Video Article

Profiling Anti-Neu5Gc IgG in Human Sera with a Sialoglycan Microarray Assay

Shani Leviatan Ben-Arye[1], Hai Yu[2], Xi Chen[2], Vered Padler-Karavani[1]

[1]Department of Cell Research and Immunology, Tel Aviv University

[2]Department of Chemistry, University of California-Davis

Correspondence to: Vered Padler-Karavani at vkaravani@post.tau.ac.il

URL: https://www.jove.com/video/56094
DOI: doi:10.3791/56094

Keywords: Behavior, Issue 125, Sialic acid, *N*-acetylneuraminic acid, Neu5Ac, *N*-glycolylneuraminic acid, Neu5Gc, carbohydrate, antibodies, lectin, glycan microarray, cancer, diagnostics, high-throughput assay

Date Published: 7/13/2017

Citation: Leviatan Ben-Arye, S., Yu, H., Chen, X., Padler-Karavani, V. Profiling Anti-Neu5Gc IgG in Human Sera with a Sialoglycan Microarray Assay. *J. Vis. Exp.* (125), e56094, doi:10.3791/56094 (2017).

Abstract

Cells are covered with a cloak of carbohydrate chains (glycans) that is commonly altered in cancer and that includes variations in sialic acid (Sia) expression. These are acidic sugars that have a 9-carbon backbone and that cap vertebrate glycans on cell surfaces. Two of the major Sia forms in mammals are *N*-acetylneuraminic acid (Neu5Ac) and its hydroxylated form, *N*-glycolylneuraminic acid (Neu5Gc). Humans cannot produce endogenous Neu5Gc due to the inactivation of the gene encoding cytidine 5'monophosphate-Neu5Ac (CMP-Neu5Ac) hydroxylase (CMAH). Foreign Neu5Gc is acquired by human cells through the dietary consumption of red meat and dairy and subsequently appears on diverse glycans on the cell surface, accumulating mostly on carcinomas. Consequently, humans have circulating anti-Neu5Gc antibodies that play diverse roles in cancer and other chronic inflammation-mediated diseases and that are becoming potential diagnostic and therapeutic targets. Here, we describe a high-throughput sialoglycan microarray assay to assess such anti-Neu5Gc antibodies in the human sera. Neu5Gc-containing glycans and their matched pairs of controls (Neu5Ac-containing glycans), each with a core primary amine, are covalently linked to epoxy-coated glass slides. We exemplify the printing of 56 slides in a 16-well format using a specific nano-printer capable of generating up to 896 arrays per print. Each slide can be used to screen 16 different human sera samples for the evaluation of anti-Neu5Gc antibody specificity, intensity, and diversity. The protocol describes the complexity of this robust tool and provides a basic guideline for those aiming to investigate the response to Neu5Gc dietary carbohydrate antigen in diverse clinical samples in an array format.

Video Link

The video component of this article can be found at https://www.jove.com/video/56094/

Introduction

Sias are acidic sugars covering glycan chains on cell-surface glycoproteins and glycolipids in vertebrates. Sia expression is modified in cancer cells[1] and correlates with progression and/or metastasis[2,3]. Two of the major Sia forms in mammals are Neu5Ac and its hydroxylated form, Neu5Gc[2]. Humans cannot synthesize Neu5Gc due to a specific inactivation of the gene encoding the CMAH enzyme . This non-human Sia metabolically incorporates into human cells as "self," originating from dietary Neu5Gc-rich foods (*e.g.,* red meat)[4,5]. Neu5Gc is present at low levels on the cell surfaces of human epithelia and endothelia, but it especially accumulates in carcinomas. Neu5Gc is recognized as foreign by the human humoral immune system[2,6]. The antigenic complexity of Neu5Gc-glycans may arise at multiple levels, including Neu5Gc modification, linkage, underlying glycans and scaffolds, and their density, all reflected by the complexity of anti-Neu5Gc antibody response in humans[6]. Some of these antibodies serve as carcinoma biomarkers and potential immunotherapeutics[7]. The advent of the chemoenzymatic synthesis of different sialoglycans[8] paved the way for the more in-depth analysis of such antibodies, facilitated by the use of glycan microarray technology[9,10]. Thus, with the facilitated preparation and manipulation of large libraries of natural and synthetic carbohydrates, glycan microarrays have become a powerful high-throughput technology for investigating the interactions of carbohydrates with a myriad of biomolecules[10,11,12,13]. In an array format, minimal amounts of materials are used, and this multivalent display of biologically relevant glycans allows for the investigation of thousands of binding interactions in a single experiment. Importantly, this technology can also be applied to biomarker discovery and to monitoring immune responses in various samples[7,12].

Successful glycan microarray fabrication requires the consideration of three important aspects: the printer robot type, glycan conjugation chemistry, and detection optics. As to the printing instrument consideration, two techniques are available: contact and non-contact printers. In contact printing, 1-48 steel pins are dipped into a multi-well source plate containing glycan solution and are spotted on functionalized glass slides by directly contacting the glass slide surface. The solution amount delivered to the slide is a function of the lingering duration on the slide surface. Usually, the samples are first pre-spotted on a glass block (to reach homogenous spots) before they are printed on the slide surface. In non-contact printers (*e.g.,* the piezo-electronic printer), the glycans are printed from a glass capillary using controlled electric signals. The electric signal can be finely calibrated to achieve more precise printing relative to contact printing. The size and morphology of the spots are also relatively more homogeneous. An additional advantage is the recycling of the sample back to the source plate after printing. Nevertheless, the major disadvantage of piezo-electronic printers is the printing tip limitation (4 or 8), resulting in a very long printing duration,

which requires special attention to slide stability, temperature, humidity, and sample evaporation. The non-contact inkjet printer requires larger sample volumes[14].

In contrast to the limited available options for printing methods, glycan conjugation chemistry is a more complex consideration, with many options to choose from. Selected immobilization chemistry must account for both the active groups on the glycans and the slide surface reactivity. The glycans to be immobilized onto a specific microarray surface, either synthetically synthesized or naturally isolated, all require an identical reactive group. In addition, the glycans need to be pure and homogenous. On the other hand, the immobilization surface and chemistry should provide reproducibility and reliable attachment density. Multiple immobilization methods have been developed with either covalent or non-covalent (physical absorption) attachment[10,11,12,13]. For highly detailed information on printed glycan microarray technology for the uninitiated investigator, refer to these excellent reviews [13,15]. Importantly, the recent Minimum Information Required for a Glycomics Experiment (MIRAGE) initiative describes guidelines for sample preparation[16] and for reporting data from glycan microarray analyses[17] to improve the standards in this growing field.

Here, we describe a detailed protocol for the fabrication of sialoglycan microarrays using a specific contact nano-printer in a 16-well format. Each of the glycans have a primary amine that mediate their covalent link to epoxy-activated glass slides. We also describe the development and analysis of one slide using various human sera samples, antibodies, and Sia-binding plant lectins. Sialoglycan microarray assays involve several major steps that include array fabrication, processing, development, and analysis. Array fabrication requires planning the array layout, preparing the glycans and source plate, programming the nano-printer, and printing the slides. Subsequently, the slides are processed, developed, and analyzed (**Figure 1**).

Protocol

Human sera samples were obtained from the Israeli Blood Bank and were used in accordance with the Helsinki declaration and Tel Aviv University Institutional Review Board.

1. Array Fabrication Planning and Layout

1. Determine the slide layout.
   NOTE: Each slide contains 16 sub-arrays divided into 16 identical blocks numbered B1 to B16 (**Figure 1B**, **Supplementary Figure 1A**).
2. Determine the pin layout. Use four pins, each printing four sub-arrays (blocks) per slide:
   Pin 1 prints Blocks 1, 2, 9, and 10;
   Pin 2 prints Blocks 3, 4, 11, and 12;
   Pin 3 prints Blocks 5, 6, 13, and 14; and
   Pin 4 prints Blocks 7, 8, 15, and 16 (**Supplementary Figure 1A**).
3. **Determine the block and 384-well plate layout.**
   NOTE: The order in which the glycans appear in each block requires careful planning. In this example array, print each sample at four replicates, design fluorescent marker spots to be located at the bottom-right corner (to facilitate spot analysis), and print standard-curve IgG (STD) spots at six increasing concentrations (**Supplementary Figure 1B**).
   1. For each printed spot, first dispense the material into four wells (one for each pin) in a 384-well plate.
      NOTE: The order of the materials in the 384-well plate relies on the designed block layout (**Supplementary Figure 1B-C**).

2. Preparation of Glycans and the Source Plate

1. Prepare glycan printing buffer (50 mL of 300 mM phosphate buffer, pH 8.4). Weigh 58.5 mg of monosodium phosphate monohydrate ($NaH_2PO_4.1H_2O$) and 3.9 g of disodium phosphate heptahydrate ($Na_2HPO_4.7H_2O$) in 40 mL of deionized water (DIW). Titrate to pH 8.4 using 4 M NaOH. Adjust the volume to 50 mL and filter through a 0.2-µm membrane for sterilization.
2. Prepare marker buffer (50 mL of 187 mM phosphate buffer, pH 5). Weigh 289 mg of monosodium phosphate monohydrate ($NaH_2PO_4.1H_2O$) and 1.94 g of disodium phosphate heptahydrate ($Na_2HPO_4.7H_2O$) in 40 mL of DIW. Titrate to pH 5 using 1 M HCl. Adjust the volume to 50 mL and filter through a 0.2 µm membrane for sterilization.
3. Prepare STD curve buffer (50 mL of PBS-glycerol: phosphate-buffered saline, pH 7.4, supplemented with 10% glycerol from a 100% glycerol stock) and filter it through a 0.2-µm membrane for sterilization.
4. Prepare each glycan stock solution at 10 mM in DIW. Verify the sialylated glycan concentrations by fluorescence detection on reverse-phase high-pressure liquid chromatography analysis[18].
5. Dilute each glycan to 100 µM in a total volume of 100 µL of glycan printing buffer in microcentrifuge tubes.
6. Prepare primary amine containing fluorescent dye. Solubilize Alexa 555-hydrazide to 1 mg/mL with marker buffer and then dilute to 1 µg/mL in a 1 mL total volume.
   NOTE: Any other primary amine-containing dye can be used with an appropriate wavelength according to the slide scanner.
   NOTE: Marker spots facilitate grid alignment in each block during analysis.
7. Prepare human IgG STD curve dilutions: prepare 0.5 mL of a stock solution of human IgG at 160 ng/µL in STD curve buffer. Serially dilute the stock 1:1 six times to obtain 80, 40, 20, 10, 5, and 2.5 ng/µL, all in STD curve buffer at a total volume of 200 µL each.
   NOTE: Other Ig isotypes can be used if suitable for the experiment (*e.g.,* human IgA, IgM, IgE, or an Ig of a different organism).
8. Place the 384-well plate on ice. Using an electronic multi-pipette, aliquot 7 µL of each glycan, marker, and STD curve IgG-four replicates of each-according to the plate layout (**Supplementary Figure 1C**). For better accuracy, load 35 µL of each sample and aliquot 7 µL into each of the four wells of the sample. Place the remaining 7 µL of each glycan back into their original tube.
9. Cover the plate with parafilm and spin down for 2 min at 250g and 12 °C. Place the plate at room temperature (RT), protected from light. Do not uncover the plate before the humidity in the room reaches 60-70%.

3. Programing the Nano-printer

1. Open the "Microarray Manager" software. Use the "Method properties" window.
2. Set up the work table layout: choose the "56 slide configuration."
   NOTE: This is a predefined configuration that allows for the printing of 56 slides. Calibrate for each type of plate, pin, and print format (**Supplementary Figure 2**, step 1).
3. Select "Pin Configuration" | "Pin Tool 1x4 9 mm."
   NOTE: This is a predefined configuration for using four pins (**Supplementary Figure 2**, step 2).
4. **Define the "Pin type." Calculate and create the pin type that is suitable for the printing.**
   NOTE: This step defines how many spots a pin would print before re-dipping in the sample 384-well and should be optimized for the different pin types and slide surface chemistry.
   NOTE: When using 946MP3 pins, consider two important issues: 1) each pin can print up to 140 homogenous spots and 2) pre-spotting is essential to drain excess liquid from the tip of the pin before printing on the slides. Therefore, program each pin to print 20 pre-spots on a pre-printing block (optimized for 946MP3 pins on epoxy-coated slides).
   1. Calculate the total number of printed spots per sample.
      NOTE: Here, each pin prints 4 spots per sub-array (block); a total of 4 sub-arrays per slides = 16 spots/slide. After 7 slides, each pin prints 16 x 7 = 112 spots. In addition, 20 pre-spots before printing gives 132 spots/dip. Therefore, the pin type is defined as "946MP3-132 spots" (4 spots per sample per block x 4 blocks x 7 slides + 20 pre-spots = 132).
   2. Create the "Pin type" (**Supplementary Figure 2**, step 3). Press "Setup" (left panel of the software) | "Micro Spotting Pins" | "New Name" (946MP3-132 spots). Define the number of spots per dip (132), the spot diameter (100 µm), the uptake (0.25 µL), the delivery (0.7 nL), and the minimum spot spacing (100 µm). Press "OK" | "OK."
   3. In the "Method properties" window, select the created pin type: "946MP3-132 spots."

5. Define the washing conditions. Set the "Wash before starting" and "Wash after Finishing" to "Normal wash" (**Supplementary Figure 2**, step 4).
6. **Define the "Plate list."**
   NOTE: This is the sampling pattern from the plate (the order of dips from the 384-well plate) according to the plate layout. To upload the plate into the plate list, set up a plate sampling pattern.
   1. Create the plate "Sampling Pattern." Go to "Templates" (**Supplementary Figure 3**) | "Sampling Patterns." Give a new name (*e.g.,* JoVE array) and select "Insert" | "Fill top-bottom" | "Pin Tool 1 x4 9 mm." Select the order of well pickups according to the pre-defined plate layout (**Supplementary Figure 1A**). Press "OK" | "OK."
   2. Load the plate sampling pattern. Click the right side of the "Plate list" cell (**Supplementary Figure 3**) | "Add plate" and select the correct plate sampling pattern (the "JoVE array" described in step 3.6.1.). Select "Normal Wash." Press "OK" | "OK."

7. Go to the "Sample Plates" and change the position offset to 1 (which determines where the plate is located on the arrayer deck).
   NOTE: The offset position of 0 is closer to the sonicator bath.
8. **Define the "Microarray Print Design." Calculate the block-to-block distances and spacing.**
   1. Consider the dimensions of the developing tool that divides the slide into 16 wells of 7 x 7 mm, with 2-mm spaces between sub-arrays (a total of 9 mm between blocks; **Supplementary Figure 4**).
   2. Consider the sub-array dimensions.
      NOTE: The spacing between spots is 0.275 mm and there are 12 columns and 18 rows in this print design; the total dimensions are: width (11 x 0.275 mm = 3.025 mm) and height (17 x 0.275 mm = 4.675 mm). Calculate the horizontal spacing: 9 mm - (11 0.275) = 5.97 mm. Calculate the vertical spacing: 9 mm - (17 x 0.275) = 4.325 mm. Calculate distance from the left side of slide: 4.43 mm + 1.07 mm = 5.5 mm. Calculate the distance from the top side of the slide: 2.95 mm + 0.55 mm = 3.5 mm (**Supplementary Figure 4**).
   3. Define the "Print Designs" (**Supplementary Figure 5**). Select "Templates" | "Print Designs" | "New."
      1. Select "Pin Setup" | "Pin Tool 1 x 4 9 mm" | "Pin - 946MP3-132 spots." In the "General" tab, change the "Name of Microarray Print" (*i.e.,* "JoVE array"). Select "Spot Speed" | "Replicate Number" (4) | "Touch point Height" (0 mm) | "Soft Touch Height" (3 mm) | "Print to all available positions" (**Supplementary Figure 5A**).
      2. In the "Microarray" tab, choose the horizontal and vertical spacing (5.9 mm and 4.325 mm from step 3.8.2) and the number of horizontal and vertical microarrays (2). Select "Print Microarray Horizontally" | "Print Duplicate Microarray" (**Supplementary Figure 5B**).
      3. In the sub-array tab, choose the maximum number of columns (12), the maximum number of rows (18), and the distance between the columns and rows (275 µm) (**Supplementary Figure 5C**).
      4. In the measurements tab, choose the distance from the left side of the slide (5.5 mm), the distance from the top of the slide (3.5 mm, calculated in step 3.8.2), the width of the print slide (25 mm), and the height of the print slide (75 mm) (**Supplementary Figure 5D**). Press "OK" | "OK."

9. Click the right side of the "Microarray Print Designs" cell and select the defined design (the "JoVE array" from step 3.8.3; **Supplementary Figure 6**).
10. Define the "Slide Count" (56), and change the "Position Offset" (0). This determines the position of the first slide to be printed on the arrayer deck (**Supplementary Figure 6**).
11. Define the "Microarray Pre-Print Design" (**Supplementary Figure 6**). Select "Glass Block" | "Pre-Print 1 x 4 mm Pin tool."
    NOTE: This spot-spacing setting allows 46,464 pre-spots (for all 4 pins) on the glass block, thus 46,464 / 4 = 11,616 pre-spots per pin. When the space on the pre-print block had been used up, the printer will stop and the block will need to be flipped over.
    NOTE: To calculate after how many samples the pre-print block is full, consider the following: every sample will be taken by 8 pin-dips in the 384-well plate to complete 56 slides (7 slides per dip), and each dip has an additional 20 pre-spots, giving a total of 160 pre-spots per sample. Therefore 11,616 / 160 = 72.6 samples. Thus, the pre-print glass block would need to be flipped over after 72 samples (to evaluate the time for flipping, measure how long it takes for one sample to complete a full run on 56 slides and then multiply by 72).

12. Select "Use PrePrint Design" | "Yes" | "Scan Barcodes" (No) | "Print Single Replicate" (No) (**Supplementary Figure 6**).
13. Click on "Validate" (**Supplementary Figure 6**).
14. Click on "Save" and save the method as an arr file (*e.g.,* "JoVE array.arr," **Supplementary Figure 6**).

4. Printing Designed Arrays

NOTE: All steps should be carried out in a clean room with a humidity of 60-70% and with appropriate gloves and clothing for protection

1. Turn on the nano-printer machine and humidifier.
2. For the wash buffer and humidification, fill with DI water. Empty the wash bottle (drain) and humidification trap carboy (**Supplementary Figure 7A**).
3. Set the humidifier to 70% humidity and start humidification.
4. **Clean all pins before printing. Prepare 50 mL of hot (65 °C) pin-tip cleaning solution (15 g of pin-tip cleaning reagent and 50 mL of 65 °C water, mixed thoroughly until the solid reagent dissolves completely).**
    1. Apply the cleaning solution to each pin tip by dipping the pin-tip brush (fine) into the pin-tip cleaning solution and brushing the surface of each pin tip.
    NOTE: Pin-tip cleaning solution is corrosive and toxic. Make sure to wear gloves and safety glasses at all times when using this solution. Soaking pins in pin-tip cleaning solution for more than a few minutes may result in permanent pin damage.
    2. Fill the ultrasonic bath with 1 L of distilled water and place the pins in a floatable pin cleaning rack in the bath. Sonicate for 5 min and remove the pins. Let dry and gently wipe with clean room special wipes.
5. Open the "Microarray Manager" printer software; upon connection set, the printer light will turn on (red/green). Press "Stop" | "Clear" | "Init" (**Supplementary Figure 7B**, steps 1-3); the print-head arm will then reset the X, Y, Z alignments.
6. To load the pins into the print head, press "Load" (**Supplementary Figure 7B**, step 4) and place the pins in the print head according to the defined configuration of the print/pin tool layout (**Supplementary Figure 7C**); this layout uses 4 pins, each printing 4 sub-arrays on each slide to get total of 16 sub-arrays per slide (**Supplementary Figure 1A**). After placing the pins, press "Init" again.
7. Assemble the slides on the arrayer deck. Open the slide box and clean each slide by blowing ultra-high-purity nitrogen gas. Place the desired number of slides on the array platform. Hold the slides with two fingers at the bottom corners and then place the slide into its position. Hold the two right corners and push the left corners gently until the slide tightly fits into its position. Push gently towards the left-bottom corner to ensure a tight fit (**Supplementary Figure 7D**).
8. Clean the pre-block glass with distilled water, 70% ethanol, and 100% ethanol and let it air dry (use only dedicated clean-room wipes). Place the pre-block glass into its position on the deck.
9. Fill a sonicator bath (**Supplementary Figure 7B**). Press on "Fill" for 55 s then press "OK." Drain the sonicator by clicking "Drain" for 20 s and then pressing "OK." Repeat the filling and draining once more and then fill again for 55 s.
10. Fill the washing bath (**Supplementary Figure 7B**): press "Prime" for 40 s and then press "OK."
11. Position the 384-well plate with aliquoted samples into its location on the arrayer deck and remove the parafilm cover.
12. Start printing.
13. When printing is complete, drain the sonicator bath (**Supplementary Figure 7B**) and turn off the humidifier.
14. Allow the slides to dry on the arrayer deck for 16-24 h without humidification.
15. Number the slides with an alcohol/solvent-resistant marking pen and store them in a vacuum-sealed box in the dark.

5. Processing and Developing the Arrays

1. Take out a slide from the vacuum-sealed box and place it in a chemical hood for 5 min, array facing up, to evaporate the excess liquid.
2. Scan the slide at the highest gain (950 PMT) to ensure that all the spots have been printed.
3. **Proceed with the chemical blocking of epoxy groups on the slide**
    1. Prepare 50 mL of ethanolamine blocking solution (0.1 M Tris-HCl, pH 8 and 0.05 M ethanolamine). Mix 140 mL of DIW with 8.8 mL of Tris-HCl (1.7 M, pH 8) and 0.45 mL of ethanolamine (16.6 M). Transfer to a 50-mL staining tube (**Supplementary Figure 8A**) and warm to 50 °C.
    2. Hydrate the slide. Place the slide in a staining tube filled with DIW, cover it with foil, and incubate for 5 min with slow and gentle shaking at RT.
    3. Block the reactive epoxy groups on the slide. Dip the slide into the 50-mL staining tube filled with pre-warmed ethanolamine blocking solution, cover with foil, and incubate for 30 min with slow and gentle shaking at RT.
    4. Discard the ethanolamine blocking solution into the appropriate biohazard trash receptacle and place the slide in a new, clean staining tube filled with 50 °C DIW. Cover with foil and incubate for 10 min with slow and gentle shaking at RT.
    5. Discard the water and transfer the slides to a glass staining holder (**Supplementary Figure 8B**). Place the glass staining holder into a swinging plate holder and centrifuge dry the slide for 5 min at 200 x g and RT.
4. **Set up a divider and proceed with the biological blocking.**
    1. Place the slide onto a 16-well divider (**Supplementary Figure 8C**).
    2. Prepare PBST washing solution: 50 mL of phosphate-buffered saline (PBS), pH 7.4 + 0.1% Tween-20.
    3. Prepare biological blocking solution (PBS-OVA): 5 mL of PBS, pH 7.4 + 1% chicken ovalbumin (10 mg/mL).
    NOTE: The choice of blocking reagent is critical for the success of the detection of anti-Neu5Gc antibodies. Ovalbumin is made in chickens which, like humans, cannot synthesize Neu5Gc, causing a lack of expression of this Sia. For the detection of Neu5Gc, even minor contaminants can dramatically reduce the sensitivity of the assay, sometimes even failing to detect any anti-Neu5Gc reactivity. For example, the most commonly used blocking reagent, bovine serum albumin (BSA), although not glycosylated by itself, contains bovine IgG contaminants that carry Neu5Gc-moieties and therefore compete with printed glycans when binding to sample with anti-Neu5Gc antibodies[19,20].

4. Prewet the wells. Using a multi-pipette, aliquot 200 µL of PBST into the slide-wells, place them in a covered humid chamber, and shake for 5 min.
   NOTE: The humidity chamber is a glass tray with a wet towel paper, covered with nylon wrap and foil to protect the samples from light.
5. Remove the PBST by flicking and tapping gently on a clean paper towel and aliquot 200 µL of PBS-OVA blocking buffer into each well. Place in a humid chamber and incubate for 1 h at RT with gentle shaking.

5. Prepare primary detection diluted in PBS-OVA blocking buffer, as listed in **Table 2**.
   NOTE: For quality control (QC) assessment of this sialoglycan microarray, use several Sia binding proteins: *Sambucus nigra* lectin (SNA), isolated from elderberry bark, is expected to recognize Siaα26; *Maackia Amurensis Lectin II* (MALII) is expected to recognize Siaα23; and chicken anti-Neu5Gc IgY is expected recognize all Neu5Gc-containing sialoglycans. In addition, use various human sera samples to profile anti-Neu5Gc IgG response. The concentration of lectins/antibodies/sera should be calibrated experimentally.
6. Flick dry PBS-OVA blocking buffer and add 200 µL of primary antibody per well (minimum volume per well: 70 µL), incubate in a humid chamber for 2 h.
7. Flick dry the primary detection and wash 4 times with PBST (between the 3rd and 4th wash, incubate the slide in PBST for 5 min in the humid chamber). Wash once with PBS.
8. Prepare the secondary antibody in PBS, as listed in **Table 2**.
9. Flick dry the PBS and add 200 µL of secondary antibody. Incubate for 1 h at RT with shaking.
10. Flick dry the secondary antibody and wash four times with PBST (between the 3rd and 4th wash, incubate the slide in PBST for 5 min in the humid chamber).
11. Carefully remove the frame and place the slides into a 50 mL slide staining tube filled with PBS and shake for 5 min.
12. Fill two slide-staining baths (**Supplementary Figure 8D**) with DIW, place the slide into the slide-holder, and dip it inside the bath. Quickly dip 10 times and then transfer to the second bath and dip 10 more times. Incubate for 10 min at RT with shaking.
13. Discard the water and transfer the slide to a glass staining holder (**Supplementary Figure 8B**; allow the slide to air dry). Place the glass staining holder in a swinging plate holder and centrifuge dry the slide for 5 min at 200 g and RT.
14. Scan the slide and then store it in a vacuum-sealed box in the dark.

6. Array Scanning and Data Analysis

1. Open the fluorescent slide scanner and let it warm for 5 min. Open the scanning and analyzing software.
2. Set the scanning parameters (**Supplementary Figure 9A**): scan at 532 nm and 100% power with a gain of 350 PMT, with one line to average and a 0-µm focus position. Use a 10.0 µm pixel size resolution.
   NOTE: Scanning parameters can be adjusted to different slide types, fluorescence, and resolution.
3. Scan the developed slide. Open the scanner door and place the slide inside, array facing down. Start scanning (green "Play" button on the software navigation panel menu, **Supplementary Figure 9B**).
4. Save the scanned slides as a tiff file.
5. Prepare slide for analysis. Select "Load array list" and upload the appropriate GAL file that maps the arrays in each block on the slide (**Supplementary Figure 9C**).
   NOTE: The GAL file contains information regarding both the identity and location (X, Y) of each spot on the slide. This file can be created by the printer software exporting a tab-delimited text file based on samples identities in the 384-well source plate and the defined print design.
6. Set the alignment and analysis parameters. Press the "Option" button on the software navigation panel menu (**Supplementary Figure 10**).
7. Define the alignment parameters. Find circular features, resize features during alignment by 50 - 350%, limit feature movement to a maximum translation of 40 µm, unflag features that fail background threshold (CPI threshold 0), estimate the wrapping and rotation when finding blocks, and automate image registration (10 pixels) (**Supplementary Figure 10**).
8. Define the analysis parameters: W1/ 532 ratio, normal standard deviation, analyze absent features, and background subtraction. Click "select" and then "Local feature background median" and set 2 pixels to exclude and a 3-pixel width of background. Press "OK." Set the scanner saturation level to default (**Supplementary Figure 10**).
   NOTE: The background subtraction method depends on the experimental design and can be modified according to specific needs.
9. Align the features maps. Set the program into block mode (press on "B"), select all blocks (Ctrl+A), position the array map grids, and align all blocks (Alt+Shift+F7) (**Supplementary Figure 11**).
10. Refine the feature alignment in each block. Zoom (press "Z" to get into zoom mode) into the upper-left block, press "B" again (block mode), and press the grid of this block. Only move the grid of this block until it aligns perfectly with the block spots and press F5 to align the features in this selected block. Repeat the grid movement and F5 alignment until the spots are perfectly circled. Do the same for every one of the 16 blocks until all grids are in place.
11. Analyze and save the data. Select all blocks (Crtl+A) and then analyze the data (Alt+A). Save the analysis results (Ctrl +U) as a .gpr file.
12. Open the saved .gpr file in a spreadsheet program and analyze the intensity of each spot based on fluorescence after local background subtraction (F532-B532).

Representative Results

Array Printing, Development, and Analysis:

Printing a sialoglycan microarray with multiple glycan samples and human IgG STD curves in 16 different blocks requires thorough calibration to ensure that all samples are printed as uniformly as possible in all 16 blocks per slide and to all slides in the same print run. Therefore, multiple calibration experiments are required before the specific printing parameters are determined, including buffer composition for each type of sample, slide type and manufacture, 384-well plate type, humidity levels, sample volume in the 384-well plate, amount of pre-printing for every type of sample, human IgG STD curve concentrations, and marker type. The durability of such printed sialoglycan array slides was validated to last for up to 10 months in a vacuum-sealed box in the dark at room temperature. In every printing experiment, uniformity is further monitored and validated through quality control experiments using Sia binding lectins and antibodies. The developing and scanning protocols had been optimized to achieve uniformity and accuracy by comparing samples within and between slides from the same print run. The slides

are developed with the 16-well divider, which ensures the proper separation between blocks, using optimized blocking conditions (chemical and biological), washes, and incubation times. The primary and secondary detection had extensively been calibrated to ensure maximum detection with minimal background and non-specific binding. Upon slide scanning and analysis, output results were transferred to a spreadsheet file and analyzed to determine the detection in each spot. Each spot was subjected to an outlier check and omitted if it did not meet QC (if the %CV is >20 and the spot is outside the range of one standard deviation away from the mean of the four replicates). Then, the mean signal intensity after the subtraction of local background was averaged for replicate spots per sample to generate the relative fluorescence unit (RFU) data point per printed sample. Once the uniformity of the IgG STD curves was validated in all tested blocks, the RFU values were normalized into ng/µL IgG, allowing for the chance to better compare samples within and between experiments.

Sia-binding High-throughput Assay:

Sialoglycan arrays can be used to detect determinants (*e.g.,* proteins, lectins, antibodies, viruses, *etc.*) that bind Sia-containing glycans. For QC after printing, Sia-binding plant lectins and anti-Neu5Gc IgY are used[19]. SNA binds α2-6-linked Sia, MALII binds α2-3-linked Sia, and anti-Neu5Gc IgY binds Neu5Gc-containing sialoglycans but does not bind Neu5Ac-containing sialoglycans[19], as shown in **Figure 2A**. The QC experiment aims to validate spot printing and identity and the lack of variability between the printed blocks using the four different pins. Block-to-block variability is monitored by developing four blocks for every primary detection per slide and by comparing blocks that were printed with each one of the four pins with the same primary detection moiety. A comparison is then done to ensure that no major differences are present.

Human sera contain diverse anti-Neu5Gc antibodies[6] with implication for various human diseases[2,21,22,23]. To evaluate sialoglycan recognition in human sera IgG, 12 sera from healthy human donors were analyzed on the printed sialoglycan microarray and developed using fluorescently labeled anti-human IgG (**Figure 2B**). Each printed block contains a human IgG STD curve that is comparable and homogenous in all blocks (**Figure 2C**). Only after validating the quality of the STD curves in each block (**Figure 2C**) are glycan spots results normalized according to the slope in the block and then multiplied by the dilution factor. As shown in **Figure 2B**, all tested human sera contain various levels of anti-Neu5Gc IgG, with almost no recognition of the matched pairs of Neu5Ac-containing sialoglycans. Furthermore, anti-Neu5Gc IgG recognition patterns are highly diverse between these 12 different sera, both in the level of intensity and of diversity.

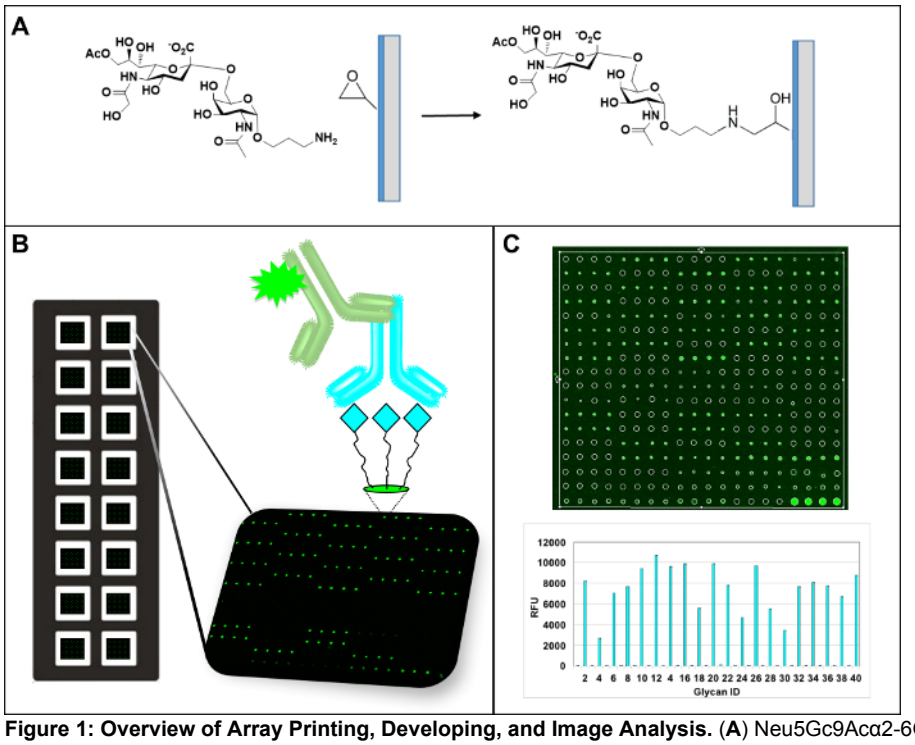

Figure 1: Overview of Array Printing, Developing, and Image Analysis. (**A**) Neu5Gc9Acα2-6GalNAcαProNH2 is shown as a representative Neu5Gc-containg Sia glycan, with a primary amine that is printed on epoxy-coated glass slides. (**B**) Printed arrays are developed with various Sia-binding proteins, followed by detection with an appropriate fluorescently labeled secondary antibody. Each sub-array can be developed individually. (**C**) Scanning the probed slide in a fluorescence scanner generates an image that is further analyzed using the scanner image software. The sub-arrays are overlaid with a grid mapping each spot on the arrays and the fluorescence detected for each spot. The results are then transferred intoa spreadsheet file. A single array in a single well is schematically represented. Please click here to view a larger version of this figure.

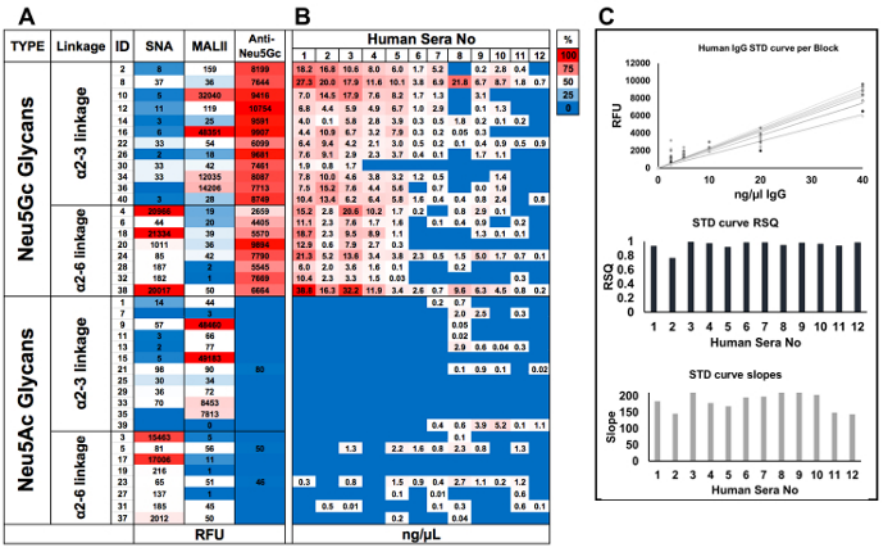

Figure 2: Profiles of Sialoglycan Microarrays Using Various Sia-binding Proteins.
Slides were developed with 16 different primary detection moieties, one in each block. (**A**) Representative 3 blocks of a QC slide showing the binding patterns of the Sia-specific plant lectins SNA and MALII and polyclonal mono-specific chicken anti-Neu5Gc IgY. The results are presented as the RFU in heatmap format for each individual block (red, white, and blue, representing the 100th, 50th, and 0th percentiles, respectively). (**B**) 12 different healthy human sera were tested at a 1:100 dilution and detected with anti-huamn IgG to profile anti-Neu5Gc IgG reactivity. RFU data were normalized to ng/µL by dividing each value with the IgG STD curve slope in each specific block and then multiplied by the dilution factor. The data is represented in heatmap format for all blocks combined (red, white, and blue, representing te 100th, 50th, and 0th percentiles, respectively). (**C**) Human IgG STD curves of the 12 blocks developed with human sera that had been used to normalize the data.
Please click here to view a larger version of this figure.

Glycan ID	Structure
1	Neu5,9Ac$_2$α3Galβ4GlcNAcβO(CH$_2$)$_2$CH$_2$NH$_2$
2	Neu5Gc9Acα3Galβ4GlcNAcβO(CH$_2$)$_2$CH$_2$NH$_2$
3	Neu5,9Ac$_2$α6Galβ4GlcNAcβO(CH$_2$)$_2$CH$_2$NH$_2$
4	Neu5Gc9Acα6Galβ4GlcNAcβO(CH$_2$)$_2$CH$_2$NH$_2$
5	Neu5Acα6GalNAcαO(CH$_2$)$_2$CH$_2$NH$_2$
6	Neu5Gcα6GalNAcαO(CH$_2$)$_2$CH$_2$NH$_2$
7	Neu5,9Ac$_2$α3Galβ3GlcNAcβO(CH$_2$)$_2$CH$_2$NH$_2$
8	Neu5Gc9Acα3Galβ3GlcNAcβO(CH$_2$)$_2$CH$_2$NH$_2$
9	Neu5,9Ac$_2$α3Galβ3GalNAcαO(CH$_2$)$_2$CH$_2$NH$_2$
10	Neu5Gc9Acα3Galβ3GalNAcαO(CH$_2$)$_2$CH$_2$NH$_2$
11	Neu5Acα3Galβ4GlcNAcβO(CH$_2$)$_2$CH$_2$NH$_2$
12	Neu5Gcα3Galβ4GlcNAcβO(CH$_2$)$_2$CH$_2$NH$_2$
13	Neu5Acα3Galβ3GlcNAcβO(CH$_2$)$_2$CH$_2$NH$_2$
14	Neu5Gcα3Galβ3GlcNAcβO(CH$_2$)$_2$CH$_2$NH$_2$
15	Neu5Acα3Galβ3GalNAcαO(CH$_2$)$_2$CH$_2$NH$_2$
16	Neu5Gcα3Galβ3GalNAcαO(CH$_2$)$_2$CH$_2$NH$_2$
17	Neu5Acα6Galβ4GlcNAcβO(CH$_2$)$_2$CH$_2$NH$_2$
18	Neu5Gcα6Galβ4GlcNAcβO(CH$_2$)$_2$CH$_2$NH$_2$
19	Neu5Acα6Galβ4GlcβO(CH$_2$)$_2$CH$_2$NH$_2$
20	Neu5Gcα6Galβ4GlcβO(CH$_2$)$_2$CH$_2$NH$_2$
21	Neu5Acα3Galβ4GlcβO(CH$_2$)$_2$CH$_2$NH$_2$
22	Neu5Gcα3Galβ4GlcβO(CH$_2$)$_2$CH$_2$NH$_2$
23	Neu5,9Ac$_2$α6GalNAcαO(CH$_2$)$_2$CH$_2$NH$_2$
24	Neu5Gc9Acα6GalNAcαO(CH$_2$)$_2$CH$_2$NH$_2$
25	Neu5Acα3GalβO(CH$_2$)$_2$CH$_2$NH$_2$
26	Neu5Gcα3GalβO(CH$_2$)$_2$CH$_2$NH$_2$
27	Neu5Acα6GalβO(CH$_2$)$_2$CH$_2$NH$_2$
28	Neu5Gcα6GalβO(CH$_2$)$_2$CH$_2$NH$_2$
29	Neu5,9Ac$_2$α3GalβO(CH$_2$)$_2$CH$_2$NH$_2$
30	Neu5Gc9Acα3GalβO(CH$_2$)$_2$CH$_2$NH$_2$
31	Neu5,9Ac$_2$α6GalβO(CH$_2$)$_2$CH$_2$NH$_2$
32	Neu5Gc9Acα6GalβO(CH$_2$)$_2$CH$_2$NH$_2$
33	Neu5Acα3Galβ3GalNAcβO(CH$_2$)$_2$CH$_2$NH$_2$
34	Neu5Gcα3Galβ3GalNAcβO(CH$_2$)$_2$CH$_2$NH$_2$
35	Neu5,9Ac$_2$α3Galβ3GalNAcβO(CH$_2$)$_2$CH$_2$NH$_2$
36	Neu5Gc9Acα3Galβ3GalNAcβO(CH$_2$)$_2$CH$_2$NH$_2$
37	Neu5,9Ac$_2$α6Galβ4GlcβO(CH$_2$)$_2$CH$_2$NH$_2$
38	Neu5Gc9Ac6Galβ4GlcβO(CH$_2$)$_2$CH$_2$NH$_2$
39	Neu5,9Ac$_2$α3Galβ4GlcβO(CH$_2$)$_2$CH$_2$NH$_2$
40	Neu5Gc9Ac3Galβ4GlcβO(CH$_2$)$_2$CH$_2$NH$_2$

Table 1: List of Printed Glycans.

Primary / Secondary	Antibody / Lectin	Stock concentration	Specificity	Working Dilution / Concentration
Primary detection	Biotinilated-MALII	1 mg/mL	Sialic acid in α2-3 linkage	1:50, 20 µg/mL
	Biotinilated-SNA	2 mg/mL	Sialic acid in α2-6 linkage	1:100, 20 µg/mL
	Chicken anti-Neu5Gc IgY	N.D	Neu5Gc Sialic acid	1:7,000
	Human Serum	100%	Numerous epitopes	1:100
Secondary Detection	Cy3-Streptavidin	0.75 mg/mL	Biotin	1:500, 1.5 µg/mL
	Cy3-anti Chicken IgY	0.75 mg/mL	Chicken IgY	1:2,000, 0.375 µg/mL
	Cy3-anti Human IgG H+L	0.6 mg/mL	Human IgG	1:1,500, 0.4 µg/mL

Table 2: List of Primary and Secondary Detection Proteins.

Supplemental Figures: Please click here to download this file.

Discussion

A successful glycan microarray fabrication requires careful planning and includes several important steps in the protocol. These include: (1) planning the block and plate layouts that define all subsequent parameters (*e.g.,* distances, spacing, amount of samples, and printing); (2) cleaning the pins and ensuring pin integrity, which is critical for controlling spot homogeneity; (3) maintaining high humidity during printing, critical to avoiding sample evaporation during long print runs, which could compromise spot homogeneity; (4) selecting proper alignment and analysis parameters, which can influence the results (*e.g.,* background subtraction method, threshold, and spot size flexibility).

The method can be further modified to meet specific experimental designs and goals. For example, the pre-spotting number can be modified to better suit each type of material to be printed on the array. The pin washing step during the print can be optimized to fit different printed materials, with either shorter or extended wash cycles. Furthermore, the amount of spots a pin prints per dip can be modified to include more or fewer spots but requires separate calibration for each type of material used on the array. The type of the fluorescence marker and its concentration can be modified, as long as it contains the proper chemical group for conjugation (*i.e.,* primary amine for epoxy-coated slides). The concentrations and types of STD curves can be extended (*e.g.,* human/mouse/other organism IgG, IgM, IgA, *etc.*). Buffer conditions can be optimized to fit different materials, preferably lacking materials with primary amine to avoid non-specific binding to the array, which would cause increased background. Importantly, for optimal detection of Neu5Gc, the biological blocking reagent must be free of Neu5Gc (*e.g.,* avoid BSA amd milk), as this may reduce Neu5Gc-sialoglycan detection and increase background[19,20]. Primary and secondary detection concentrations should be optimized to avoid high background and non-specific binding. In addition, scanning parameters (*e.g.,* power, gain, and resolution) can be modified to reduce background or enhance low signals. Finally, alignment and analysis parameters can be modified to suit high background or differently shaped features. Further information and guidelines regarding the recommended standards for reporting glycan microarray data has recently been described by the MIRAGE initiative[17], which can eventually facilitate data sharing and interpretation.

In summary, glycan microarrays provide a robust tool for investigating glycan-biomolecule interactions and can be adapted to various biological samples. Anti-Neu5Gc antibodies play different roles in human disease[23]. For example, in cancer, anti-Neu5Gc antibodies play dual roles: on the one hand, they serve as potential biomarkers, but on the other hand, they serve as potential therapeutics[7,21,24]. These antibodies also contribute to the exacerbation of atherosclerosis[25], the efficacy of xenotransplantation[20,26], and the effect of glycosylated biotherapeutics[27]. Thus, profiling anti-Neu5Gc antibodies in various human samples is of growing interest, and high-throughput assays, such as the one described here, can contribute to furthering our understanding of their roles in human health and disease.

Disclosures

The authors have nothing to disclose.

Acknowledgements

This work was supported in part by a Research Career Development Award from the Israel Cancer Research Fund, a grant from the Israeli National Nanotechnology Initiative and the Helmsley Charitable Trust for a Focal Technology Area on Nanomedicines for Personalized Theranostics (V.P-K), and National Institutes of Health grant R01GM076360 (to X.C.).

References

1. Padler-Karavani, V. Aiming at the sweet side of cancer: Aberrant glycosylation as possible target for personalized-medicine. *Cancer Lett.* **352** (1), 102-112, (2014).
2. Amon, R., Reuven, E.M., Leviatan Ben-Arye, S., Padler-Karavani, V. Glycans in immune recognition and response. *Carbohydr Res.* **389**, 115-122, (2014).
3. Häuselmann, I., Borsig, L. Altered tumor-cell glycosylation promotes metastasis. *Front Oncol.* **4**, 28 (2014).
4. Tangvoranuntakul, P. *et al.* Human uptake and incorporation of an immunogenic nonhuman dietary sialic acid. *Proc Natl Acad Sci USA.* **100** (21), 12045-12050, (2003).

5. Bardor, M., Nguyen, D.H., Diaz, S., Varki, A. Mechanism of uptake and incorporation of the non-human sialic acid N-glycolylneuraminic acid into human cells. *J Biol Chem.* **280** (6), 4228-4237, (2005).
6. Padler-Karavani, V. *et al.* Diversity in specificity, abundance, and composition of anti-Neu5Gc antibodies in normal humans: potential implications for disease. *Glycobiology.* **18** (10), 818-830, (2008).
7. Padler-Karavani, V. *et al.* Human xeno-autoantibodies against a non-human sialic acid serve as novel serum biomarkers and immunotherapeutics in cancer. *Cancer Res.* **71** (9), 3352-3363, (2011).
8. Cao, H., Chen, X. General consideration on sialic acid chemistry. *Methods Mol Biol.* **808**, 31-56, (2012).
9. Deng, L., Chen, X., Varki, A. Exploration of sialic Acid diversity and biology using sialoglycan microarrays. *Biopolymers.* **99** (10), 650-665, (2013).
10. Liang, C.H., Hsu, C.H., Wu, C.Y. Sialoside Arrays: New Synthetic Strategies and Applications. *Top Curr Chem.* **367**, 125-149, (2015).
11. Song, X., Heimburg-Molinaro, J., Smith, D.F., Cummings, R.D. Glycan microarrays of fluorescently-tagged natural glycans. *Glycoconj J.* **32** (7), 465-473, (2015).
12. Muthana, S.M., Gildersleeve, J.C. Glycan microarrays: powerful tools for biomarker discovery. *Cancer Biomark.* **14** (1), 29-41, (2014).
13. Rillahan, C.D., Paulson, J.C. Glycan microarrays for decoding the glycome. *Annu Rev Biochem.* **80**, 797-823, (2011).
14. Heimburg-Molinaro, J., Song, X., Smith, D.F., Cummings, R.D. Preparation and analysis of glycan microarrays. *Curr Protoc Protein Sci.* **12** (10), (2011).
15. Park, S., Gildersleeve, J.C., Blixt, O., Shin, I. Carbohydrate microarrays. *Chem Soc Rev.* **42** (10), 4310-4326, (2013).
16. Struwe, W.B. *et al.* The minimum information required for a glycomics experiment (MIRAGE) project: sample preparation guidelines for reliable reporting of glycomics datasets. *Glycobiology.* **26** (9), 907-910, (2016).
17. Liu, Y. *et al.* The minimum information required for a glycomics experiment (MIRAGE) project: improving the standards for reporting glycan microarray-based data. *Glycobiology.* (2016).
18. Hara, S., Yamaguchi, M., Takemori, Y., Furuhata, K., Ogura, H., Nakamura, M. Determination of mono-O-acetylated N-acetylneuraminic acids in human and rat sera by fluorometric high-performance liquid chromatography. *Anal Biochem.* **179** (1), 162-166, (1989).
19. Padler-Karavani, V. *et al.* Cross-comparison of protein recognition of sialic acid diversity on two novel sialoglycan microarrays. *J Biol Chem.* **287** (27), 22593-22608, (2012).
20. Padler-Karavani, V., Varki, A. Potential impact of the non-human sialic acid N-glycolylneuraminic acid on transplant rejection risk. *Xenotransplantation.* **18** (1), 1-5, (2011).
21. Samraj, A.N. *et al.* A red meat-derived glycan promotes inflammation and cancer progression. *Proc Natl Acad Sci USA.* **112** (2), 542-547, (2015).
22. Pearce, O.M., Läubli, H. Sialic acids in cancer biology and immunity. *Glycobiology.* **26** (2), 111-128, (2016).
23. Alisson-Silva, F., Kawanishi, K., Varki, A. Human risk of diseases associated with red meat intake: Analysis of current theories and proposed role for metabolic incorporation of a non-human sialic acid. *Mol Aspects Med.* **51**, 16-30, (2016).
24. Pearce, O.M. *et al.* Inverse hormesis of cancer growth mediated by narrow ranges of tumor-directed antibodies. *Proc Natl Acad Sci USA.* **111** (16), 5998-6003, (2014).
25. Pham, T. *et al.* Evidence for a novel human-specific xeno-auto-antibody response against vascular endothelium. *Blood.* **114** (25), 5225-5235, (2009).
26. Reuven, E.M. *et al.* Characterization of immunogenic Neu5Gc in bioprosthetic heart valves. *Xenotransplantation.* **23** (5), 381-392, (2016).
27. Ghaderi, D., Taylor, R.E., Padler-Karavani, V., Diaz, S., Varki, A. Implications of the presence of N-glycolylneuraminic acid in recombinant therapeutic glycoproteins. *Nat Biotechnol.* **28** (8), 863-867, (2010).

