Cross-platform comparison of glycan microarray formats

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**Carbohydrates participate in almost every aspect of biology
from protein sorting to modulating cell differentiation and
cell–cell interactions. To date, the majority of data gathered on
glycan expression has been obtained via analysis with either
anti-glycan antibodies or lectins. A detailed understanding of
the specificities of these reagents is critical to the analysis of
carbohydrates in biological systems. Glycan microarrays are
increasingly used to determine the binding specificity of
glycan-binding proteins (GBPs). In this study, six different
glycan microarray platforms with different modes of glycan
presentation were compared using five well-known lectins;
concanavalin A, *Helix pomatia* agglutinin, *Maackia amurensis*
lectin I, *Sambucus nigra* agglutinin and wheat germ agglutin-
in. A new method (universal threshold) was developed to
facilitate systematic comparisons across distinct array plat-
forms. The strongest binders of each lectin were identified
using the universal threshold across all platforms while identi-
fication of weaker binders was influenced by platform-specific
factors including presentation of determinants, array compos-
ition and self-reported thresholding methods. This work com-
piles a rich dataset for comparative analysis of glycan array
platforms and has important implications for the implementa-
tion of microarrays in the characterization of GBPs.**

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Introduction

Glycan-binding proteins (GBPs), also referred to as carbohydrate-
binding proteins, mediate a broad range of biological processes
through interactions with glycans, including bacterial infection,
inflammation and metastasis (Varki et al. 2009). On all known
cell types, glycans cover the majority of the cell surface
(Lanctot et al. 2007). Discovering the binding specificities for
GBPs is crucial to understanding their biological roles and to
our use of these proteins as analytical reagents (Kuno et al.
2005; Pilobello et al. 2005, 2007; Stowell et al. 2008, 2010).
Glycan microarrays, in which libraries of natural or synthetic
glycans are immobilized onto a solid support, are a powerful
method for determining the binding specificities of GBPs
(Fukui et al. 2002; Stallforth et al. 2008; Liu et al. 2009;
Oyelaran et al. 2009; Smith et al. 2010; Heimburg-Molinaro
et al. 2011; Rillahan and Paulson 2011; Park et al. 2013).
Multiple platforms exist for glycan microarrays covering a
broad range of glycan determinants and different presentations
of glycans. Since all glycan microarray platforms represent a
limited number of glycans relative to the number of naturally
occurring structures, the GBP binding observed will be related
to the platform used. Strong evidence exists that the presentation
of glycans within different formats can have a profound effect on
the apparent-binding affinity and specificity of GBPs (Brewer
et al. 2002; Unverzagt et al. 2002; Oyelaran et al. 2009; Smith
et al. 2010; Rillahan and Paulson 2011; Kilcoyne et al. 2012),
resulting in differences in the binding profiles for GBPs exam-
ined on different platforms. Recent work by Padler-Karvani et al.
compared two sialoglycoside arrays in which the modes of
presentation were similar (amine-derivatized glycans coupled to
activated glass slides), but structures of glycans differed signifi-
cantly (Padler-Karavani et al. 2012). In this work, large agree-
ment between datasets was found, but it was concluded that
array format may affect outcome and suggested that a broader
comparison of platforms is necessary to vet this conclusion.

Herein, we present the results of a broad comparison
between microarray platforms from the six microarray labora-
tories participating in our cross-comparison study. The
experiments were designed along the lines of glycoprofiling
studies in which the same sample was sent to multiple mass
spectrometry laboratories for analysis (Wada et al. 2007) and
were intended to examine whether differences in analytical
platforms across laboratories had a significant effect on
reported GBP profiles. The microarrays in this study con-
tained a wide diversity of glycans and varied in size, presen-
tation and composition (Figure 1, Table I). Aliquots from
the same protein lots for five well-known lectins (Table II),
concanavalin A (Con A), *Helix pomatia* agglutinin (HPA),
Maackia amurensis lectin I (MAL-I), *Sambucus nigra* agglutinin
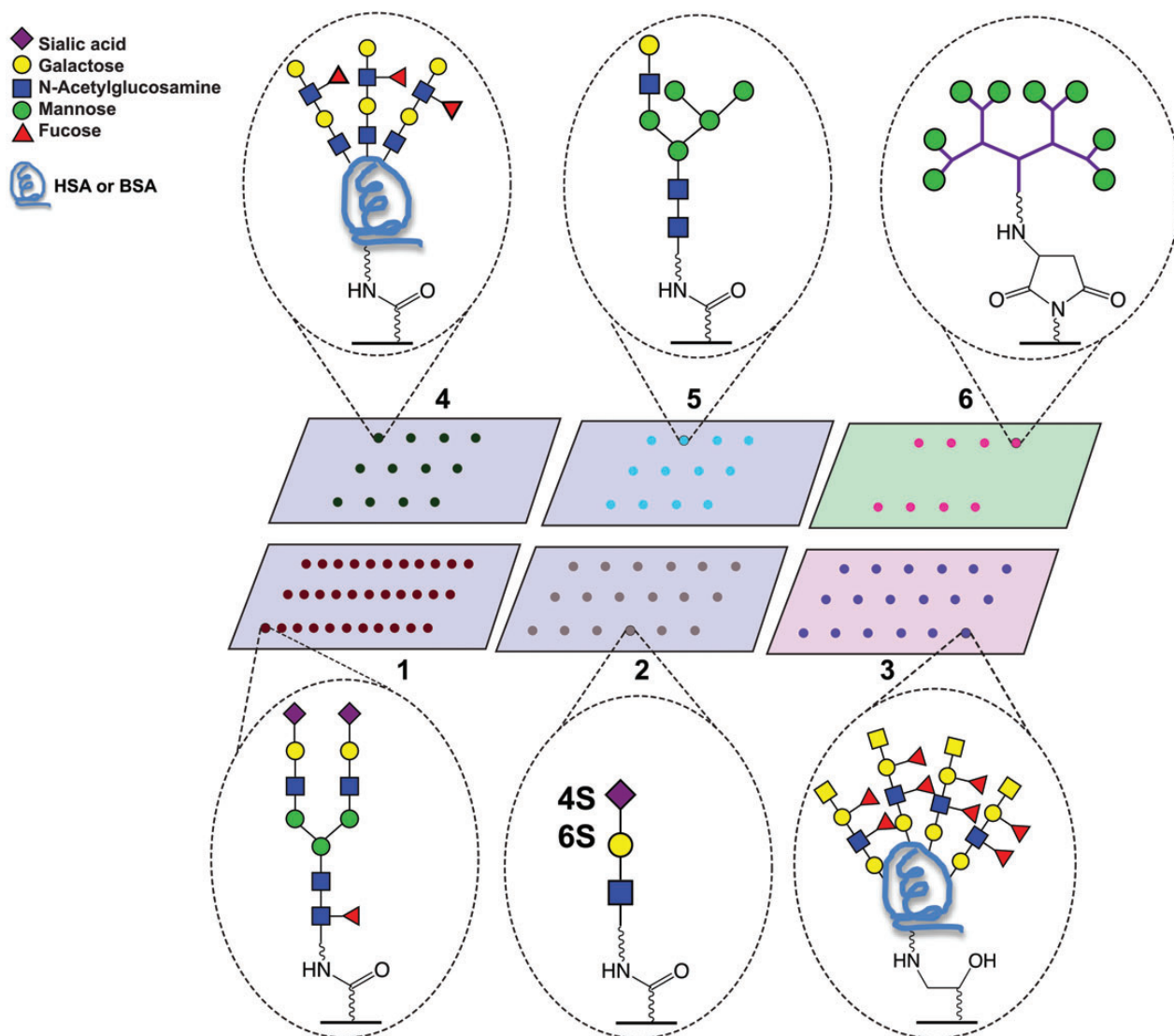
Fig. 1. Graphic representation of glycan array formats. Array 1-CFG; Array 2-Huflejt; Array 3-Gildersleeve; Array 4-Joshi; Array 5-Reichardt; Array 6-Pieters. Gray surface: Nexterion H® (NHS) slide, pink surface: epoxide slide, green surface: PamChip® (maleimide). Representative structures are shown.

Table I. Summary of glycan microarrays from six research groups participating in this study

Array	Research groups	Number of array components	Type of slides	Glycan or neoglycoprotein concentration (μM)	References
1	CFG	611	Nexterion H®	100	
2	Huflejt	378	Nexterion H®	50	Vuskovic et al. (2011)
3	Gildersleeve	317	Epoxide	5–55	Zhang and Gildersleeve (2012)
4	Joshi	52	Nexterion H®	16–298	Kilcoyne et al. (2012)
5	Reichardt	39	Nexterion H®	50	Serna et al. (2010)
6	Pieters	20	PamChip®	500–5000	Branderhorst et al. (2008)

(SNA) and wheat germ agglutinin (WGA) were sent to each group. Comparison of the results from all groups concur with earlier work (Padler-Karavani et al. 2012), arguing that for strong binders there is good agreement between platforms, but for medium to weak binding interactions, which may be physiologically relevant, platforms and practices have a profound effect on the observed glycan-protein interactions. This has implications for interpreting glycan microarray data to determine the specificity of GBP-glycan-binding interactions.

Table II. List of five biotinylated lectins tested in this study

Name	Abbreviation	Vendor	Catalog number	Lot number
Concanavalin A	Con A	EY Laboratories	BA-1104-5	290210-5
Helix pomatia agglutinin	HPA	Sigma-Aldrich	L6512	028K3810V
Maackia amurensis lectin I	MAL-I	Vector Laboratories	B-13l5	W0224
Sambucus nigra agglutinin	SNA	Vector Laboratories	B-1305	W0425
Wheat germ agglutinin	WGA	EY Laboratories	BA-2101-5	290227

Table III. Binary comparison of overlapping determinants between the six arrays

Array	1	2	3	4	5	6
1	545	192	80	31	14	4
2	192	302	68	29	0	4
3	80	68	221	35	11	5
4	31	29	35	50	0	3
5	14	0	11	0	39	0
6	4	4	5	3	0	5

Determinant count ignores differences in linker type, coupling chemistry and glycan density.

Results and discussion

Experimental design for cross-array comparison

Six glycan microarray research groups participated in this study which was coordinated by the laboratory of Dr. Lara Mahal (New York University): the Consortium for Functional Glycomics (http://www.functionalglycomics.org/static/consortium/ resources/ resourcecoreh.shtml, last accessed March 31, 2014) (CFG, Emory University School of Medicine, Array 1) and the laboratories of Dr. Margaret Huflejt (New York University School of Medicine, Array 2), Dr. Jeffrey Gildersleeve (National Cancer Institute, Array 3), Dr. Lokesh Joshi (National University of Ireland Galway, Array 4), Dr. Niels-Christian Reichardt (CICbiomaGUNE, Array 5) and Dr. Roland Pieters (Utrecht University, Array 6). Each group was sent five identical aliquots of biotinylated lectins (Con A, HPA, MAL-I, SNA and WGA; Table II) and highly purified bovine serum albumin (BSA, Boval) to minimize differences in binding due to lot-dependent lectin variation or glycan contaminants of BSA. The lectins used in this study are widely utilized throughout the biomedical community to examine glycosylation in mammalian systems (Van Damme et al. 1998). It should be noted, however, that the physiological ligands of these lectins may not be mammalian glycans and the platforms examined contained predominantly mammalian sugars. This study was designed to compare glycan-binding profiles obtained using different array platforms (i.e. different printing procedures, slide chemistries, glycans, glycan presentation and practices such as binding time, wash buffers, scanning protocols and analysis) to obtain information on the reliability of glycan profiles across laboratories and to identify issues that might play a role in glycan microarray interpretation. Each group was asked to run three select concentrations of each lectin on their glycan microarrays (50, 10 and 1 µg/mL), with an additional two concentrations of their choosing in the linear range of their system. Fluorophore-labeled streptavidin was used for detection of the binding across all platforms tested. Data were then sent to the laboratory of Dr. Lara Mahal, which has expertise in microarray datasets (Pilobello et al. 2005, 2007), for analysis.

Comparison of array platforms used in this study

To understand the similarities and differences between the arrays, we determined the level of overlap in glycan determinants between the six arrays. For simplicity, we ignored differences in linker type, coupling chemistry and glycan density in defining determinants, resulting in fewer determinants than glycan structures printed (Table III). No determinants were

Fig. 2. Venn diagram of determinants for Arrays 1–3. Number of determinants (overlapping and unique) are indicated.

shared by all six arrays, showcasing the diversity inherent in these platforms. A Venn diagram representing the determinant overlap of the three largest arrays (Arrays 1–3) is shown in Figure 2. Of the 789 unique determinants represented by the three arrays, only 61 are shared between all three arrays, corresponding to 7.7% of the total. Examination of the two largest arrays (Array 1 and Array 2) showed the most total determinants in common (192 total, 64% of determinants on Array 2). However, the strongest overlap in determinants was observed between Arrays 3 and 4 (70% of Array 4) with 35 of the 50 determinants on Array 4 represented on the larger Array 3. Of these 35 common glycan structures, 23 were neoglycoproteins purchased from the same company, providing a direct comparison of the effects of slide chemistry and practices on binding.

With the exception of Array 6, which had only five determinants, each array contained determinants exclusive to that array. Each array platform had unique features and analytical protocols (Figure 1; Supplementary Methods). Array 1 is version 5.0 of the CFG array and is the largest of the group, with 611 glycan structures linked via a primary amine to an NHS-derivatized glass slide (Blixt et al. 2004; Heimburg-Molinaro et al. 2012). This array had the most diversity of determinants displayed and many of these were only found on this array. Array 2 used identical coupling and slide chemistries but differed from Array 1 in

the size (378 glycan structures) and in the concentration of glycan deposited (Vuskovic et al. 2011). Array 5 also used similar coupling chemistry but was unique in that it was generated through a mixture of chemical synthesis and enzymatic elaboration, with 17 synthesized glycans elaborated with galactosyl- and sialyltransferases to create an array of 39 determinants. This array, while less diverse, had strong representation of larger N-linked glycan structures and contained a unique set of hybrid N-linked glycans (Serna et al. 2010). In contrast to these arrays, Arrays 3 and 4 both utilized neoglycoproteins, rather than glycan-amine monomers, to present the carbohydrate moieties. Array 3, printed on epoxide slides, is the larger of the two (317 glycan structures), and used the neoglycoprotein platform to vary the displayed density of some determinants (Zhang and Gildersleeve 2012). For example, Array 3 contained a unique series of Tn-antigen containing peptides. Array 4 was printed on an NHS-derivatized surface and contained 52 glycoproteins (Kilcoyne et al. 2012). Array 6 was highly focused and contained only five carbohydrate structures, but these structures were presented at varying valencies using a glycodendrimer approach (Parera Pera et al. 2010). These six arrays represent a wide variation in glycan microarray technology summarized in Table I; however, they are not all inclusive and several array types including neoglycolipid arrays (Fukui et al. 2002) and glycans on gold surfaces (Zhi et al.

2008) are not represented in this study. Overall, the divergence of represented determinants on the six arrays represented in this study complicates comparative analysis.

Quality control and data analysis of glycan array datasets

Representative images of the microarrays upon binding of WGA are shown in Figure 3 (see Supplementary data, Figure S1 for images from all lectins). As a quality control measure for the data provided, we examined the Pearson correlation coefficients between all concentrations of a single lectin for each dataset, with the exception of Array 6. We reasoned that if the appropriate binding determinants were present, all concentrations of a particular lectin on a single array platform should give a conserved pattern of relative binding, reflected in fluorescence intensities, that is statistically significant ($P < 0.001$). Data that failed to meet this threshold indicated problems in the quality of one or more arrays and were therefore excluded from our analysis (Supplementary data, Table S1).

Comparison of glycan-binding profiles

In defining the binding of a GBP to its cognate glycans, identifying both the structures bound and their relative affinities is crucial. One major challenge for a cross-platform comparison is selecting an appropriate method for comparing signals on various
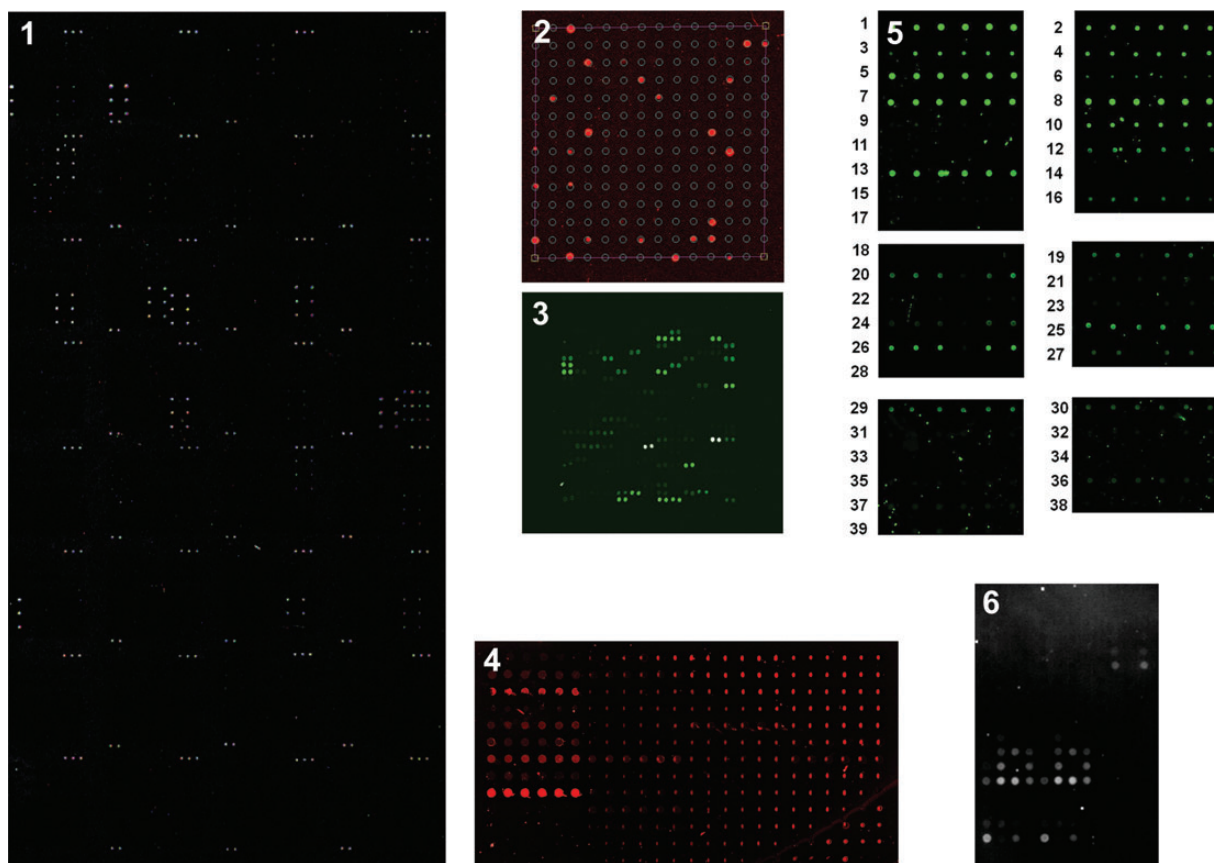

Fig. 3. Representative images of six glycan arrays for WGA binding. Microarrays are not to scale and, in some cases, are false-colored dependent on the platform. Due to differences in platforms, concentrations of biotinylated WGA used vary: Arrays 1 and 3 (10 µg/mL WGA), Array 2 (100 µg/mL), Array 4 (0.8 µg/mL), Array 5 (1 µg/mL) and Array 6 (5 µg/mL).

arrays. One simple approach involves comparing signals from each array at a particular lectin concentration (e.g. 1 µg/mL). Unfortunately, different arrays have different sensitivities such that no single concentration would be suitable for all the various array platforms used in this study. In addition, this approach does not account for the fact that the maximal signal attainable for a given spot can vary depending on the specific glycan (i.e. one glycan may reach saturation at an RFU of 10,000 while another saturates at 60,000). A second strategy would involve assaying lectins at a range of concentrations and calculating apparent K_d values for each glycan that generates suitable signals (Liang et al. 2007). The advantage of this approach is that it provides quantitative or semi-quantitative measures of avidity for comparison across arrays that are independent of absolute signal levels and array sensitivity. The disadvantage is that this approach requires at least three lectin concentrations for a rough estimate of apparent K_d values and 5–10 concentrations for a more accurate calculation. Moreover, one must ensure that the set of lectin concentrations used in the study spans a range that will allow determination of both saturating signal levels and half saturating signals. The optimal set of concentrations is difficult to predict a priori and varies greatly from one array platform to another. Although this was our original intent, we found that this approach was difficult to implement with the data from our cross-platform comparison as many of the datasets did not have sufficient data within an appropriate range. A third strategy would be to use the z-score method of Cholleti et al. (2012). In this method, binding glycans are defined by a z-score (with P-value <0.15), which is calculated with the sum of the fluorescence values for all the concentrations

of the glycan structure. We found that this metric, used by the CFG for the larger more diverse Array 1, breaks down when smaller arrays with less diversity are examined. In arrays where a significant number of glycans are bound by a lectin, the statistical difference from the mean of a positive binder may not be significant. Due to the size and diversity differences between the six platforms, z-score-based methods were not suitable for this study. A fourth method would be to have each array group apply their own approach for defining bound glycans (self-reported threshold). This method allows each group to apply the statistical method that is deemed most appropriate for their particular array platform. Although the self-reported threshold method is probably the best to determine binders on each array, we wanted a metric that could be used to examine the data across all arrays to ensure the least biased comparison of the array results.

For a more systematic comparison of results, we developed a universal threshold method. We then compared this with the self-reported thresholding methods used by each group. We excluded Array 6 from this analysis as it contained too few determinants (5 determinants, 20 structures) to apply this method. For the universal threshold, we defined positive binders for each lectin on an array platform as glycans for which the average fluorescence was >10% of the maximum signal observed for that lectin for arrays in the linear range (Figure 4). Arrays in which the maximum fluorescence intensity observed was between 20 and 80% of the highest fluorescence observed for that lectin across all arrays were taken to be in the linear range. This assumes that on the array with the highest maximum (usually the highest concentration tested) the array has reached
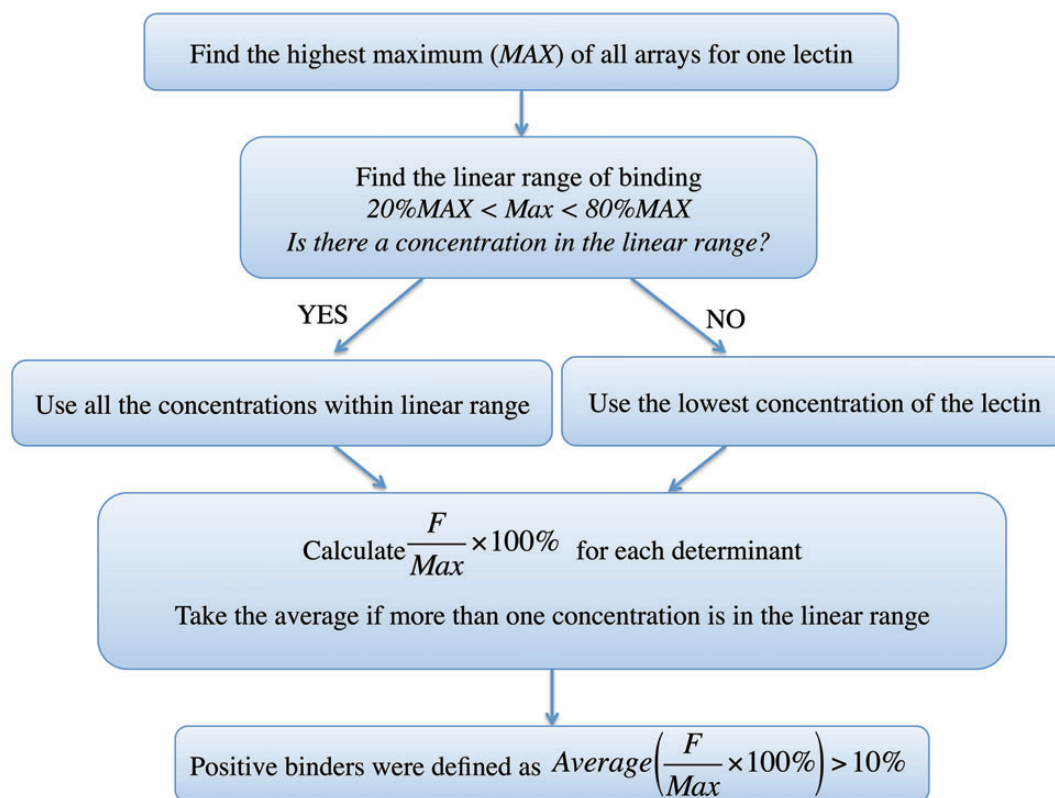

Fig. 4. The process of identifying positive binders using the universal threshold.

saturation. In many cases, only one concentration was found to be in the linear range using this criteria. If no array was determined by this method to be in the linear range, the lowest concentration data were used. A detailed example of these calculations for MAL-I from Array 4 is given in Supplementary data.

The universal threshold allowed only the strongest binding determinants to be detected and was insensitive to weak or moderate binders. For example, using the universal threshold only 18 positive binders were identified for Con A on Array 1. However, none of the more complex biantennary glycans, which are known to be ligands of Con A, were observed. In contrast, using the self-reported threshold of Array 1 (z-score method), which includes all concentrations of lectin, 72 glycan structures bound to Con A including several of the expected biantennary glycans (Supplementary data, Table S2). However, the universal threshold provided a comparative metric for identifying the strongest binding events on each platform allowing us an additional unbiased analysis of the datasets.

In general, the binding motifs observed for the lectins across the datasets included in the analysis were those expected based on previous work and the literature (Van Damme et al. 1998; Manimala et al. 2006). The disparities in array size and divergence of determinants on the different platforms make it difficult to quantify the similarities and differences in binding between the arrays. We present a discussion of the glycan binding observed in the five largest arrays, and corresponding data from Array 6, using our universal threshold method. Self-reported threshold data for these lectins is discussed when it sheds light on discrepancies.

Concanavalin A. We excluded the data from Array 2 for Con A based on our quality control (QC) criteria using Pearson correlation (Supplementary data, Table S1). Based on our

universal threshold method, agreement between the remaining four large datasets on the nature of the strongest binding determinant was high. All four arrays identified Man3 as one of the top five binders (Table IV; Supplementary data, Table S2). Other high mannose determinants (Man5–Man9) were also identified as strong binders on arrays that contained them. Man3 was the only terminal mannose determinant on Array 4. Beyond high mannose, a simple biantennary *N*-glycan was observed on Array 1, more complex biantennary glycans were seen on Array 5 and hybrid structures were strong hits on both Arrays 3 and 5. Self-reported structures from Array 1 included more biantennary glycan structures in agreement with the self-reported data from Arrays 3 and 5 (Supplementary data, Table S2).

More divergent data were observed for simpler structures with overall weaker binding, most often observed in the self-reported data. For example, the α-linked mannose monosaccharide was a self-reported binder with Con A on Array 3 but only at high densities of α-mannose (20 glycans/BSA). Mannose was also seen as a binder on Array 6. Binding to the mannose determinant increased with increasing display density, plateauing at tetrameric display of the ligand (Supplementary data, Figure S2). In contrast, no signal was observed on Array 1 for α-mannose with the same lot of Con A. This determinant was not present on Arrays 4 and 5. A second example of divergence in the self-reported data was seen with glucose. Several terminal α-glucose containing determinants were observed as ligands on Array 3, a neoglycoprotein array. Although similar determinants were printed on Array 1, no positive binding was observed. Array 4 did not contain α-glucosides but Con A did bind to a β-glucosyl determinant. Although this binding interaction was not observed in the other neoglycoprotein array (Array 3), which also contained β-glucosyl determinants, it has been seen in other studies and may be due to linker effects (Kilcoyne et al. 2012). Concordant with Array 3, binding to β-glucose was not observed in Array 6,

Table IV. Top five binders of Con A obtained with the universal threshold for Arrays 1–5 ranked by average (F/MAX × 100%)

GID	Determinants
(a) Array 1	
214	Manα1-6(Manα1-3)Manα-Sp9
212	Manα1-2Manα1-6(Manα1-3)Manα1-6(Manα1-2Manα1-2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAcβ-Sp12
51	Manα1-6(Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAcβ-Sp13
216	Manα1-6(Manα1-3)Manα1-6(Manα1-2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAcβ-Sp12
215	Manα1-2Manα1-2Manα1-6(Manα1-3)Manα-Sp9
(b) Array 3	
277	Manα1-6(Manα1-3)Manβ-BSA
270	Manα1-2Manα1-6(Manα1-3)Manα1-6(Manα1-2Manα1-2Manα1-3)Manβ1-4GlcNAc-β1-4GlcNAc-BSA
269	Manα1-2Manα1-6(Manα1-3)Manα1-6(Manα1-2Manα1-3)Manβ1-4GlcNAc-β1-4GlcNAc-BSA
268	Manα1-6(Manα1-3)Manα1-6(Manα1-2Manα1-2Manα1-3)Manβ1-4GlcNAc-β1-4GlcNAc-BSA
265	Manα1-6(Manα1-3)Manα1-6(Manα1-3)Manβ1-4GlcNAc-β1-4GlcNAc-BSA
(c) Array 4	
24	Manα1-3(Manα1-6)Man-BSA
3	Ovalbumin
7	Glcβ-4AP-BSA
11	Fucα1-2Galβ1-3GlcNAcβ1-3Galβ1-4Glc-BSA
(d) Array 5	
14	Manα1-3(Manα1-6)Manα1-6(GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc
6	Manα1-3(Manα1-6)Manα1-3(Manα1-3(Manα1-6)Man1-6)Manβ1-4GlcNAcβ1-4GlcNAc
3	Manα1-3(Manα1-6)Manβ1-4GlcNAcβ1-4GlcNAc
4	Manα1-6(Manα1-3)Manα1-6(Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc
15	GlcNAcβ1-2Manα1-3(Manα1-6)Manβ1-4GlcNAcβ1-4GlcNAc

The terminal mannose trisaccharide is highlighted in grey. *Note*: Array 5 does not have the trisaccharide alone. GID, glycan ID number.

which did not contain the α-glucose determinant. No glucosyl determinants were present on Array 5. Although in both of these cases Array 1 did not observe binding for the same batch of Con A, it is interesting to note that both determinants have been annotated as positive signals on previous versions of Array 1 using other preparations of Con A (Supplementary data).

Helix pomatia agglutinin. We excluded the data from Arrays 2 and 5 based on our QC criteria as before (Supplementary data, Table S1). All three of the remaining datasets identified GalNAcα1-3Gal as the strongest binding motif (Supplementary data, Table S3). In general, determinants bearing terminal α-GalNAc residues, including the monosaccharide (Tn antigen), were observed on all arrays that contained them. Arrays 1 and 3 also found α-GlcNAc residues as a binding determinant for HPA, arguing that the orientation of the 4-position of the terminal sugar is not crucial to glycan binding, in line with work by Sanchez et al. (2006). This determinant was not on the smaller Array 4. Some differences were seen between the platforms. Array 3, observed significant binding to Galβ1-3GalNAcα-structures (TF antigen) that were presented in the context of a peptide (i.e. attached to serine). When it was presented via a non-peptide linker on Array 3 or 1 (which also has the TF antigen), no significant binding was observed. This determinant was not present on Array 4. GalNAcβ- was another determinant observed only in the context of Array 3. Binding to the TF antigen and GalNAcβ- have been observed previously for HPA (Piller et al. 1990; Sanchez et al. 2006). Array 4 found Galβ- as a binding determinant, which was uncorroborated by the other datasets.

Maackia amurensis lectin I. We excluded MAL-I's data from Arrays 2 and 5 based on our QC criteria as before (Supplementary data, Table S1). MAL-I is widely regarded as a binder of terminal Neu5Acα2-3 residues linked to type-2 lactosamine sequences, but MAL-I can also bind 3-*O*-sulfated lactosamines (Bai et al. 2001; Porter et al. 2010; Geisler and Jarvis 2011). On all of the arrays, the sialylated determinant was observed in the cohort of strongest binders as determined by the universal threshold (Supplementary data, Table S4). Modifications of *N*-acetylneuraminic acid, including *N*-glycolyl, the amino variant KDN and the 9-*O*-acetyl modification, were all tolerated as observed by the arrays containing these determinants. In addition, MAL-I also bound Neu5Acα2-8Neu5Acα2-3Gal containing species. On Array 1, both the 2,3-sialylated and 3-*O*-sulfated lactosamine sequences were bound by MAL-I. On average, the top five binding sulfated determinants had signals ~2-fold greater than the signals of the top five binding sialylated determinants on Array 1. The 3-*O*-sulfated determinant identified in Array 1 was not present on Array 3 or 4. These arrays did contain (3S)Galβ1-4(Fucα1-3/4)GlcNAcβ-, a determinant presents on Array 1 which was not recognized on any of the array formats, indicating that the fucose substitution disrupts lectin binding. This highlights the obvious cautionary point that one can only observe binding to determinants that are present on an array. Binding to some LacNAc determinants was observed on Arrays 3 and 4, but not on Array 1. LacNAc was clearly a weak binder for MAL-I and observation of it on Array 3, where only 3 of 12 LacNAc

determinants were positive using the universal threshold and one for the self-reported, and Array 4, where binding was only observed in the self-reported set, is most likely due to either the multivalent presentation of this ligand by the neoglycoproteins or the fact that neither Array 3 nor 4 had the best ligand, lowering the highest level of binding observable, which in turn lowers the threshold.

Sambucus nigra agglutinin. No data were excluded from this analysis, see Supplementary data, Table S1. In all arrays, except for Array 4, structures containing terminal Neu5Acα2-6Galβ- were the top binders for SNA, consistent with the known literature for this lectin (Supplementary data, Table S5). All of the binding glycans from Array 1 contained this motif and KDN, 9-*O*-acetyl and *N*-glycolyl variants were also observed. The universal threshold examination of Array 4 found a Neu5Acα2-3Galβ1-4GlcNAc determinant as the top binder, although the α2-6 species were observed in their self-reported data. Although there are three α2-3 determinants on Array 4, only one structure showed binding, arguing that this may be an artifact. However, Array 2 also observed this determinant as a strong binder. Neither Array 1 nor 3 observed any α2-3 sialoside binding.

A variety of other determinants were observed for this lot of SNA by different arrays. Array 3 found terminal GalNAcα/β, Galβ-1-3GalNAc, Galα1-4Gal and Man6 determinants as binders using the universal threshold method. These glycans were listed as weak binders in the self-reported data. Array 4 also reported Galα1-3Galβ1-4GlcNAc as a binder for SNA. A hybrid structure with terminal galactose was observed on Array 5; Array 2 detected binding to a disulfated GalNAc structure. Binding to these determinants might be caused by minor isolectins of SNA contained in this lot of lectin. While SNA-I, which binds terminal Neu5Acα2-6Galβ-, is the major isolectin of SNA; SNA-II, which binds to terminal Gal and GalNAc (La Belle et al. 2007), may also be present and has been observed in SNA preparations (data not shown).

Wheat germ agglutinin. We excluded the data from Array 4 based on our QC criteria as before (Supplementary data, Table S1). WGA is known to strongly bind chitin-type glycans (GlcNAcβ1-4GlcNAc) and has weaker interactions with terminal sialosides (Monsigny et al. 1980; Wright 1992). In all datasets, glycans with terminal β-GlcNAc were observed among the cohort of top binders (Supplementary data, Table S6). Binding to GalNAcβ1-4 determinants was also observed on Arrays 1, 2 and 3; this determinant was not present on Array 5. The binding of WGA to sialylated determinants showed array dependent divergence. Array 1 found predominant binding to Neu5Acα2-6 sialosides. Array 5 also saw binding to these determinants; however, the α2-3 sialosides were not on this array. Array 2, which contained both α2-3 and α2-6 sialic acid determinants, demonstrated binding only to the Neu5Acα2-3 motif. In contrast, Array 3 observed positive binding to a small subset of glycans containing either motif but only with the self-reported threshold.

Effect of presentation on apparent binding

One reason for differences in observed binders is the difference in presentation modes across the arrays. To better quantify variations in binding due to presentation, we studied the agreement

between the largest neoglycoprotein array (Array 3) and the largest directly coupled glycan array (Array 1). The two arrays have 80 binding determinants in common (Figure 2), although each can have multiple presentations of that same determinant which may vary in linker structure and glycan density (Supplementary data, Table S7). The Gildersleeve laboratory (Array 3) received 42 compounds from the CFG (Array 1) and coupled these to BSA, for these compounds, the glycan determinant and a portion of the linker are identical. We quantified the overlap between the two arrays as follows: For each lectin, we determined the number of compounds on each array that met either the universal or self-reported threshold and were between the 80 common determinants. If one or more structures containing the common determinants were positive on both arrays, we considered it a positive match (positive). If they were negative on both arrays, we considered it a negative match (negative) and if the data were divergent (i.e. one or more positive on one array, all negative on the other) we considered it a mismatch (Table V). For example, when the universal threshold was applied for HPA, 7 of the 80 shared determinants were bound on both arrays, 71 shared determinants showed no binding to HPA on either array, and two determinants were only positive on Array 3.

In general, we found good concordance between the datasets using the universal threshold method (i.e. the vast majority of glycans were either positive on both arrays or negative on both arrays). Nevertheless, some mismatches were observed, mainly with shorter glycan or monosaccharide determinants. The majority of these were positive binders found on Array 3, but not on Array 1. Upon further analysis, several factors may contribute to the mismatches. First, Array 3 tended to produce more positive signals with monosaccharides. For example, GlcNAc-β monosaccharide was bound by WGA on Array 3 but not Array 1. Second, there were a number of mismatch cases where Array 1 showed some positive binding, but the signal was not strong enough to satisfy the criteria for the universal threshold. For example, 3′SLacNAc (Neu5Acα2-3Galβ1-4GlcNAcβ-) was bound by MAL-I on Array 3 but did not meet the threshold on Array 1 using the universal threshold method. However, it was observed as a binding glycan using the self-reported method. Differences in glycan density could account for the apparently

stronger binding to determinants on Array 3. For example, GD2 (Neu5Acα2-8Neu5Acα2-3(GalNAcβ1-4)Galβ1-4Glcβ-) was positive with HPA on Array 3 when displayed at a conjugation ratio of 10 per BSA; however, it was negative at a ratio of 4 per BSA. Therefore, the higher density variant was a mismatch, whereas the lower density variant was in agreement. Since the CFG provided GD2 for printing on Array 3, the difference in HPA binding is more consistent with a density dependent effect than a linker effect. It should be noted that the methodology used to assign glycans as positive or negative had a substantial impact on the results. In particular, there were more mismatches between arrays when using the self-reported methods, as different criteria were used by different groups to threshold positives and these methods yielded more positive binders overall than the more stringent metric applied in this work.

Glycan density was a factor for all the arrays, as exemplified by the interaction between the monosaccharide β-GlcNAc and the dimeric lectin WGA (Nagata and Burger 1974). This specific molecule was present on 4 of 5 arrays that passed QC for this lectin. However, binding to β-GlcNAc by this specific lot of WGA was seen on only 2 of these arrays. Binding of WGA to the simple monosaccharide was not observed on either Array 1 or 2, which present the determinant conjugated directly to the SCHOTT Nexterion H® surface. In contrast, β-GlcNAc densely presented on a neoglycoprotein array (Array 3, $n = 21$ GlcNAc residues/protein) was easily observable. Array 6, in which the monosaccharide GlcNAc was attached to a maleimide slide surface (PamChip®) as a monomer or as a series of localized dendrimeric structures showed differential binding based on the presentation mode. The monomeric GlcNAc, which most closely mimics Array 1 and 2 presentations, showed little binding. In contrast, dimeric presentation of the GlcNAc determinant shows a binding pattern that is identical to tetrameric and octameric variants (Figure 5). This suggests that presenting different densities of glycans may be important in examining glycan binding. Such presentations may enable multiple binding pockets to be engaged or may contribute to altered off-rates, as
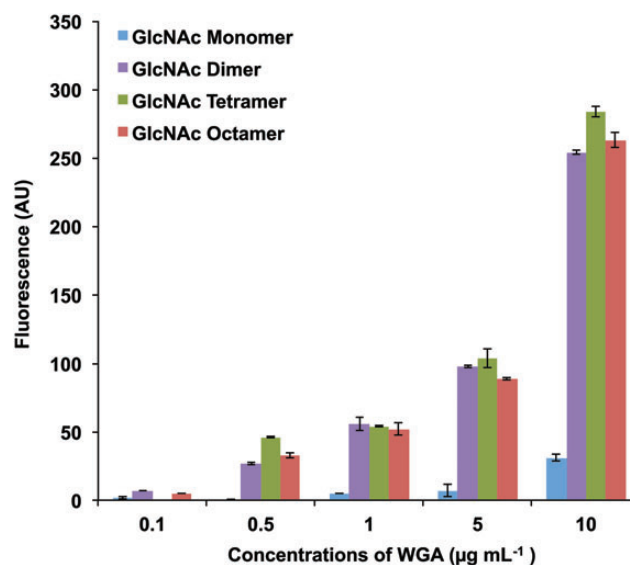
Table V. Number of positive (Pos.), negative (Neg.) or mismatched (Mis.) determinants seen between Arrays 1 and 3

Lectin	Universal threshold			Self-reported		
	Pos.	Neg.	Mis.[a]	Pos.	Neg.	Mis.
Con A	0	80	0	0	77	3
HPA	7	71	2	7	67	6
MAL-I	7	67	6	8	68	4
SNA	4	71	5	4	71	5
WGA	2	77	1	2	61	17

All numbers are out of 80 total shared determinants.
[a]In each case, the mismatch was positive on Array 3 using the universal threshold. The two HPA mismatches were self-reported as "strong binders" by Gildersleeve. Of the six MAL-I mismatches, four were listed as "medium" binders and two were listed as "non-binders" by Gildersleeve. All five SNA mismatches were listed as "weak binders" by Gildersleeve. The one WGA mismatch was self-reported as a "medium binder" by Gildersleeve.

Fig. 5. Binding profile of WGA on Array 6.

has been seen in mucin binding where surface constrained glycan presentation occurs (Dam and Brewer 2008; Parera Pera et al. 2010).

Conclusions

Glycan microarrays are a powerful tool to rapidly explore the binding specificities of GBPs. They provide a critical initial evaluation of binding specificity without the bias inherent in other technologies such as surface plasmon resonance (SPR), frontal affinity chromatography (FAC) or isothermal calorimetry (ITC), which require preliminary knowledge of GBP specificity. This technology is important for the discovery of physiological ligands of GBPs, allowing the rapid development of hypothesis about glycan-binding partners, which can be further validated using these other, more specialized techniques. In this study, we sought to examine whether differences in analytical platforms across laboratories had a significant effect on reported GBP profiles from glycan microarrays. Datasets from six glycan array research groups for five well-characterized lectins were obtained and analyzed. We anticipate that this rich collection of data will be valuable to other groups as they seek to create universal annotation methods and standards across platforms in the community. In our comparison, we draw several conclusions from this work. First, in general the strongest binding determinants were observed for these five lectins as long as the epitope was present. This finding validates the utility of these arrays in identifying the main epitopes recognized by GBPs, showcasing the power of this technology to rapidly identify binders for unknown GBPs. Second, the method by which positive signals are assigned on an array (i.e. thresholding) can have significant effects on interpretation of results for moderate and weaker binders. Case in point, our universal threshold method did not annotate potentially important weaker binding interactions on the arrays. For example, this metric overlooked the majority of biantennary glycan structures as binders for Con A. In contrast, self-reported thresholds were generally more sensitive but these were tied to the system analyzed (see Supplementary data). Annotation of the datasets using array-specific metrics, such as the z-score analysis-based methodology used by Array 1 (Cholleti et al. 2012) generally captured more binders, pushing the threshold to lower affinity ligands. Variability between microarray platforms was highest using the self-reported threshold presumably due to the detection of these weaker binding events, which may be more dependent on glycan presentation.

A third conclusion is that presentation of determinants on the array matters most for weaker binding interactions, which may still be physiologically relevant. The effect of presentation on glycan binding is a well-studied phenomenon (Brewer et al. 2002). GBPs, such as plant and mammalian lectins or antibodies, exhibit multivalent binding towards multivalent molecules (avidity), which can lead to observed binding affinities that are relatively high. In nature, avidity occurs both at the level of the GBP (i.e. multimeric lectins) and at the level of glycan presentation on the cell surface. Thus, it is not surprising that on the surface of a microarray how a glycan is presented to the GBP, the relative density, the local glycan packing and even the linker (which can affect packing) can alter what is observed (Stallforth et al. 2008; Godula and Bertozzi 2012; Kilcoyne et al. 2012). The effect of local ligand spacing is most clearly seen in this work in the comparison of the response of WGA to varying presentations of a monosaccharide, β-GlcNAc. No binding was observed for either monomeric β-GlcNAc (Array 6) or on the directly coupled arrays of Arrays 1 and 2, where spacing was dictated by the density of NHS-esters on the slide. In contrast, dendrimerized β-GlcNAc (Array 6, $n = 2$ or more) or β-GlcNAc presented on a densely conjugated neoglycoprotein showed clear binding to this glycan, arguing that for weaker determinants, presentation or local concentration plays a key role.

Our analysis reveals several important considerations in the use of glycan microarrays as a tool for determining GBP specificities. First, diversity is a critical factor in determining binding specificity. A clear example of this is seen in the identification of sulfation, rather than sialylation, as the strongest binding determinant for MAL-I. To get the most accurate preliminary identification of GBP specificity requires an array that covers a significant portion of the glycome. Expanding glycan microarrays to cover the mammalian glycome is an enormous undertaking. At present, it is estimated that there are a minimum of 3000 glycan determinants in the mammalian glycome alone (glycosaminoglycans excluded) (Cummings 2009). By comparison, the largest array to date (Array 1) has only 611 structures, some of which are replicates with different linkers. Overcoming the synthetic challenge of covering the glycome will require a concerted effort by the glyco-community.

A second consideration is that identifying strong binders using arrays does not guarantee that it is the best or the physiological ligand, and these two may not be synonymous. Conclusions regarding physiological relevance will always require independent biological validation. A third consideration is the presentation of ligands. This can have a profound effect on binding and diversification of glycan presentation in array platforms is another challenge for the community. Inter-ligand spacing in three dimensions is a critical factor in glycan recognition where varying the presentation of even a simple determinant can have profound consequences (Oyelaran et al. 2009; Godula and Bertozzi 2012; Grant et al. 2014). On the cell surface, there is a three-dimensional architecture to glycan presentation on proteins or lipids, which is then clustered in two dimensions by its confinement on the lipid bilayer. How this maps onto recognition of glycans by human immune lectins, bacterial adhesins, viral lectins and other important GBPs is still not well understood. This is complicated by the fact that even weak interactions, such as those between glycolipids on opposing cell membranes, can have profound biological effects due to avidity (Hakomori 2010). Teasing apart physiological interactions is thus a complicated endeavor in which microarrays can provide a crucial starting point. Arrays that use lipid architectures (Fukui et al. 2002), vary presentation densities (Houseman and Mrksich 2002; Ngundi et al. 2006; Chevolot et al. 2007; Liang et al. 2007; Mercey et al. 2008; Song et al. 2008; Oyelaran et al. 2009; Tian et al. 2012) and alter inter-ligand spacing (Zhang, Campbell, et al. 2010; Zhang, Li, et al. 2010; Godula and Bertozzi 2012; Narla and Sun 2012) are beginning to untangle these higher order questions about glycan binding. More work is needed to create specific two and three-dimensional presentations that mimic cell surfaces

including fluidity to gain greater insights into glycan-binding mechanisms. The utility of specific glycan microarrays will be dependent on the nature of the GBP to be examined and the question to be asked. In some cases, multiple microarray platforms may be required to obtain the best understanding of glycan binding in a system and much microarray development remains to get a comprehensive view of GBP specificity.

Supplementary data

Supplementary data for this article are available online at http://glycob.oxfordjournals.org/.

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Conflict of interest

None declared.

Abbreviations

BSA, bovine serum albumin; Con A, Concanavalin A; FAC, frontal affinity chromatography; GBPs, glycan-binding proteins; HPA, *Helix pomatia* agglutinin; ITC, isothermal calorimetry; MAL-I, *Maackia amurensis* lectin I; QC, quality control; SNA, *Sambucus nigra* agglutinin; SPR, surface plasmon resonance; WGA, wheat germ agglutinin.

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