

Carbohydrate Antigen Microarrays

Denong Wang

Abstract

This chapter describes one of my laboratory's working protocols for carbohydrate-based microarrays. Using a standard microarray spotter, we print carbohydrate antigens directly on the nitrocellulose-coated bioarray substrates. Because these substrates support noncovalent immobilization of many spotted antigens, in general no chemical modification of the antigen is needed for microarray production. Thus, this bioarray platform is technically simple and applicable for high-throughput construction of carbohydrate antigen microarrays. A number of nitrocellulose-coated glass slides with different technical characteristics are commercially available. Given the structural diversity of carbohydrate antigens, examining each antigen preparation to determine the efficacy of its immobilization in a given type of substrate and the surface display of the desired glycoepitopes in a microarray assay is essential.

Key words: Carbohydrate microarrays, Glycoconjugate, Glycoprotein, Glycolipid, Glycoepitope, Glycomics, Nitrocellulose, Polysaccharide

1. Introduction

Although recognition of carbohydrate antigens began in the early twentieth century with the study of microbial polysaccharides (1), the scope of investigation into these antigens has since been substantially extended (2). Conceptually, carbohydrate antigens are carbohydrate-containing macromolecules that can evoke and react with carbohydrate-specific antibodies. In terms of their structural characteristics, carbohydrate antigens are polysaccharides with solely carbohydrate moieties and various forms of glycoconjugates. The latter include natural glycoproteins and glycolipids of living cells, as well as synthetic antigens produced by coupling mono- and oligosaccharides to a larger carrier molecule.

Carbohydrate moieties are unsurpassed in generating structural diversity and are prominent in surface displays. They may be specifically or selectively recognizable by either soluble or membrane-bound cellular proteins. In the higher eukaryotic species, the expression of cellular glycoconjugates, and especially their complex carbohydrate structures, is frequently cell type or tissue specific. In microbes, many sugar chains, including those displayed on the surface of a microbial cell and those secreted outside it, have been recognized as “signatures” of specific pathogens. Exploring the biological information content of sugar chains is a current focus of glycomics research.

A number of experimental approaches have been developed to construct carbohydrate-based microarrays to facilitate the exploration of sugar chain diversity and its biomedical significance (3–13). These carbohydrate microarrays are all solid-phase binding assays for carbohydrates and their interaction with other biological molecules. In spite of their technological differences, they share a number of common characteristics and technical advantages. First, they have the capacity to display a large panel of carbohydrates in a limited chip space. Second, the amount needed to spot each carbohydrate is drastically smaller than that required for a conventional molecular or immunological assay. Third, the microarray-based assays have higher detection sensitivity than most conventional molecular and immunological assays; this increased sensitivity is due to the fact that the binding of a molecule in solution phase to an immobilized microspot of ligand in the solid phase minimally reduces the molar concentration of the molecule in solution (14). Therefore, it is much easier to have a binding equilibrium take place in a microarray assay and result in a high sensitivity.

This chapter presents the method of nitrocellulose-based immobilization of carbohydrate-containing macromolecules. This method is suitable for the high-throughput construction of carbohydrate antigen microarrays (2, 3, 6, 10, 15), and thus is readily applicable for the large-scale immunological characterization of carbohydrate antigens and anticarbohydrate antibodies and the interaction of carbohydrates and other receptors.

2. Materials

2.1. Apparatus

1. Microspotting: Cartesian PixSys 5500C (Cartesian Technologies, Irvine, CA).
2. Bioarray Substrates: FAST Slides (Schleicher & Schuell, Keene, NH).
3. Scanner: ScanArray 5000A Microarray Scanner (PerkinElmer, Torrance, CA).

2.2. Software

1. Array Design: CloneTracker (Biodiscovery, Marina del Rey, CA).
2. Array Printing: Cartesian AxSys (Cartesian Technologies, Irvine, CA).
3. Array Scanning: ScanArray Express (PerkinElmer, Torrance, CA).
4. Array Data-processing and Statistic Analysis: JMP-Genomics (SAS Institute Inc., Cary, NC).

2.3. Reagents and Buffers

1. Species-specific anti-immunoglobulin antibodies and their fluorescent conjugates, Cy3, Cy5, or FITC (Sigma, St. Louis, MO; BD-Pharmingen, San Diego, CA).
2. Dilution buffer: Saline (0.9% NaCl).
3. Rinsing solution: 1× phosphate-buffered saline (PBS), pH 7.4 with 0.05% (vol/vol) Tween 20.
4. Blocking solution: 1% bovine serum albumin (BSA) (wt/vol) in 1× PBS with 0.05% (wt/vol) NaN_3 .

3. Methods

The key steps in our carbohydrate microarray applications are:

1. Designing and constructing sugar arrays.
2. Microspotting molecules onto bioarray substrates.
3. Immunostaining arrays.
4. Microarray scanning and data analysis.
5. Validating microarray data using conventional immunological assays.
6. Identifying carbohydrate-based biomarkers using carbohydrate microarrays.

Each of these steps is described below.

3.1. Designing and Constructing Carbohydrate Antigen Arrays

The full surface of a microscope slide can be used to construct a “repertory” carbohydrate microarray with ~20,000 spots capacity for biomarker discovery. A multichamber, subarray system can also be used to construct customized, carbohydrate microarrays for defined purposes (16). For example, each glass slide is separated into 8 subarrays with 488 microspots spotted per subarray; each spot is approximately 200 μm , and the spots are situated at 300- μm intervals, center to center. A single slide of this design is suitable for eight microarray assays. Our laboratory has been using the latter approach more often in our research and clinical applications.

Repeats and dilutions: We usually print carbohydrate antigens at an initial concentration of 0.1–0.5 mg/ml. The amount of antigen or antibody solution printed on the chip substrate is in the range of 0.5–1.0 nL per microspot. The carbohydrate antigens are further diluted at 1:3, 1:9, and 1:27. A given concentration of each preparation is repeated at least three times.

Antibody isotype standard curves: Antibodies of IgG, IgA, and IgM isotypes of corresponding species are diluted in 1× PBS, pH 7.4, and printed at given concentrations to serve as standard curves in the microarray format. Usually, they are applied at an initial concentration of 0.1 mg/ml and further diluted at 1:5, 1:25, 1:125, and 1:625. This design allows antibody signals that are captured by spotted carbohydrate antigens to be quantified by comparing the signal levels with the standard Ig curves of corresponding antibody isotypes. In addition, such standard curves are useful for microarray data normalization and cross-chip scaling of microarray detection.

3.2. Printing Carbohydrate Arrays onto Nitrocellulose Slides

Using Cartesian Technologies' PixSys 5500C, a high-precision robot designed for cDNA microarrays, carbohydrates of various complexities (see Note 1) are picked up by quill pins dipped into carbohydrate solutions and printed onto bioarray substrates. The complementary Cartesian AxSys software is used to instruct movement of pins about the dispense platform and the printing process. Using the nitrocellulose-coated glass slides as bioarray substrates, carbohydrate antigens are immobilized to the nitrocellulose by physical–chemical adsorption involving noncovalent interactions, such as H bonding, ionic, and hydrophobic interactions with the substrate. The specific steps are the following.

1. Prepare samples of carbohydrate antigens in 0.9% NaCl at 0.1–0.5 mg/ml and transfer them in 96-well plates (see Notes 2 and 3).
2. Place the 96-well plates containing the samples and standards on the Cartesian arrayer robot for printing.
3. Adjust the print program so that the carbohydrate antigens and antibodies are printed at spot sizes of ~150 μm and at 375-μm intervals, center to center.
4. Spot each antigen and antibody as triplet replicates in parallel.
5. Air dry the printed carbohydrate microarrays and store at room temperature overnight (see Note 5).

3.3. Immunostaining Carbohydrate Microarrays

The staining procedure for carbohydrate microarrays is basically identical to the routine procedure for immunohistology. Immunostaining steps of carbohydrate arrays are listed below (see Notes 4, 6, and 7).

1. Rinse printed microarray slides with 1× PBS, pH 7.4, and 0.05% Tween 20 for 5 min.

2. Block slides with 1% BSA in PBS containing 0.05% NaN_3 at room temperature for 30 min.
3. Stain each subarray with 50 μl of test sample, which is diluted in 1% BSA PBS containing 0.05% NaN_3 and 0.05% Tween 20.
4. Incubate the slide in a humidified chamber at room temperature for 60 min.
5. Wash slides five times with $1\times$ PBS, pH 7.4, and 0.05% Tween 20.
6. Stain slides with 50 μl of titrated secondary antibodies. Antihuman (or other species) IgG, IgM, or IgA antibodies with distinct fluorescent tags, Cy3, Cy5, or FITC, are mixed and then applied on the chips.
7. Incubate the slide in a humidified chamber with light protection at room temperature for 30 min.
8. Wash slides five times.
9. Place slide in a 50-ml falcon centrifuge tube and spin at 1,000 rpm for 2 min to remove washing buffer.
10. Cover slides in a histology slide box to prevent fluorescent quenching of signal by lights.

3.4. Microarray Scanning and Data Analysis

We scan microarray with ScanArray5000A Microarray Scanner (PerkinElmer Life Science) following the manufactory user manual. Fluorescence intensity values for each array spot and its background were calculated using ScanArray Express software. A positive staining result is considered if the mean fluorescent intensity value of microspot is significantly higher than the mean background of the identically stained microarray with the same fluorescent color.

Further microarray data analysis requires specialized software package and guidance of bioinformatics and statistical experts. It is important to conceptually understand the functions of the bioinformatics tools in use in order to correctly interpret the results. Our general approach is to process carbohydrate array datasets using JMP-Genomics software from SAS Institute. In brief, antigen-specific antibody reactivity is shown as microarray scores, which are the \log_2 transformed microarray values (mean and/or mean minus background). Then, we utilized an antigen-by-antigen ANOVA model to obtain statistically significant differences. Data from triplicate spots for each antigen were included in the ANOVA model for that antigen. A cutoff to detect significant differences is determined by applying a multiple testing correction to statistical results from the ANOVA model.

3.5. Validation and Further Investigation of Microarray Observations

A microarray finding may require further validation by other experimental approaches. We usually confirm our results by at least one of the alternative immunoassays, such as ELISA, Dot blot, Western Blot, flow cytometry, and immunohistology. However, the epitopes

or antigenic determinants displayed by a carbohydrate antigen in a specific biarray substrate may not be necessarily identical to those that are displayed by other assay systems. Thus, there is possibility that a “chip hit” is not reproduced by other assays. In such circumstances, one may conduct multiple carbohydrate array assays to confirm the initial microarray observation.

3.6. Identification of Carbohydrate-Based Biomarkers Using Carbohydrate Microarrays

Our laboratory is interested in carbohydrate-based biomarkers, especially the immunogenic sugar moieties that play key roles in the host recognition of complex carbohydrates and immune responses to carbohydrate antigens. To facilitate these investigations, we explore the use of multiple platforms of carbohydrate microarrays. These include the carbohydrate antigen microarrays described here, a method for photogeneration of oligosaccharide arrays (11, 17), and methods for constructing carbohydrate cluster microarrays (13). Investigations using these technologies have led to the discovery of autoimmunogenic sugar moieties of SARS-CoV (10), a highly potent immunological target of *Bacillus anthracis* exosporium (*In this Issue, Proteomics*, 7(2), pNA, and 11) and a number of glycan markers of human tumors (18).

Conceptually, we take the advantage that the immune systems of many animal species are able to recognize subtle changes of sugar moieties displayed by cells or by soluble antigens. Technically, we make the use of complementary methods to diversify and extend the repertoires of glycoepitopes in bioarray substrates. It is important to note that carbohydrate microarrays constructed by various methods may differ in their technical features and suitability for a given practical application. For example, they may differ in the detection specificity. The carbohydrate antigen microarray discussed here would be antigen specific but not epitope specific, if the native carbohydrate antigens were spotted. This is owing to the fact that many carbohydrate antigens, such as polysaccharides, glycoproteins, and glycolipids, display more than one antigenic determinant, including glyco- and nonglycoepitopes. Examining the finer details of the binding properties would require the use of microarrays of pure saccharide sequences. The mono- and oligosaccharide array-based binding assays, such as sister chapters in this book, may be employed, in combination with saccharide competition assays, to decipher precise saccharide components of a specific antigenic determinant or glycoepitope (6, 11, 13).

4. Notes

1. Antigen preparations suitable for this high-throughput biochip platform: Carbohydrate antigens of multiple structural configurations, including polysaccharides, natural glycoconjugates, oligosaccharide–protein, and oligosaccharide–lipid conjugates, are applicable for platform of carbohydrate antigen microarrays (3, 6, 10). In addition to printing carbohydrate microarrays, this platform is also applicable for producing protein microarrays (15). As with carbohydrate microarrays, there is no need to chemically conjugate a protein for its surface immobilization. However, it is recommended that each antigen preparation be tested on chip substrate for the efficacy of immobilization and expression of antigenic determinants.
2. Preservation of polysaccharides and glycoconjugates: Purified polysaccharides are generally stored as dried powder at room temperature. They can also be preserved in saline solutions (0.9% NaCl) containing a droplet of chloroform and stored at 4°C for a long period of time. Glycoconjugates are structurally diverse. Conditions for their storage are likely variable although it is practical to store them as smaller aliquots in Revco freezers (–80°C) to avoid multiple freezing and thawing of given preparation.
3. Printing of samples: Before loading sample solutions onto the arrayer, it is important to spin the solution in an Eppendorf centrifuge at maximum speed (at least 15,000×g) for at least 15 min. Before and after each arraying experiment, it is recommended to examine and clean the printing pins following a routine pin cleaning protocol recommended by the corresponding printing pin manufacturers. A test slide is usually implemented to optimize quality of printing. The water supply of the Cartesian arrayer should constantly be checked during the arraying experiment to ensure adequate flow to the wash chamber.
4. Examination of the presence of samples on array and their antigenic structures: It is essential to examine whether proteins, synthetic peptides, and carbohydrates are successfully “printed” and whether desired epitopes or antigenic determinants are preserved on the chips. The printed microarrays can be incubated with antibodies, receptors, or lectins known to react with the printed substance. The reaction is detected either by conjugating directly a fluorochrome to the “detector” or by a second-step staining procedure.
5. Storage of printed carbohydrate and protein microarrays: The arrays are usually air dried and stored at room temperature. For long-term preservation, the chips can be sealed in a vacuum-dried plastic bag with desiccant and stored at –20°C.

6. Staining considerations: After the last wash between each staining step, it is important to completely withdraw the wash buffer from inside reaction chambers. Otherwise, the remaining buffer may lower antibodies' concentration to be analyzed.
7. Biosafety procedures: When working with chemicals, suitable protective wear, such as lab coat and disposable gloves, are advised. When human serum specimens are involved, experiments must be conducted in accordance to the standard biosafety procedures instituted by CDC and WHO.
8. Scanning and data collection: Training with the technical experts of PerkinElmer is necessary before performing microarray scanning and data collection using the ScanArray Express software package.

Acknowledgments

Development of the carbohydrate microarrays described in this chapter was partially supported by NIH grants R21AI45326 and U01CA128416 to D. Wang.

References

1. Dochez, A. R., and Avery, O. T. (1917) The elaboration of specific soluble substance by pneumococcus during growth, *J. Exp. Med.* 26, 477–493.
2. Wang, D. (2004) Carbohydrate Antigens, *In: Encyclopedia of Molecular Cell Biology and Molecular Medicine*, (ed. Robert A. Meyers) II, 277–301.
3. Wang, D., Liu, S., Trummer, B. J., Deng, C., and Wang, A. (2002) Carbohydrate microarrays for the recognition of cross-reactive molecular markers of microbes and host cells, *Nat Biotechnol* 20, 275–281.
4. Willats, W. G., Rasmussen, S. E., Kristensen, T., Mikkelsen, J. D., and Knox, J. P. (2002) Sugar-coated microarrays: A novel slide surface for the high-throughput analysis of glycans, *Proteomics* 2, 1666–1671.
5. Fazio, F., Bryan, M. C., Blixt, O., Paulson, J. C., and Wong, C. H. (2002) Synthesis of sugar arrays in microtiter plate, *J Am Chem Soc* 124, 14397–14402.
6. Fukui, S., Feizi, T., Galustian, C., Lawson, A. M., and Chai, W. (2002) Oligosaccharide microarrays for high-throughput detection and specificity assignments of carbohydrate-protein interactions, *Nat Biotechnol* 20, 1011–1017.
7. Houseman, B. T., and Mrksich, M. (2002) Carbohydrate arrays for the evaluation of protein binding and enzymatic modification, *Chem Biol* 9, 443–454.
8. Park, S., and Shin, I. (2002) Fabrication of carbohydrate chips for studying protein-carbohydrate interactions, *Angew Chem Int Ed Engl* 41, 3180–3182.
9. Adams, E. W., Ratner, D. M., Bokesch, H. R., McMahan, J. B., O'Keefe, B. R., and Seeberger, P. H. (2004) Oligosaccharide and glycoprotein microarrays as tools in HIV glycobiology; glycan-dependent gp120/protein interactions, *Chem Biol* 11, 875–881.
10. Wang, D., and Lu, J. (2004) Glycan arrays lead to the discovery of autoimmunogenic activity of SARS-CoV, *Physiol Genomics* 18, 245–248.
11. Wang, D., Carroll, G. T., Turro, N. J., Koberstein, J. T., Kovac, P., Saksena, R., Adamo, R., Herzenberg, L. A., Herzenberg, L. A., and Steinman, L. (2007) Photogenerated glycan arrays identify immunogenic sugar moieties of *Bacillus anthracis* exosporium, *Proteomics* 7, 180–184.
12. Zhou, X., and Zhou, J. (2006) Oligosaccharide microarrays fabricated on aminoxyacetyl functionalized glass surface for characterization of carbohydrate-protein interaction, *Biosensors & Bioelectronics* 21, 1451–1458.

13. Zhou, X., Turchi, C., and Wang, D. (2009) Carbohydrate cluster microarrays fabricated on three-dimensional dendrimeric platforms for functional glycomics exploration, *Journal of proteome research* 8, 5031–5040.
14. Ekins, R., Chu, F., and Biggart, E. (1990) Multispot, multianalyte, immunoassay, *Ann Biol Clin* 48, 655–666.
15. Wang, D. (2003) Carbohydrate microarrays, *Proteomics* 3, 2167–2175.
16. Wang, R., Liu, S., Shah, D., and Wang, D. (2005) A practical protocol for carbohydrate microarrays, *Methods in Molecular Biology (Totowa, NJ, United States)* 310, 241–252.
17. Carroll, G. T., Wang, D., Turro, N. J., and Koberstein, J. T. (2006) Photochemical micro-patterning of carbohydrates on a surface, *Langmuir* 22, 2899–2905.
18. Newsom-Davis, T. E., Wang, D., Steinman, L., Chen, P. F., Wang, L. X., Simon, A. K., and Sreaton, G. R. (2009) Enhanced immune recognition of cryptic glycan markers in human tumors, *Cancer research* 69, 2018–2025.