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Application of Lectin Microarray to Crude Samples: Differential Glycan Profiling of Lec Mutants

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We recently developed a novel system for lectin microarray based on the evanescent-field fluorescence-detection principle, by which even weak lectin-oligosaccharide interactions are detectable without a washing procedure. For its practical application, cell glycan analysis was performed for Chinese hamster ovary (CHO) cells and their glycan profile was compared with those of their glycosylation-defective Lec mutants. Each of the cell surface extracts gave a significantly different profile from that of the parental CHO cells in a manner reflecting denoted biosynthetic features. Hence, the developed lectin microarray system is considered to be fully applicable for differential glycan profiling of crude samples.

Key words: evanescent, flow cytometry, glycan profiling, lectin, Lec mutant, microarray.

Glycans are extensively concerned with fundamental phenomena in living organisms. In fact, more than half of eukaryotic proteins are subjected to glycosylation (*1*). As a result, most of the tumor antigens and cell differentiation markers so far identified are glycoproteins and glycolipids. In this context, cell surface glycans are regarded as a "face" representing each cell type and state. Hence, acquisition of structural information on glycans is critical for elucidation of the relationships between glycan structures and cell-specific functions. For this purpose, however, previously developed approaches requiring multiple steps are too time-consuming, and thus laborious; *i.e.*, typically consisting of the isolation of glycoproteins, cleavage of glycans from the core protein, labeling of the liberated glycans, and detailed analysis by means of the combination of tandem-mass spectrometry and glycosidase digestion.

We recently developed a novel procedure for glycan profiling involving lectin microarray (*2*). The method is based on the evanescent-field fluorescence-detection principle, which requires no washing procedure. Therefore, it has a critical advantage over other methods described previously (*3–5*) in that it enables the detection of relatively weak lectin-oligosaccharide interactions even in mixed samples. As one of the first trials at application of the newly developed method to such "crude samples," differential cell surface glycan analysis was carried out, and its performance was compared with that of conventional flow cytometry. As target cells, Chinese hamster ovary (CHO) cells and their glycosylation-defective Lec mutants (*6–11*), designated as Lec1 (with a defect in GlcNAc-T1), Lec2 (CMP-sialic acid transporter) and Lec8 (UDP-Gal transporter), were chosen, of which the biosynthetic features have been well characterized.

For experiments, the Lec mutant and parental CHO cells were grown in 15-cm dishes, and then harvested by centrifugation after trypsin treatment. The cells (5×10^6) were extensively (more than three times) washed with PBS to remove ingredient containing glycoprotein, and then suspended in 50 μl of PBS. Then, cell surface proteins were labeled with 1 μl of Cy3-succimidyl ester (100 mg/ml DMF) at 4°C for 60 min. After the labeling reaction, the cells were washed three times with PBS, and then suspended in 200 μl of PBS containing 1% Triton X-100 for their ultrasonic disruption. The thus solubilized, Cy3-labled membranous fractions were recovered by centrifugation (15,000 rpm, 20 min), and an aliquot (20 μl) of each supernatant was diluted with 180 μl of probing buffer described previously (*2*), and then subjected to lectin microarray.

Figure 1 shows the results of lectin microarray analysis of CHO cells (wild type) and Lec mutants (note that the signal at each lectin spot corresponds to that derived from 5×10^5 cells). Apparently, the profiles differed from one another. For comparison with the parental CHO cells more quantitatively, the relative ratio of the signal for each Lec mutant is summarized to Table 1. Lec1 showed decreased signals for extensive lectins; *e.g.*, MAL (α2-3Sia-binder), ECA and RCA120 (LacNAc-binders), PHA(L) and PHA(E) (branched complex-type *N*-glycan-binders), and WGA, DSA and LEL (chitin-binders), while significant increases in lectin signal were detected for GNA and HHL (Man-binders). We evaluated the extents of similarity among the glycan profiles by means of correlation coefficients, which are often used for spectral matching in MS^n analysis (*12–14*). For instance, the similarity is considered to be "high" enough if the correlation coefficient is higher than 0.9, whereas it is considerably "low" if the coefficient is lower than 0.5.

As a result, the total resemblance between CHO and Lec1 cells is relatively low in terms of the correlation coefficient (0.57; note that in this study the net intensities of

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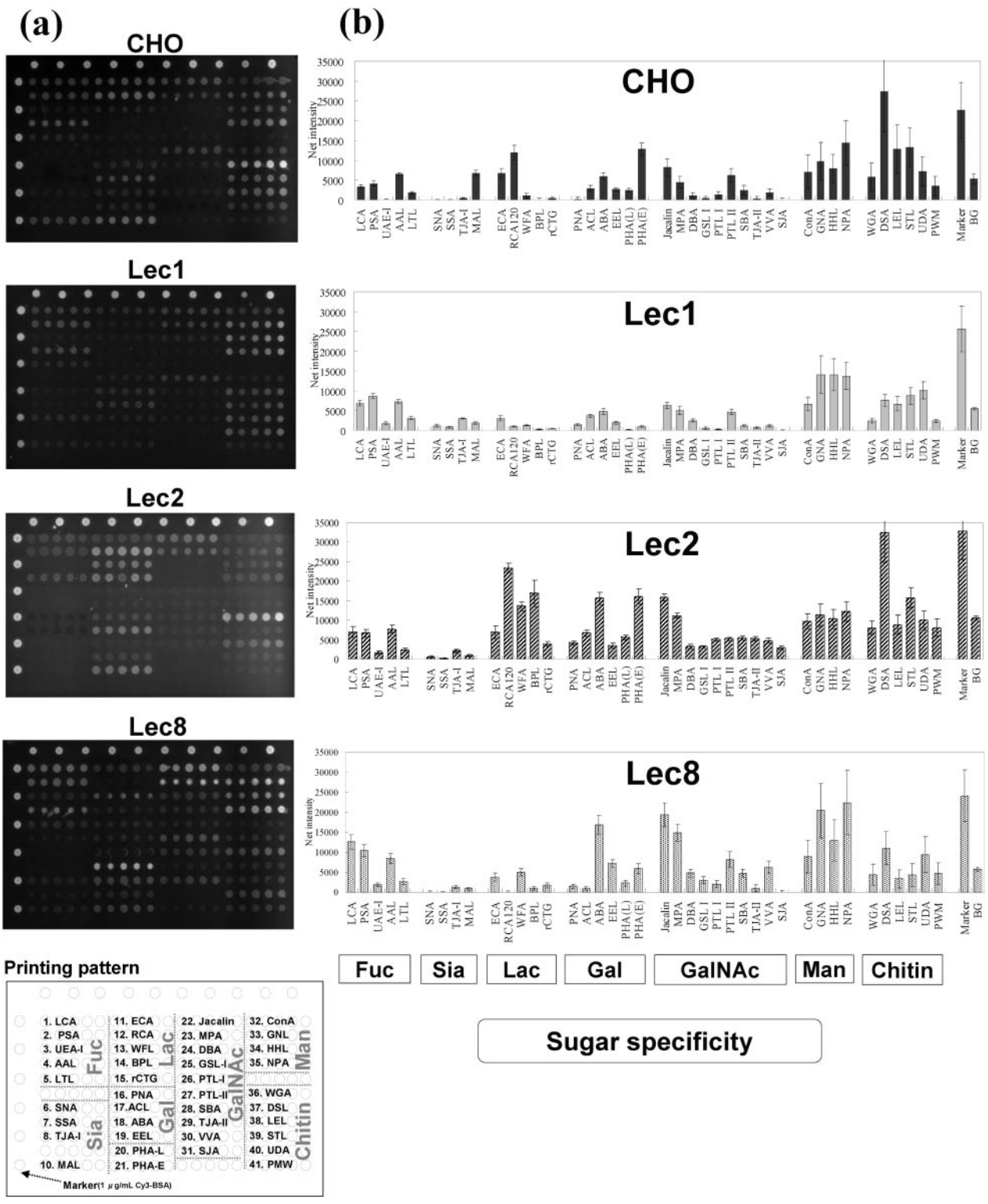

Fig. 1. **Glycan profiles of the Lec mutant and wild type CHO cells investigated by lectin microarray.** (a) Real images of scanned pictures obtained on lectin microarray. (b) Vertical bar graph representation of signals for the arrayed 40 lectins. For preparation of the probing solution, 5×10^6 cells were suspended in 50 μl of PBS, to which 1 μl of Cy3-succimidyl ester (100 mg/ml in DMF) was added, and the labeling reaction was allowed to proceed for 60 min at 4°C in the dark. The cells were extensively washed with PBS and then suspended in 200 μl of PBS containing 1% Triton X-100 for ultrasonic extraction. After centrifugation at 15,000 rpm for 20 min, a 20 μl aliquot of each supernatant was applied to a lectin microarray after dilution with 180 μl of probing buffer as described previously (*2*). Net intensity is the value excluding the absolute signal intensity minus the background (BG) signal for each spot. Error bars represent the SD for five replicate spots. As a positional marker (Marker), 1 μg/ml of Cy3-labeled BSA was used. For lectin abbreviations, see Ref. *2*. The details of the oligosaccharide specificities of these lectins will be published elsewhere.

Table 1. **Relative signal changes in each Lec mutant for the critical lectins (data from Fig. 1b).**

Sugar specificity	Lectin	CHO net intensity	Relative ratio of net intensity to CHO		
			Lec1	Lec2	Lec8
Fuc	LCA	3,300	2.06 (2.22)	2.12 (1.55)	3.82 (3.05)
	PSA	4,200	2.07 (2.23)	1.60 (1.17)	2.48 (1.98)
	AAL	6,500	1.12 (1.21)	1.18 (0.87)	1.29 (1.03)
	LTL	1,800	1.72 (1.85)	1.33 (0.98)	1.50 (1.20)
Sia	MAL	6,800	0.28 (0.30)	0.15 (0.11)	0.13 (0.11)
Lac	ECA	6,800	0.46 (0.49)	1.01 (0.74)	0.56 (0.45)
	RCA120	11,900	0.08 (0.09)	1.97 (1.44)	0.00 (0.00)
	WFA	1,100	1.18 (1.27)	12.36 (9.05)	4.55 (3.63)
Gal	ACL	2,900	1.28 (1.37)	2.31 (1.69)	0.34 (0.28)
	ABA	6,000	0.80 (0.86)	2.60 (1.90)	2.82 (2.25)
	EEL	2,700	0.74 (0.80)	1.30 (0.95)	2.67 (2.13)
	PHA(L)	2,500	0.08 (0.09)	2.24 (1.64)	0.92 (0.73)
	PHA(E)	12,800	0.08 (0.08)	1.26 (0.92)	0.46 (0.37)
GalNAc	Jacalin	8,200	0.77 (0.83)	1.94 (1.42)	2.37 (1.89)
	MPA	4,500	1.13 (1.22)	2.49 (1.82)	3.31 (2.64)
	DBA	1,100	2.27 (2.44)	2.91 (2.13)	4.36 (3.48)
	PTL I	1,300	0.23 (0.25)	3.92 (2.87)	1.62 (1.29)
	PTL II	6,300	0.75 (0.80)	0.83 (0.60)	1.30 (1.04)
	SBA	2,400	0.50 (0.54)	2.25 (1.65)	1.96 (1.56)
	VVA	1,900	0.58 (0.62)	2.53 (1.85)	3.32 (2.65)
Man	ConA	7,100	0.93 (1.00)	1.37 (1.00)	1.25 (1.00)
	GNA	9,800	1.44 (1.55)	1.15 (0.84)	2.08 (1.66)
	HHL	8,000	1.76 (1.90)	1.29 (0.94)	1.63 (1.30)
	NPA	14,400	0.96 (1.03)	0.85 (0.62)	1.56 (1.24)
Chitin	WGA	5,800	0.41 (0.45)	1.40 (1.02)	0.76 (0.61)
	DSA	27,300	0.28 (0.30)	1.19 (0.87)	0.40 (0.32)
	LEL	12,900	0.52 (0.56)	0.68 (0.50)	0.26 (0.21)
	STL	13,300	0.66 (0.71)	1.18 (0.86)	0.32 (0.26)
	UDA	7,200	1.40 (1.51)	1.39 (1.02)	1.31 (1.04)
	PWM	3,500	0.69 (0.74)	2.31 (1.69)	1.34 (1.07)

The numerical values in parentheses are the relative ratios to ConA (ConA = 1.00).

40 lectins were used to calculate correlation coefficients by means of Microsoft Excel (Microsoft Japan, Tokyo); $S_{xy}/(S_x * S_y)$. $S_{xy} = 1/40\{(x_1 - x)(y_1 - y) + (x_2 - x)(y_2 - y) + \cdots + (x_{40} - x)(y_{40} - y)\}$. $S_x^2 = 1/40\{(x_1 - x)^2 + (x_2 - x)^2 + \cdots + (x_{40} - x)^2\}$. $S_y^2 = 1/40\{(y_1 - y)^2 + (y_2 - y)^2 + \cdots + (y_{40} - y)^2\}$. x and y are the average net intensities of the CHO and Lec mutant cells, respectively). From the macroscopic viewpoint, the difference between Lec1 and CHO cells is more evident in *N*-glycan–related lectins, *e.g.*, PHA(L), PHA(E), GNA and HHL, whereas the difference in *O*-glycan–related lectins, *e.g.*, Jacalin, MPA, DBA, PTL-II, SBA and VVA, is relatively small.

In contrast, the Lec2 profile is relatively similar to that of the parental CHO cells (correlation coefficient, 0.81) except for the denoted reduction in MAL signal (α2-3Sia-binder). This observation is quite reasonable considering the fact that CHO cells dominantly express α2-3Sia rather than α2-6Sia[15], while Lec2 can only translocate CMP-sialic acid into Golgi compartment at 2% of the rate in the case of the wild-type (*8*, *9*). Due to the substantial absence of sialic acid, Lec2 cells showed a significant increase in the signals for LacNAc-binders (RCA120, WFA and BPL). In the absence of sialic acid, general enhancement of the signals for *O*-glycan-binders was also observed.

Lec8 showed the biggest difference among the Lec mutants examined in this work from the wild type (correlation coefficient, 0.46), whereas the difference in Lec1 (0.57) mentioned above was restricted to *N*-glycan–related lectins. Lec8 showed significant decreases in the signals for MAL, ECA, RCA120, ACL and PHA(E), which recognize non-reducing terminal sialic acid or galactose. In addition, the signals for poly(LacNAc)-binders, DSA and LEL, were much reduced. On the other hand, the signals for WFA, ABA, EEL, NPA and SBA were significantly increased. The reason for their enhanced affinity is not clear at the moment. Notably, Lec8 showed general increases in the signals for GalNAc-binding lectins. This is explained in part by the absence of galactose in the core 1 structure of *O*-glycans. As regards ABA, it was characterized as a Galβ1-3GalNAc (T antigen)–binding lectin. However, this lectin was recently shown to have another binding site, *i.e.*, for GlcNAc (*16*). Therefore, this may explain the enhanced signal in the Lec8 mutant.

For evaluation of the above results obtained by lectin microarray, we took two approaches: one was glycosidase digestion and the other was flow cytometric analysis. For the former, each cell preparation was treated with neuraminidase from *Arthrobacter ureafaciens* (Marukin Bio, Inc., Kyoto), 0.05 unit/ml, at 37°C for 60 min. The resultant cells were washed extensively, and then subjected to Cy3 labeling as described above. As a result, a substantial change in the glycan profile was observed for CHO cells

(correlation coefficient, 0.79). In contrast, a significant difference was not observed for any of the three Lec mutants: the correlation coefficients between before and after neuraminidase digestion were 0.97 for Lec1, 0.97 for Lec2 and 0.96 for Lec8. This result coincides with the above observation that Lec mutants are entirely devoid of sialic acid, and thus no drastic change in their glycan profiles was observed even after neuraminidase treatment.

For flow cytometry, we employed 11 lectins as well as anti-Tn (αGalNAc) antibody. Representative results for the Lec1 mutant and wild type CHO cells are shown in Fig. 2 (data for Lec2 and Lec8 not shown). Apparently, both lectin microarray (Fig. 2a) and flow cytometric analyses (Fig. 2b) gave essentially the same results, although the former method can only target cell surface glycoproteins, while the latter probes both glycoproteins and glycopilids. In brief, substantially no binding was observed in Lec1 for PHA(L) and PHA(E) as mentioned above, whereas CHO cells exhibited detectable signals for these lectins. A sialic acid–binding lectin, MAL, showed a decreased signal in all Lec mutants, whereas the wild type showed a significant signal (note that for flow cytometry, FITC-MAM was performed). The signal for the LacNAc-binder RCA120 was considerably decreased in Lec1 as well as Lec8 compared with in CHO cells, whereas the signal for this lectin increased significantly in response to the absence of, *i.e.*, on unmasking of, sialic acid in Lec2. The signal for a poly(LacNAc)-binder, DSA, also decreased substantially in agreement with the data obtained on lectin microarray.

On the other hand, neither signals for SBA, anti-Tn antibody nor ConA showed a significant change between CHO cells and the Lec1 mutant. However, the observation that ConA-binding is not altered in the Lec1 mutant indicates an apparent discrepancy, because the absence of GlcNAc-T1 results in the disappearance of complex-type *N*-glycans, and thus should lead to increased expression of high-mannose type *N*-glycans. However, this lectin, while showing the highest affinity to a set of high-mannose type *N*-glycans, also exhibits significant affinity to biantennary complex-type *N*-glycans, in particular to agalactosylated biantennary *N*-glycan (*17*, *18*). Therefore, it is possible that such a compensating effect explains this observation. Apparently, further structural study is necessary on glycans expressed on CHO and Lec mutant cells as well as detailed investigation of lectin specificity. For the latter, we have already carried out comprehensive analysis of lectin-oligosaccharide interactions by means of frontal affinity chromatography (*19*). Although detailed data have not been presented, fully reasonable results have been obtained for both the Lec2 and Lec8 mutants. The results of neuraminidase treatment also agreed with those of flow cytometric analysis. For more precise comparison of glycan profiles between distinct samples, some considerations are necessary, which include not only the quantities of particular glycans but also their affinities to lectins. As described in the previous paper, the developed microarray can also be used for antibodies, *i.e.*, in terms of hybrids (lectin and antibody) (*2*). Thus, by focusing on particular proteins, for which antibodies are available, more quantitative differential analysis will be possible. More detailed structural analysis, such as with glycosidase treatment and mass spectrometry analysis, is necessary to fully support the interpretation made of the present

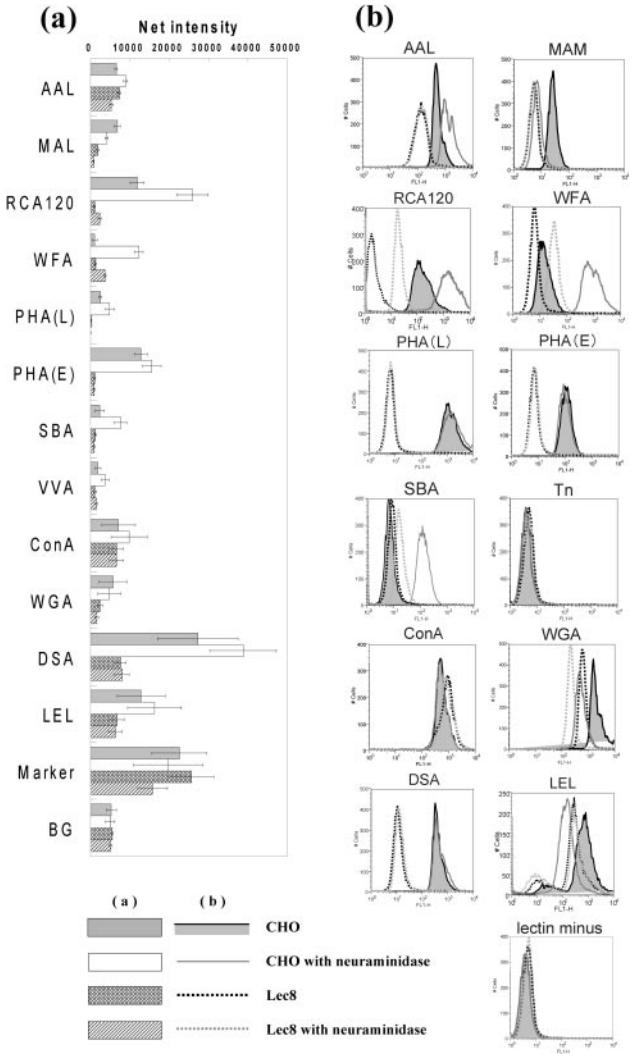

Fig. 2. **Comparison of the results obtained on lectin microarray with flow cytometry for Lec1 and wild type CHO cells.** (a) A horizontal bar graph representation of the fluorescent signals (Net intensity) observed on lectin microarray. Data are originally from Fig. 1. (b) Histogram representation of flow cytometric analysis with 11 lectin and anti-Tn antigen antibody probes. The same number of cells (5×10^6 cells) was incubated with fluorescein isothiocyanate (FITC)–conjugated lectin (1/100 dilution of each commercial product, see below) for 30–60 min at 4°C in the dark, washed with 1% BSA in PBS including 0.1% NaN_3, and then fixed with 0.5% paraformaldehyde in PBS. For single cell preparation, each cell suspension was passed through a mesh of 40 μm diameter. The fluorescence intensity of 10,000 cells was measured for each preparation using a FACS Caliber™ (BD Biosciences) (*20*). The commercial sources of the FITC-conjugated lectins were as follows: ConA, WGA, DSA, SBA, PHA(E), PHA(L), RCA120 and MAM, (J-OIL MILLS, Inc., Tokyo), WFA and LEL (Vector Laboratories, Burlingame, CA 94010) and AAL (Seikagaku Co., Tokyo).

results. In this context, MS analysis of the oligosaccharides released from glycoproteins of each mutant as well as parental cells is now in progress (to be published elsewhere).

Clearly, the developed lectin microarray has a great advantage over conventional flow cytometry in its throughput, because a number of lectin-binding experiments can

be carried out at one time in a rapid (4 h after cell preparation) and sensitive (0.1 μg of purified protein/well or 10^5 cells/well required) manner. Thus, we conclude that the developed system is versatile and applicable even to crude samples containing glycoproteins in a similar manner to conventional flow cytometry with much improved performance. The best use of this method would be to see the effect of either a defect or introduction of glycogenes in certain cells or model organisms. Inversely, some specific glycogenes would be identified through systematic comparison of glycan profiles between two target cells or animals (*e.g.*, normal and abnormal) targeting both *N*-glycans and *O*-glycans in the context of "differential glycomics."

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