. 2. Representative spectra of fractionated serum incubated on IMAC ProteinChip Arrays using sinapinic acid as the matrix, high-mass range.

the robot pipets to collect the sample the first time, it will not collect all the material. The addition of 50 μL U1 and mixing allows the residual material to be obtained and added to the first 50 μL. This step can be omitted if performing the protocol manually; in this case all of the material should be pipetted and applied to the resin.

14. To avoid cross-contamination between wells use adhesive sealing film on the microplate during the mix step. Remove the film before applying a vacuum, and replace with a new piece for each mixing.
15. Do not aliquot wash buffer 6 into the buffer tray until wash buffer 5 is being applied to the resin. This ensures that evaporation of the volatile organic solvents will not be an issue.
16. Different metals can be used to charge the IMAC3 array surface. In the authors' experience, copper binds the greatest number of proteins and, therefore, is useful for general protein-expression profiling. Magnesium, manganese, gallium, and iron generally demonstrate the least binding to proteins.
17. For purposes of assay monitoring, one should process a standard sample (pooled human serum is available from a variety of commercial sources) with the samples of interest. The spectra of the standard may look different from the spectra of the study samples because of differences in serum preparation by the commercial vendor. However, the spectra of the standard should look similar from assay run to assay run.

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Protein Arrays for Serodiagnosis of Disease

Tito Bacarese-Hamilton, Andrea Ardizzoni, Julian Gray, and Andrea Crisanti

Summary

Protein microarrays offer the possibility to circumvent most of the current limitations in the serodiagnosis of allergy, autoimmune, and infectious disease by allowing the simultaneous, multiparametric determination of specific subclasses of antibodies directed against many pathogenic antigens. Microarray immunoassays have been developed with these characteristics. A first-generation assay, for the serodiagnosis of infectious disease, allows the determination of IgG and IgM antibodies to various viral and bacterial antigens. In addition, a second-generation assay, designed for the serodiagnosis of allergic disease, permits the determination of IgE antibodies to various allergens implicated in allergic disease. Slides printed with antibody dilution curves and antigen are first incubated with serum samples and then subsequently with secondary antibodies. For detection of human IgG and IgM, fluorescently labeled secondary antibodies are employed. However, because of low-level concentrations of circulating IgE antibodies, a more sensitive protocol is required for human IgE detection. Here, fluorescence is delivered via the coupling of the secondary antibody to tyramide signal-amplification reagentry. Human IgG, IgM, or IgE bound to the printed antigens can then be revealed by confocal scanning microscopy and quantified with internal calibration curves. Generation of analytical and clinical data have demonstrated that the microarray test format provides equivalent performance to enzyme-linked immunosorbent assay (ELISA) tests and offers a significant advantage in convenience and cost when compared to traditional test formats.

Key Words:

Protein microarray; immunoassay; signal amplification; serodiagnosis; allergy; infectious diseases.

1. Introduction

In vitro immunoassays form the basis of many clinical diagnostic tests. Assays capable of antigen-specific antibody detection, in the form of ELISA-based tests ***(1)***, are widely available and are routinely employed in the clinical laboratory. However, certain characteristics of these assays, notably large sample/reagent volume require-

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ments, procedure-intensive protocols, and the absence of true parallelism, render them unsuitable for clinical applications that require the ability to determine numerous parameters in a timely and cost-effective manner ***(2,3)***. With the establishment of robust, automated microdeposition technology allowing the construction of high-density ordered arrays of molecules (microarrays) ***(4)***, the capability now exists that, in overcoming most of the current ELISA limitations ***(5)***, could support a truly parallel, multiparametric, immunoassay platform. Such miniaturized microspot ligand-binding assays were first described over a decade ago by Ekins et al. in the "ambient analyte theory," which demonstrated the feasibility of miniaturizing standard ligand-binding chemistry and predicted superior sensitivity for this assay format over others ***(6–8)***. The realization of miniaturized immunoassays, however, has been held back by the absence of the necessary instrumentation that is essential for their development and analysis. Driven by interest in high-density nucleic acid arrays for gene-expression studies, these tools have now become available as standard laboratory equipment and can be used for the generation of protein microarrays ***(9)***. Microdeposition technology, fluorescent scanners, and associated software are in place, enabling image generation and signal quantification from printed arrays of proteins. Early progress in protein arraying has seen the development of comparative fluorescence assays for measuring the concentrations of several specific proteins and antibodies in complex solutions ***(10–12)***. Furthermore, remarkable advances have been made in the development of antigen microarrays for the detection of specific antibodies in clinical samples ***(13–16)***. Results to date show that the microarray test format provides equivalent performance to ELISA (**ref.** ***13*** and **Table 1**), indicating that microarray tests can be incorporated in a fully automated random-access immunoassay platform for routine use in clinical chemistry laboratories.

Here, the authors document methods for the construction and analysis of two distinct microarray-based immunoassays. The first, tailored to the serodiagnosis of infectious disease, permits the simultaneous, quantitative determination of IgG and IgM antibodies to the ToRCH panel (*Toxoplasma gondii*, rubella virus, cytomegalovirus [CMV], and herpes simplex virus [HSV] types 1 and 2) in human serum. The second, designed for the serodiagnosis of allergic disease, permits the simultaneous, quantitative determination of serum IgE antibodies to various common allergens.

2. Materials

2.1. Equipment

1. Computer-controlled high-speed robot (Total Array System; Biorobotics).
2. S5000 scanner including ScanArray™ and QuantArray™ software (PerkinElmer).
3. Equipment for ELISA:
   (i) Microtiter plate washer: Autura plate washer (Mikura).
   (ii) Microtiter plate reader: Kinetic microplate reader, v max (Molecular Devices).
4. Spectrophotometer: DU 640 (Beckman Coulter).

2.2. Common Reagents and Materials

1. Silanized glass microscope slides (CEL Associates).
2. 96- or 384-well microtiter plates (Thermo Life Sciences).

3. Gene frames, 65 μL (1.5 × 1.6 cm) (Abgene).
4. Signal control: Alexa 546 dye (Molecular Probes, product no A—10237) in 2X phosphate-buffered saline (PBS) containing 0.1 mL/L Tween-20.
5. Negative control: 10 g/L bovine serum albumin (BSA) in 2X PBS containing 0.1 mL/L Tween-20.

2.3. Buffers

1. PBS 1X: 0.2 g/L KCl, 1.44 g/L Na_2HPO_4, 0.24 g/L KH_2PO_4, 8 g/L NaCl, pH 7.4.
2. Serum sample, horseradish peroxidase (HRP)-streptavidin conjugate and conjugated antibody diluent: 2X PBS containing 10 g/L BSA and 0.1 mL/L Tween-20.
3. Slide wash buffer: 1X PBS with 0.1 mL/L Tween-20.
4. Print buffers (*see* **Note 1**) for IgG/IgM and microbial antigens: 1X PBS containing 0.1 mL/L Tween-20 and polyvinylpyrrolidone (PVP, 10 g/L) (for human IgG and HSV1 antigen); sodium dodecyl sulphate (SDS, 0.1 g/L) (for human IgM); sucrose (100 g/L) (for CMV antigen).
5. Human IgE print buffer: 1X PBS containing 0.1 mL/L Tween-20 and 1 g/L SDS.
6. Slide blocking solution: 20 g/L BSA in 1X PBS.

2.4. Determination of Serum IgG/IgM

1. Succinimidyl Alexa 546/Alexa 594 fluorophores (Molecular Probes).
2. Microbial preparations: *T. gondii*, CMV, HSV types 1 and 2 (Radim); rubella virus antigen (grade K1S) (Microbix Biosystems).
3. Human IgG and IgM (reagent grade) (Sigma).
4. Human sera (BioMedical Resources).
5. Anti-human IgG monoclonal antibody (Radim).
6. Goat anti-human IgM antibody (OEM Concepts).

2.5. Determination of Serum IgE

1. Allergen extracts: *Dermatophagoides pteronyssinus* (D1), *Dermatophagoides farinae* (D2), *Alternaria alternata* (M6), *Olea europaea* (T9), *Artemisia vulgaris* (W6), *Dactylis glomerata* (G3), and house dust (H2) (Allergon).
2. Human IgE purified from myeloma plasma (Calbiochem).
3. Human sera (Radim).
4. HRP-streptavidin conjugate (0.1 mg/mL), Alexa 546-tyramide conjugate, amplification buffer, and 300 mL/L hydrogen peroxide as provided in the TSA™ kit no. 23 (Molecular Probes).
5. Biotinylated anti-human IgE at a final concentration of 1 mg/mL (KPL).

3. Methods

The methods detailed below describe (1) the silanization of glass slides, (2) the preparation of proteins and antibodies for printing, (3) the printing of proteins and antibodies onto silanized slides, (4) the processing of arrayed slides for IgG/IgM determination, (5) the processing of arrayed slides for IgE determination, (6) the scanning of processed slides, and (7) the quantitative analysis of the image data.

3.1. Silanization of Glass Slides

Commercial aminosilane-coated slides provided by Cel Associates are routinely used in the authors' laboratory. Some commonly used silanization methods have been documented ***(17)***.

3.2. Preparation of Proteins and Antibodies for Printing

The arrays employed in the authors' investigations are usually designed as 7 × 7 matrices. This format incorporates IgG and IgM antibodies and microbial antigens, that is, the ToRCH panel (*see* **Fig. 1A**), or alternatively IgE antibodies and allergens (*see* **Fig. 2A**). The respective antibodies are printed at known concentrations and serve as internal calibration curves. The panels also feature signal and negative controls.

3.2.1. Preparation of Human IgG and IgM for Printing

1. Human IgG, as provided by Sigma, is in the form of a salt-free lyophilized powder and can be stored at 2–8°C. Reconstitution is in 8.7 g/L NaCl to a final concentration of 1 mg/mL, and the protein concentration is determined after reconstitution by absorbance at 280 nm.
2. Once reconstituted, the IgG is aliquoted and stored at –20°C. Repeated freezing and thawing is not recommended.
3. Human IgM is supplied by Sigma as a 10 mg aliquot in 6.1 g/L Tris-HCl, 11.7 g/L NaCl, pH 8.0, containing 0.9 g/L sodium azide as a preservative. As for the IgG, the protein concentration is determined by absorbance at 280 nm. This solution is stored at 2–8°C. If turbidity should occur on prolonged storage, the solution can be clarified by means of centrifugation before use.
4. The IgG calibration curve (2–50 μg/mL) is prepared by diluting the human IgG preparation in 1X PBS containing Tween-20 (0.1 mL/L) and PVP (10 g/L).
5. The IgM calibration curve (0.4–8 μg/mL) is prepared by diluting the human IgM preparation in 1X PBS containing Tween-20 (0.1 mL/L) and SDS (0.1 g/L). The authors' recommendation is that the human IgG and IgM antibodies not be reused once diluted in their respective printing buffers.

3.2.2. Preparation of Microbial Antigens

All the ToRCH microbial antigens are initially diluted in 1X PBS containing Tween-20 (0.1 mL/L) and printed at a concentration of 50 μg/mL. In addition, PVP (10 g/L) is included in the HSV1 antigen solution and sucrose (100 g/L) in the CMV antigen solution. All microbial antigens are stored as aliquots at –80°C.

3.2.3. Preparation of Controls for the IgG/IgM Microarray

A solution of 10 g/L BSA in 2X PBS containing 0.1 mL/L Tween-20 is employed as a negative control. A solution of Alexa 546 dye (Molecular Probes) in the signal-control buffer is employed as a signal control.

3.2.4. Preparation of Human IgE

1. Human IgE provided by Calbiochem is diluted in 1X PBS to a concentration of 100 μg/mL and stored at –20°C, according to the manufacturer's instructions. The resulting solution should not undergo freeze–thaw cycles. To avoid this problem, the solution is aliquoted prior to storage.
2. The IgE calibration curve (2–200 ng/mL) is prepared by diluting the human IgE preparation in 1X PBS containing Tween-20 (0.1 mL/L) and SDS (1 g/L). As with the human IgG and IgM antibodies, the human IgE should not be reused once diluted in its printing buffer.

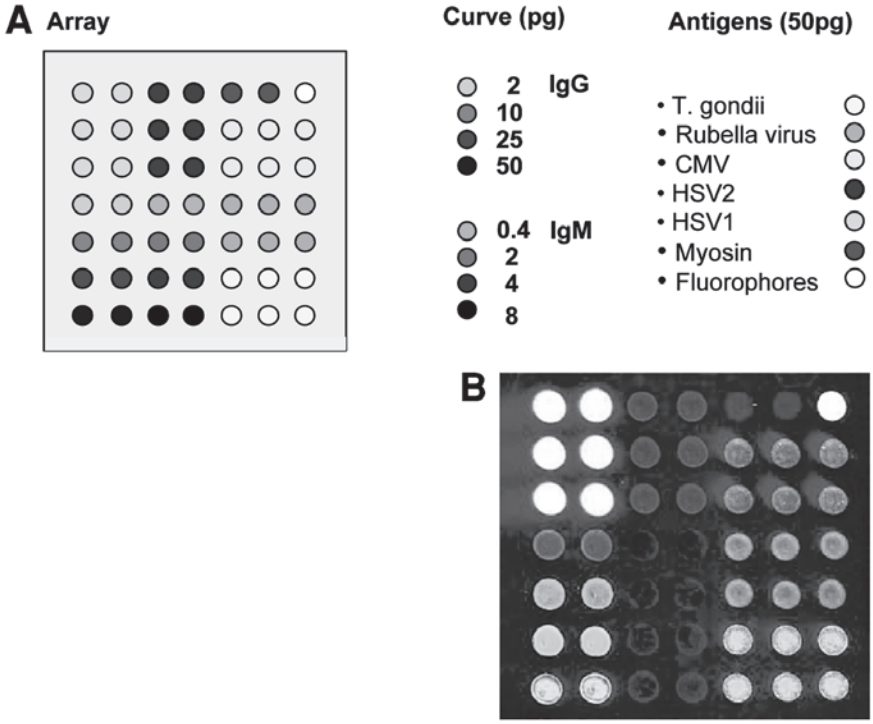

Fig. 1. Schematic array format and fluorescent scan for the microbial array. **(A)** Schematic representation of the microbial array format. **(B)** Fluorescent scan of microbial array incubated with serum and developed with Alexa 546-labelled secondary antibody.

3.2.5. Preparation of Allergens

1. Allergens are obtained as 1-g samples and are reconstituted in 1X PBS, pH 7.2, to a concentration of 10 mg/mL and stirred overnight at room temperature.
2. After centrifugation at 503*g* for 30 min at 10°C, the allergen extracts are filtered in four stages: first through Whatman paper (125-mm diameter, cat. no. 1001125), then through 5 μm filter disks (Pall Corp, cat. no. 4199), then through 0.45 μm filter units (Schleicher & Schuell, cat. no. 10462 450), and finally through 0.2 μm filter units (Schleicher & Schuell, cat. no. 10462 200).
3. Extracts are stored frozen at –20°C as 1.5-mL aliquots.

3.2.6. Preparation of Controls for the IgE Microarray

A solution of 10 g/L BSA in 2X PBS containing 0.1 mL/L Tween-20 is employed as a negative control. A solution of Alexa 546 dye (Molecular Probes) in the signal-control buffer is employed as a signal control. This choice of fluorophore is based on the fact that the signal-amplification technique used to process the slides makes use of the same fluorophore coupled to the tyramide.

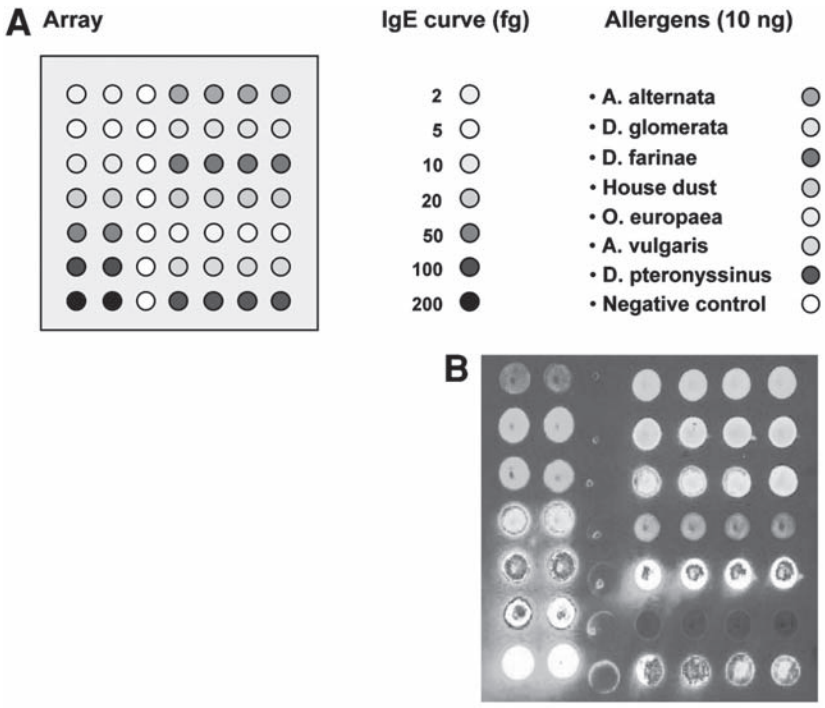

Fig. 2. Schematic array format and fluorescent scan for the allergen array. **(A)** Schematic representation of the allergen array format. **(B)** Fluorescent scan of allergen array incubated with serum and developed using the tyramide amplification protocol.

3.3. Printing of Proteins and Antibodies onto Silanized Slides

3.3.1. Preparation of the Source Plate

All the solutions for printing (antibodies/antigens/allergens) are transferred into a microtiter plate (96- or 384-well). In the authors' laboratory, 384-well plates are commonly employed for this purpose. Depending on the number of replicate arrays required, a corresponding number of pins are employed and the appropriate number of wells filled with each printing solution. At least 50 μL of solution is added to each well. The solutions are prepared and transferred into the source plate just before the start of the print run.

3.3.2. Printing

The aforementioned antibodies, antigens, or allergens are contact printed on silanized glass slides by means of computer-controlled high-speed robotics. The samples are transferred from 384-well microtiter plates to glass slides by use of

Table 1
Comparison of Microarray and ELISA for Serum Reactivity

	ELISA		Microarray			
Allergens	Positive	Negative	Positive	Negative	Sensitivity %	Specificity %
D. pteronyssinus	11/22	11/22	13/22	9/22	90.9	72.7
D. farinae	8/16	8/16	9/16	7/16	87.5	75
O. europaea	7/22	15/22	8/22	14/22	71.4	80
A. alternata	3/20	17/20	4/20	16/20	100	94.1
A. vulgaris	2/22	20/22	2/22	20/22	50	95
D. glomerata	6/22	16/22	7/22	15/22	83.3	87.5

ELISA, enzyme-linked immunosorbent assay.

The clinical sensitivity is calculated by expressing the number of samples that are positive by microarray immunoassay as a percent of the number of samples positive for the disease (i.e., positive by ELISA).

The clinical specificity is calculated by expressing the number of samples that are negative by the microarray immunoassay as a percent of the number of samples negative for the disease (i.e., negative by ELISA).

As an example, for Dactylis glomerata:

	Test positive	Test negative
Disease present: 6	5	1
Disease absent: 16	2	14

Therefore:
Sensitivity = 5/6 = 83.3%
Specificity = 14/16 = 87.5%

stainless steel solid pins (200 μm diameter). Each pin is estimated to transfer approx 1 nL of sample to the slide and produces spots with a pitch of 0.6 mm. After deposition of each print solution, pins are washed first with dH_2O, then with 70% ethanol, and finally dried.

3.3.3. Storage of Printed Slides

When the printing process has been completed, the slides remain inside the robot printer cabinet for at least 12 h. This stable and controlled environment (approx 55% humidity and 25°C) is conducive to the binding of the antibodies and the antigens/allergens to the slide surface. Printed slides are stored in the dark in boxes containing silica gel bags. It is critical that the slides are kept dry; slides that have been exposed to moisture on storage give imprecise and varying signals.

3.4. Processing of Microarrayed Slides for the Determination of IgG/IgM in Human Serum

3.4.1. Preliminary Procedures

3.4.1.1. Serum Samples

Human sera are first analyzed for their reactivity against *T. gondii*, rubella virus, HSV1, HSV2, and CMV antigens by means of ELISAs (e.g., Radim EIA Well). The assays are performed according to the manufacturer's instructions. Serum samples are diluted 1:200 in serum sample diluent (*see* **Subheading 2.3.**) for determination of antibody reactivity to the ToRCH antigens. Samples are categorized as positive, negative, or equivocal according to the manufacturer's instructions.

3.4.1.2. Fluorescent Labeling of Antibodies (*see* **Note 2**)

Antibodies are labeled with the fluorophore of choice using a protein-labeling kit, for example, Alexa Fluor 546 protein-labeling kit (Molecular Probes). The efficiency of the labeling process is determined by measuring the molar ratio of dye incorporated in the protein.

3.4.1.3. Preparation of the Slides for Processing

The first step in the processing of slides is the sticking of an adhesive frame (GeneFrame) around the printed array. This frame serves two purposes: first, samples and reagents are contained within the array area; second, the frame limits the developing area to where the array is printed, thereby minimizing reagent use.

To block nonspecific binding to the activated slide surface, the printed slides are incubated for 60 min at room temperature with the slide blocking solution (*see* **Subheading 2.3.**). Staining jars with at least 50 mL of blocking solution are commonly used for this purpose.

3.4.2. Processing of Slides

After blocking and gentle rinsing with wash buffer (five times 1 mL) (*see* **Subheading 2.3.**), sera (150 μL) are pipetted and incubated on the arrayed slides for 15 min at room temperature in a humid chamber. The use of humid chambers ensures that evaporation of samples/reagents is kept to a minimum. After the serum incubation, slides are rinsed with wash buffer (five times 1 mL).

To reveal the IgG and IgM bound to the printed antigens, two different protocols are followed:

(i) IgG bound to the printed antigens are detected by incubating the slides for 5 min at room temperature with Alexa 546-labeled anti-human IgG (150 μL) monoclonal antibody, diluted in the appropriate buffer.

(ii) IgM bound to the printed antigens are revealed by incubating the slides for 5 min at room temperature with Alexa 594-labeled goat immunoglobulins (150 μL) directed against the human IgM μ chain. It is possible to perform both processes simultaneously by incubating with a mixture of an anti-IgG and an anti-IgM-antibodies labeled with the different fluorophores.

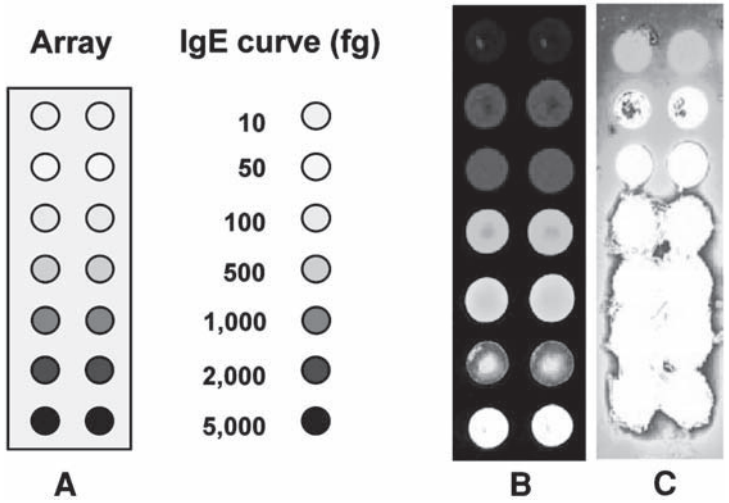

Fig.3. Detection of IgE by the non-amplified and amplified protocols. **(A)** Schematic of IgE dilution curve. **(B)** Detection of IgE by Alexa 546-labelled anti-human IgE. **(C)** Detection of IgE by biotinylated anti-human IgE followed by HRP-streptavidin and Alexa 546 tyramide conjugate.

The slides are rinsed with wash buffer (five times 1 mL) and dried at 37°C prior to reading in the fluorescent scanner.

3.5. Processing of Microarrayed Slides for Determination of IgE in Human Serum

For this purpose, a protocol incorporating signal amplification has been identified and is described below (*see* **Subheading 3.5.2.**) and in **Fig. 3**. The amplification protocol is required to increase test sensitivity because human IgE circulates at very low concentrations.

3.5.1. Preliminary Procedures

3.5.1.1. Serum Samples

Undiluted serum samples are first analysed with a commercial ELISA (e.g., Radim S.p.A.). ELISAs are performed and samples categorized according to the manufacturer's instructions.

3.5.1.2. Preparation of the Slides for Processing

See **Subheading 3.4.1.3.**

3.5.2. Processing of Slides

Slides are incubated with serum (150 µL) for 60 min at room temperature and then thoroughly rinsed with wash buffer (five times in 1 mL). A 60-min incubation with a biotinylated anti-human IgE (150 µL) at a final concentration of 1 mg/L in the appropriate diluent is performed at room temperature. After rinsing with wash buffer (five

times 1 mL), slides are incubated for a further 60 min at room temperature with a HRP-streptavidin conjugate diluted 1:100 in the appropriate diluent (*see* **Subheading 2.3.**). The resulting streptavidin-biotinylated antibody complexes are detected by incubating the slides for 15 min at room temperature with Alexa 546-tyramide conjugate, diluted 1:100 with Molecular Probes' diluent (namely amplification buffer) containing 0.015 mL/L hydrogen peroxide (*see* **Note 3**). On completion of the final processing step, the Gene-Frame is removed and slides are dried at 37°C for 10–15 min. Slides are then ready to be scanned.

Note: The HRP-streptavidin and Alexa 546-tyramide conjugates, hydrogen peroxide, and the amplification buffer are all provided in the TSA kit no. 23 supplied by Molecular Probes.

3.6. Scanning of Processed Slides

Scanning is performed using ScanArray™ software, according to the manufacturer's instructions. Images (**Figs. 1B** and **2B**) are optimized using fine-tuning capabilities provided (notably, adjustment of laser/photomultiplier tube [PMT] settings) and stored as bitmap files.

3.7. Quantitative Analysis of Image Data

Quantification is achieved using QuantArray™ software according to the manufacturer's instructions. Background signal and fluorophore crosstalk (if appropriate) are automatically subtracted.

Data are stored as Excel files. Calibration curves incorporated in each protein array are fitted using the appropriate curve-fitting routine and provide a basis on which to correlate the signal generated against a concentration of the analyte under determination. Signals from printed antigens are then interpolated from these calibration curves.

4. Notes

1. Printing solutions: Tween-20 is the surfactant of choice; print quality is much improved by the addition of 0.01% Tween-20 to all print solutions. PVP is used for printing some antigens because it retains a hydration shell surrounding the molecules in solution, allowing them to maintain their tridimensional structure. Sucrose is used for similar reasons; furthermore, sucrose is critical for the stability of the CMV antigen.
2. Signal generation: Fluorescent end points are used as the signal generation option of choice. Other end points have been described (such as colorimetric, chemiluminescent) but direct labeling of proteins with fluorophores to generate high specific activity and stable labels is easy to perform, and instrumentation is widely available for the determination of the emitted signal. It is preferable to choose fluorophores with spectral properties >500 nm because many proteins in blood fluoresce <500 nm, and background signals are much reduced at these wavelengths. The Alexa range of dyes (Molecular Probes) and the Cy dyes (Amersham Biosciences) can be recommended for this type of protein array immunoassay.
3. Signal amplification using tyramide reagentry: In the presence of hydrogen peroxide, HRP activates the dye-labeled tyramide derivatives creating extremely reactive short-lived tyramide radicals that bind to nucleophilic residues on the slide surface. This results in significant amplification of the bound signal. Use of this type of chemistry can increase signal generation by 50- to 100-fold.

Appendix

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