

A Practical Protocol for Carbohydrate Microarrays

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Summary

We have established a high-throughput biochip platform for constructing carbohydrate microarrays. Using this technology, carbohydrate-containing macromolecules of diverse structures, including polysaccharides, natural glycoconjugates, and mono- and oligosaccharides coupled to carrier molecules, can be stably immobilized on a glass chip without chemical modification. Here, we describe a practical protocol for this technology. We hope that anyone who has access to a standard cDNA microarray facility will be able to explore this technology for his or her own research interest. We also provide an example to illustrate that the carbohydrate microarray is also a discovery tool; this is particularly useful for identifying immunologic sugar moieties, including complex carbohydrates of cancer cells and sugar signatures of previously unrecognized microbial pathogens.

Key Words: Antigens; antibodies; carbohydrates; glycans; glycoconjugates; microarrays; microspotting; nitrocellulose; polysaccharides; SARS-CoV.

1. Introduction

Like nucleic acids and proteins, carbohydrates are another class of crucial biological molecules (1–3). Because of their unique physicochemical properties, carbohydrates are superior to other biological molecules in generating structural diversity. In aqueous solutions, such as bodily fluids, carbohydrate chains are prominently displayed on the surfaces of cell membranes or on the exposed regions of macromolecules. Carbohydrates are, therefore, suitable for storing biological signals in the forms that are identifiable by other biological systems.

Recent studies have demonstrated that cell-surface expression of specific complex carbohydrates is associated with various stages of embryonic development and cell differentiation (4–7). Abnormalities in the expression of complex

carbohydrates are found in cancer (8,9), retrovirus infection (10,11), and diseases with genetic defects in glycosylation (12). Sugar moieties are also abundantly expressed on the outer surfaces of the majority of viral, bacterial, protozoan, and fungal pathogens. Many sugar structures are pathogen-specific, which makes them important molecular targets for pathogen recognition, diagnosis of infectious diseases, and vaccine development (1,3,13–15). Exploring the biological information contained in sugar chains is, therefore, an important topic of current postgenomic research.

Our group has focused on development of a carbohydrate-based microarray technology to facilitate exploration of carbohydrate-mediated molecular recognition and anti-carbohydrate immune responses (16–18). This technology takes advantage of existing cDNA microarray systems, including the spotter and scanner, for efficient production and use of carbohydrate microarrays (*see Note 1*). We have demonstrated that the current platform is able to overcome a number of technical difficulties, by showing that (1) carbohydrate molecules can be immobilized on a nitrocellulose-coated glass slide without chemical conjugation, (2) the immobilized carbohydrates are able to preserve their immunological properties and solvent accessibility, (3) the system reaches the sensitivity, specificity, and capacity to detect a broad range of antibody specificities in clinical specimens, and (4) this technology can be applied to investigate carbohydrate-mediated molecular recognition and anti-carbohydrate antibody reactivities on a large scale.

In this chapter, we provide a practical protocol for this high-throughput carbohydrate microarray system. We summarize the key steps of carbohydrate microarray applications, including (1) design and construction of sugar arrays, (2) microspotting molecules onto nitrocellulose-coated glass slides, (3) immunostaining and scanning of arrays, (4) analysis of microarray data, and (5) validation of microarray data using conventional immunological assays. We focus on an eight-chamber subarray system to produce carbohydrate microarrays on a relatively smaller scale, which is more frequently applied in our laboratory's routine research activities. Lastly, we present an example to illustrate the application of this system in addressing biomedical questions.

2. Materials

2.1. Apparatus

1. Microspotting: Cartesian Technologies' PIXSYS 5500C (Irvine, CA) or GMS 417 Arrayer, Genetic Microsystems, Inc. (Woburn, MA).
2. Supporting substrate: FAST Slides (Schleicher & Schuell, Keene, NH).
3. Microarray scanning: ScanArray 5000 Standard Biochip Scanning System (Packard Biochip Technologies, Inc., Billerica, MA).

2.2. Software

1. Array design: CloneTracker (Biodiscovery, Inc., Marina del Rey, CA).
2. Array printing: AxSys™ (Cartesian Technologies, Inc., Irvine, CA).
3. Array scanning and analysis: ScanArray Express (PerkinElmer, Torrance, CA).

2.3. Antibodies and Lectins

1. Horse anti-SARS-CoV anti-sera (gift of Dr. Jiahai Lu, Sun-Yatsen University, Guangdong, China).
2. *Phaseolus vulgaris* L. (PHA-L) (EY Laboratories, Inc., San Mateo, CA).
3. Streptavidin-Cy3 and streptavidin-Cy5 conjugates (Amersham Pharmacia, Piscataway, NJ).
4. Species-specific anti-immunoglobulin antibodies and their fluorescent conjugates, Cy3, Cy5, or fluorescein isothiocyanate (FITC) (Sigma, St. Louis, MO; BD-PharMingen, San Diego, CA).

2.4. Reagents and Buffers

1. Dilution buffer: saline (0.9% NaCl).
2. Rinsing solution: 1X phosphate-buffered saline (PBS) (pH 7.4) with 0.05% (v/v) Tween-20.
3. Blocking solution: 1% (w/v) bovine serum albumin (BSA) in PBS with 0.05% (w/v) Tween-20, 0.025% (w/v) NaN₃.

3. Methods

Our previous experimental investigations have led to the establishment of the current high-throughput carbohydrate microarray platform (16) (see Note 1). The methods described below outline: (1) design and construction of an eight-chamber subarray system, (2) microspotting carbohydrate-containing molecules onto nitrocellulose-coated glass slides, (3) immunostaining and scanning of microarrays, (4) analysis of microarray data, and (5) validation of microarray findings by conventional immunological assays. Figure 1 is a schematic view of this high-throughput microarray system.

3.1. Design and Construction of the Chip

We have designed an eight-chamber subarray system to construct customized carbohydrate microarrays. As illustrated in Fig. 2, each microglass slide contains eight separated subarrays. The microarray capacity is approx 600 microspots per subarray. A single slide is, thus, designed to enable eight microarray assays. A similar design with array capacity of approx 100 microspots is also commercially available (Schleicher & Schuell, Keene, NH).

1. Each microglass slide contains eight identical subarrays. There is chip space for 600 microspots per subarray, with spot sizes of approx 200 μ and at 300-μ inter-

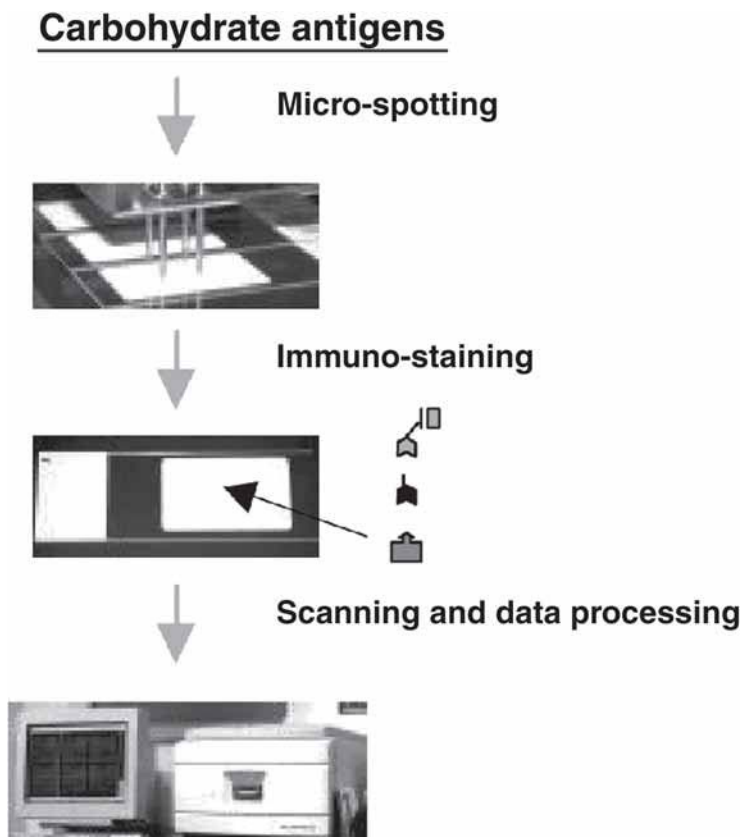


Fig. 1. A high-throughput platform of the carbohydrate-based microarrays. A high-precision robot designed to produce cDNA microarrays was utilized to spot carbohydrate antigens onto a chemically modified glass slide. The microspotting capacity of this system is approximately 20,000 spots per chip. The antibody-stained slides were then scanned for fluorescent signals with a Biochip Scanner that was developed for cDNA microarrays. The microarray results were subsequently confirmed by at least one of the conventional alternative assays.

vals, center to center. A single slide is, therefore, designed to allow eight detection reactions.

2. Repeats and dilutions: We usually print carbohydrate antigens at the initial concentration of 0.1–0.5 mg/mL. The absolute amount of antigens or antibodies printed on the chip substrate ranges from 0.1–0.5 ng per microspot. They are further diluted at 1:3, 1:9, and 1:27. A given concentration of each preparation is repeated at least three times to allow statistic analysis of detection of identical preparation at a given antigen concentration.

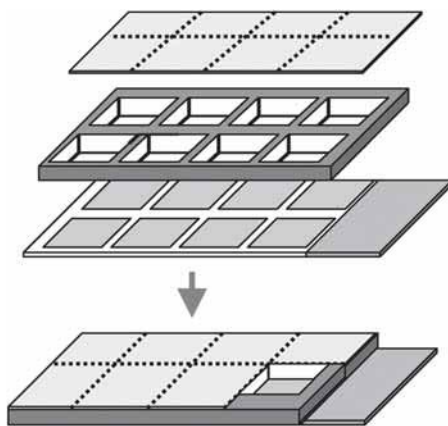


Fig. 2. Schematic of the eight-chamber subarrays. Each microglass slide contains eight subarrays of identical content. There is chip space for 600 microspots per subarray, with spot sizes of approx $200\ \mu$ and at $300\text{-}\mu$ intervals, center to center. A single slide is, therefore, designed to enable eight detections.

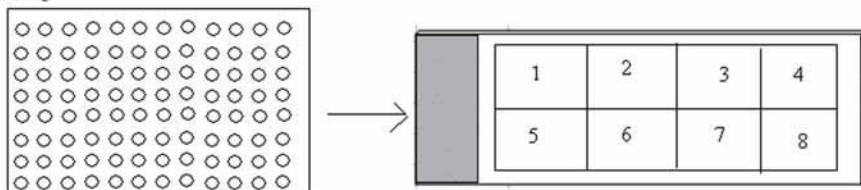
3. Antibody isotype standard curves: antibodies of immunoglobulin (Ig)G, IgA, and IgM isotype of corresponding species are printed at given concentrations to serve as standard curves in the microarray format. This design allows quantification of the antibody signals that are captured by spotted carbohydrate antigens. In addition, such standard curves are useful for microarray data normalization and cross-chip scaling of microarray detection.

3.2. Microspotting of Carbohydrates Onto Nitrocellulose-Based Substrate

Using Cartesian Technologies' PIXSYS 5500C (Irvine, CA), a high-precision robot designed for cDNA microarrays, carbohydrate antigens of various complexities are picked up by dip quill pins from antigen/antibody solutions and printed onto nitrocellulose-coated FAST slides in consistent amounts (Schleicher & Schuell, Keene, NH). The complementary AxSys software (Cartesian Technologies, Inc., Irvine, CA) is used to control the movement of pins during the dispensing and printing process.

1. Prepare samples of carbohydrate antigens in 0.9% NaCl and transfer them into 96-well plates (*see* **Notes 2** and **3**).
2. Place the 96-well plates containing samples on the Cartesian arrayer robot.
3. Adjust program so that carbohydrate antigens are printed at spot sizes of approx $150\ \mu\text{m}$ and at $375\ \mu\text{m}$ intervals, center to center.
4. Each antigen or antibody is spotted as triplet replicates in parallel.

1 Spotting



2 Rinse, 1XPBS 5 min

3 Block, 1% BSA PBS, RT, 30 min

4 Staining

Chip#	Chamber	40 μ L/subarray		
		Serum, 90 min RT	2nd Ab, 30min RT	3rdAb, 30min RT
100803-5	5	Horse anti-SARS serum 1:20	Rabbit anti-Horse IgG-BI	AV-Cy3 1:500
	6	Horse anti-SARS serum 1:100	↓ 1:200 dil	
	7	Horse anti-Pn18 serum 1:20		
	8	Horse anti-Pn18 serum 1:100		

Fig. 3. Schematic of staining process of SARS-CoV immunoarray. (1) Spotting: A high-precision robot transfers the samples, SARS-CoV proteins, and glycans of various complexities, from 96-well plate to nitrocellulose-coated glass slides. (2) Staining: Before staining, the slides are rinsed with 1X phosphate-buffered saline (PBS), and blocked with 1% bovine serum albumin (BSA)-PBS containing 0.05% NaN_3 and 0.05% Tween-20. They are subsequently incubated with horse anti-SARS sera. The primary antibodies captured by microarrays are detected using biotinylated anti-horse immunoglobulin (Ig)G, and visualized by Cy3-streptavidin.

5. The printed carbohydrate microarrays are air-dried and stored at room temperature without desiccant before further use (*see Note 5*).

3.3. Immunostaining of 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 and 6 and Fig. 3*).

1. Rinse printed microarray slides with 1X PBS (pH 7.4) with 0.05% Tween-20 for 5 min.
2. Block slides with 1% BSA in PBS containing 0.05% Tween-20, 0.025% NaN_3 , at RT for 30 min.
3. Stain each subarray with 40 μ L 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 90 min.
5. Wash slides five times with 1X PBS (pH 7.4) with 0.05% Tween-20.

6. Stain slides with 40 μL of titrated secondary antibodies. Anti-human (or other species) IgG, IgM, or IgA antibodies with distinct fluorescent tags, Cy3, Cy5, or FITC, are mixed and then applied to 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. Air-dry the washed slides.
10. Cover slides in a histology slide box to prevent fluorescent quenching by light.

3.4. Microarray Scanning and Data Processing

1. Scan microarray with ScanArray Express Microarray Scanner (PerkinElmer Life Science) following the manufacturer's instructions.
2. Fluorescence intensity values for each array spot and its background were calculated using Packard Bioscience's QuantArray software analysis packages or the updated ScanArray Express software. A staining result is considered positive if the mean fluorescent intensity value of the microspot is significant higher than the mean background of the identically stained microarray with the same fluorescent color (see Note 7).

3.5. Validation and Further Investigation of Microarray Observations

It is highly recommended that microarray findings be verified using other experimental approaches. We usually confirm our results by at least one of the alternative immunoassays, such as enzyme-linked immunosorbent assay (ELISA), dot blot, Western blot, flow cytometry, or immunohistology (16,18).

3.6. Constructing Glycan Arrays to Probe Immunologic Sugar Moieties of SARS-CoV

The following example illustrates the use of carbohydrate microarray technology in an important research area. In this case, the above described eight-chamber subarray system was applied to construct a glycan array. This glycan array was then applied to identify immunogenic sugar moieties of a human coronavirus (SARS-CoV).

SARS-CoV is a newly identified human viral pathogen that caused an outbreak of severe acute respiratory syndrome (SARS). Although substantial efforts have been made to study the etiological agent of the disease, the carbohydrate structures of SARS-CoV remain largely uncharacterized. This information is, however, very important for devising a vaccination strategy against SARS-CoV, as well as understanding the pathogenesis of SARS.

To investigate this, we constructed glycan arrays to display carbohydrate antigens of defined structures and then applied these tools to detect carbohydrate-specific antibody "fingerprints" that were elicited by a SARS vaccine.

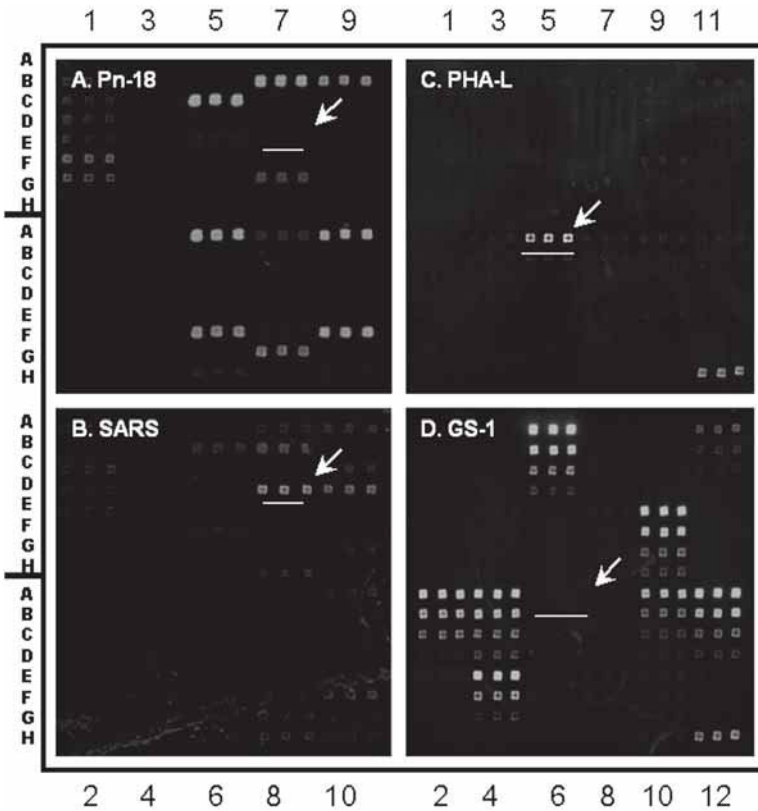


Fig. 4. Glycan arrays are used to characterize the antibody profiles of vaccinated animals (Glycan array I) and to scan for asialo-orosomucoid (ASOR)-specific immunological probes (Glycan array II). Antigen preparations spotted on each glycan array and their array location are summarized in Supplemental Tables S1 and S2 of reference (18) (available at the Physiological Genomics Web site).

Our rationale was that if SARS-CoV expressed antigenic carbohydrate structures, then immunizing animals using the whole virus-based vaccines would have elicited specific antibodies for these structures. In addition, if SARS-CoV displayed a carbohydrate structure that mimics host cellular glycans, then vaccinated animals may develop antibodies with autoimmune reactivity to their corresponding cellular glycans.

In **Fig. 4**, we show representative results. First, we detected an antibody reactivity specific for the carbohydrate moieties of an abundant human serum glycoprotein asialo-orosomucoid (ASOR) (**Fig. 4B**). Second, we found that lectin PHA-L (*Phaseolus vulgaris* L.) is specific for a defined complex carbohy-

drate ASOR (Fig. 4C,D). Third, we applied this probe to examine whether SARS-CoV expresses antigenic structures that imitate the host glycan. We confirmed that only the SARS-CoV-infected cells express PHA-L reactive antigenic structure (data not shown). Therefore, we obtained immunologic evidence that a carbohydrate structure of SARS-CoV shares antigenic similarity with host glycan complex carbohydrates.

The biological significance of this finding remains to be further explored. For example, what is the possible involvement of autoimmune responses in SARS pathogenesis? ASOR is an abundant human serum glycoprotein, and the ASOR-type complex carbohydrates are also expressed by other host glycoproteins (19,20). Thus, the human immune system is generally nonresponsive to these “self” carbohydrate structures. However, when similar sugar moieties were expressed by a viral glycoprotein, their cluster configuration could differ significantly from those displayed by a cellular glycan, thereby generating a novel “non-self” antigenic structure. A documented example of such an antigenic structure is a broad-range HIV-1 neutralization epitope recognized by a monoclonal antibody 2G12. This antibody is specific for a unique cluster of sugar chains displayed by the gp120 glycoprotein of HIV-1(21). It is, thus, important to examine whether naturally occurring SARS-CoV expresses the ASOR-type autoimmune reactive sugar moieties. In addition, a number of cellular receptors that bind the ASOR complex carbohydrate have been identified (20,22). This study provided clues to explore the possible roles of carbohydrate-mediated receptor-ligand interactions in SARS-CoV infection, especially in determining host-range and tissue-tropic characteristics of the virus.

In summary, our laboratory has established a simple, precise, and highly efficient experimental approach for the construction of carbohydrate microarrays (16–18). This approach makes use of existing cDNA microarray systems, including the spotter and scanner, for carbohydrate array production. A key technical element of this array platform is the introduction of nitrocellulose-coated microglass slides to immobilize unmodified carbohydrate antigens on the chip surface noncovalently. This technology has achieved the sensitivity to recognize the profiles of human anti-carbohydrate antibodies with as little as a few microliters of serum specimen, and reached the chip capacity to include the antigenic preparations of most common pathogens (approx 20,000 microspots per biochip). We describe in this chapter a practical protocol of this platform of carbohydrate microarrays. However, we would like to take this opportunity to refer our readers to other technology platforms of carbohydrate microarrays or glycan chips (23–28). This progress, especially the availability of different technological platforms to meet the multiple needs of carbohydrate research, marks an important developmental stage of chemical genomics research, approaching the era of the glycome.

4. Notes

1. Antigen preparations suitable for this high-throughput biochip platform: Carbohydrate antigens of multiple structural configurations, including polysaccharides, natural glycoconjugates, and oligosaccharide-protein and oligosaccharide-lipid conjugates, are applicable for this microarray platform (16,18,25). In addition to printing carbohydrate microarrays, the current platform is also applicable for producing protein microarrays (17). 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: 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.
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 15,000g) for at least 15 min. Before and after each arraying experiment, we recommend examining and cleaning the printing pins.

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 presence of samples on array: To verify that proteins, synthetic peptides, and carbohydrates are successfully 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 plastic bag with desiccant and stored at 4°C.
6. Staining considerations: After the last wash between each staining step, it is important to completely withdraw the wash buffer inside the reaction chambers; otherwise, the remaining buffer may lower the concentration of the antibodies to be analyzed.
7. Data analysis: Training with the technical experts of PerkinElmer is necessary before performing microarray data analysis using the software package.
8. 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 as instructed by the Centers for Disease Control and Prevention (CDC) and the World Health Organization (WHO).

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