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Concept, Strategy and Realization of Lectin-based Glycan Profiling

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Lectins are a diverse group of carbohydrate-binding proteins. Each lectin has its own specificity profile. It is believed that lectins exist in all living organisms that produce glycans. From a practical viewpoint, lectins have been used extensively in biochemical fields including proteomics due to their usefulness as detection and enrichment tools for specific glycans. Nevertheless, they have often been underestimated as probes, especially compared with antibodies, because of their low affinity and broad specificity. However, together with the concept of glycomics, such properties of lectins are now considered to be suitable for the task of 'profiling' in order to cover a wider range of ligands. Recently there has been rapid movement in the field of proteomics aimed at the investigation of glycan-related biomarkers. This is partly because of limitations of the present approach of simply following changes in protein-level expression, without paying sufficient attention to the fact and effects of glycosylation. The trend is reflected in the frequent use of lectins in the contexts of glycoprotein enrichment and glycan profiling. However, there are many aspects to be considered in using lectins, which differ considerably from antibodies. In this article, the author, as a developer of two unique methodologies, frontal affinity chromatography (FAC) and the lectin microarray, describes critical points concerning the use of lectins, together with the concept, strategy and means to achieve advances in these emerging glycan profiling technologies.

Key words: biomarker, chromatography, frontal affinity, glycan profiling, glycomics, lectin microarray.

Abbreviations: CE, capillary electrophoresis; FAC, frontal affinity chromatography; ITC, isothermal calorimetry; LC, liquid chromatography; LOD, limit of detection; MS, mass spectrometry; RI, radioisotope.

CURRENT STATUS OF UNDERSTANDING ABOUT GLYCANS

'Glycomics' is an emerging field in the post-genome/proteome era, which is directed toward not simply structural identification, but also to the elucidation of glycan functions in particular biological phenomena, including cell–cell interaction events, such as differentiation, development, morphogenesis, embryogenesis, immunity, infection and tumourigenesis including metastasis (*1–5*). This emergence is a quite reasonable consequence considering the fact that all living organisms comprise cells, which are, with no known exceptions, covered abundantly with diverse forms of glycoconjugates (*6*). It is an unavoidable fact in proteomics that >50% of proteins expressed in eukaryotes are glycosylated (*7*), and thus, it is logical to include the study of glycosylation in individual fields of biology.

In the light of recent research, glycans should no longer be considered less important than nucleic acids and proteins, which have been favoured traditionally. In many instances the ultimate biological recognition processes have been shown to be mediated by glycans.

Similarly, glycomics should be considered to be just as important as genomics and proteomics.

BASIC APPROACHES TO STRUCTURAL GLYCOMICS

The most fundamental issue of glycomics is resolving what structural approaches to adopt for these highly heterogeneous and complex molecules having multiple branches and linkage isomers. Moreover, glycans are usually attached to either proteins or lipids in the form of glycoconjugates. These are the basic reasons why there is still no established procedure for automated glycan synthesis and glycan sequencing. Currently, glycans are probably best characterized in a 'matching manner' with respect to authentic standards. This type of structural analysis is referred to as 'glycan profiling'. In general, glycan profiling requires speed, sensitivity and high-throughput, rather than time-consuming laborious structure determination. Although there is a conventional method for *de novo* determination of complete covalent structures, e.g. permethylation analysis, much faster and highly sensitive alternatives for purposes of glycan profiling are eagerly awaited (*8–12*).

There are four key technologies available for glycan profiling (Fig. 1). The first method introduced in this

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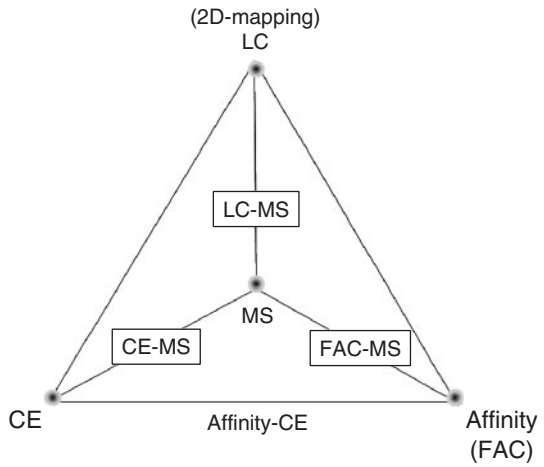

Fig. 1. **The four major separation technologies for structural glycomics: MS (mass spectrometry), LC (liquid chromatography), CE (capillary electrophoresis) and affinity techniques (FAC, frontal affinity chromatography).** Appropriate combinations of these techniques have also been developed, such as LC–MS, FAC-MS, affinity-CE and CE–MS.

field is multi-dimensional liquid chromatography (LC), which is usually coupled with prior labelling with a fluorescence reagent, typically 2-aminopyridine (*13*). The method was first established for neutral N-linked glycans as 2-D mapping (*14*) and later for those including acidic glycans as 3-D mapping (*15*). The separation principle is based on differences in the chemical structures of glycans; *i.e*. in normal-phase (hydrophilic interaction) chromatography the separation substantially depends on the size of each glycan (roughly, the number of hydroxyl groups), while in reversed-phase chromatography the separation is based on the hydrophobicity of each glycan, though the actual mechanism is much more complex. For acidic glycans, anion-exchange chromatography is used as the third mode of separation. The method is basically applicable to all kinds of glycans once they are pyridylaminated, and has proved to be able to discriminate >500 glycans. It should also be mentioned that the method is indispensable for preparative purposes, and that coupling with conventional glycosidase treatments is highly effective. As a significant defect, the method requires a relatively long time for each chromatographic analysis (>30 min), and thus has in general low throughput.

The second approach is mass spectrometry (MS). This powerful technique is an aspect of advanced biotechnology. Its applicability to complex glycans was first achieved by the development of a tandem MS technique, generally called MS^n (*10–12*, *16–19*). Since individual glycans tend to show a characteristic degradation pattern, they are effectively differentiated usually in the process of up to MS^3 or MS^4. Essentially, each MS experiment provides only a set of *m/z* values, which explain theoretically possible composition in terms of elementary saccharides, *e.g*. hexose (Hex, $m/z = 162$), *N*-acetylhexosamine (HexNAc, $m/z = 203$), deoxyhexose (dHex, $m/z = 142$) and *N*-acetylneuraminic acid (NeuAc, $m/z = 291$). Thus MS analysis only affords possible combinations of Hex/HexNAc/dHex/NeuAc, and therefore cannot alone reveal component saccharide identities. Nevertheless, the method is highly useful for both confirmation and estimation of glycan structures including glycosaminoglycans (*20–22*). The method is, first, superior in its accuracy (resolution). However, its practice needs, at least at the present level, pretreatments (*i.e*. liberation of glycans from protein and lipid and subsequent modification with an appropriate labelling reagent, such as 2-aminopyridine). Direct application to clinical samples will require great improvements. It should also be noted that MS is utilized only for detection, not for preparative purposes.

The third approach, capillary electrophoresis (CE) is based on a different separation principle from the ones described previously (*23–25*). Compared with multi-dimensional LC, CE has advantages in speed and sensitivity, while the method is used, together with MS, predominantly for analytical purposes. Considering the extremely high number of theoretical plates (*e.g*. >100,000) and speed of CE, it seems likely that multi-dimensional LC could well be substituted by multi-dimensional CE. From a practical viewpoint, however, coupling of multiple separation modes is of practical difficulty, because the method usually deals with an extremely small volume of analyte solution (*i.e*. nl level) though recent advances in CE technology enables combination of a CE device with an MS detector system (*i.e*. CE–MS; *26*).

The final approach is to use lectins for structural analysis of glycans (*27*). This approach is rather exceptional considering that the other approaches are based on established separation sciences, and have already been utilized in various fields of life science. In this context, these approaches are simple applications to structural glycomics. On the other hand, this final approach, described in most detail in this mini-review, utilizes special tools, *i.e*. lectins. Lectins have long been used for detection and purification of glycans in various research areas (*28*). Attempts to separate various types of glycans have been elaborated in several groups (*29–31*). However, practical use of lectins had never been achieved before a highly quantitative, sensitive and rapid analytical method emerged in the form of reinforced frontal affinity chromatography (FAC; *32–37*), of which the principle and theory were originally derived by Kasai (*38*). FAC provides values for the strength of binding between analyte (glycan) and ligand (lectin) [the latter being immobilized on a matrix gel (*e.g*. agarose)] in terms of a dissociation constant (K_d) or an affinity constant (K_a), where $K_d = 1/K_a$. Since the recently developed system for automated FAC (*39–42*) enables 100 analyses per day, systematic data on lectin sugar-binding specificity may be accumulated rapidly (*36*, *37*, *39*, *43–52*) and lectin specificity can now be discussed in terms of K_d in a more systematic manner than ever.

LECTIN MAKES A TREND IN STRUCTURAL GLYCOMICS

Consistent with such a trend to utilize a variety of lectins, an increase in the number of papers dealing

with lectins is a feature in the field of proteomics in the last few years (Fig. 2). As of January 27, 2008, PubMed (http://www.ncbi.nlm.nih.gov/sites/entrez?db=pubmed) hits with the query 'glycomics OR glycome' total 214, among which those with 'lectin' terminology account for as many as 55 (26%). As can be seen from Fig. 1, the increase in 'Lectin/Glycomics' papers is obvious in the last 4 years. This trend runs parallel to the development of an automated FAC machine (designated FAC-1; *27*), which appeared immediately after the first statements using the term 'glycome' around the year 2000 (*53–56*). It is noteworthy that the concept 'proteome', which was first presented by Wasinger (*57*), was accompanied with the supporting technologies to put forward the practical approach, *i.e*. separation of proteins by 2D-polyacraylamide gel electrophoresis followed by structural identification by MS. The same situation should be applied to glycomics, which is performed with the four basic technologies described previously—LC, CE, MS and lectin-affinity. However, as lectin-affinity is an emerging technology, rapid advances can be expected in its sensitivity, resolution, reproducibility and applications (*e.g*. combinations with other methodologies).

So far, a number of lectins derived from diverse origins have been identified, which show a variety of carbohydrate-specificities in their biological contexts. Lectin is a technical term for 'select' in Latin, and in its generally accepted definition—'a protein which shows affinity to a group of carbohydrates'. Any such protein, which had previously been categorized as some other functional protein (such as enzyme, receptor and cytokine) is entitled to become a new lectin, if its sugar-binding ability can be demonstrated.

METHODS FOR INTERACTION ANALYSIS OF LECTINS AND GLYCANS: A BASIS OF LECTIN STUDY

Since the use of lectins assumes their specific recognition of some glycans, separation technologies based on lectin affinity should be properly evaluated, considering both their merits and demerits, *e.g*. sensitivity, resolution, reproducibility, speed, throughput, feasibility, etc. Major analytical methods so far reported are listed in Table 1. First, the method requires accuracy in the determination of affinity strength between lectins and glycans with

Table 1. **Quantitative methods to determine K_d for lectin-carbohydrate interactions.**

Methods	Examples of analyses	Ref
Equilibrium dialysis	ConA and PA-oligosaccharides	(*58*)
FAC-RI[a]	ConA and ovalbumin *N*-glycans	(*59*)
FAC-FD[b]	Galectins and PA-oligosaccharides	(*36*)
	mJRLs and PA-oligosaccharides	(*48*)
FAC-MS[c]	Chorela toxin and ganglioside series derivatives	(*60*)
	Galectin-3 and lacto-*N*-biose libraries	(*61*)
ITC[d]	Galectins and synthetic glycans	(*62*)
	Artocarpin and deoxytrimannoside derivatives	(*63*)
SPR[e]	C-type macrophage lectin and glycopeptides	(*64*)
	Plant lectins and asialofetuin glycopeptide	(*65*)
	Galectin-4 and glycosphingolipids	(*66*)
FP[f]	Galectins and their inhibitors	(*67*)

[a]Frontal affinity chromatography-radio isotope detection method. [b]Frontal affinity chromatography-fluorescence detection method. [c]Frontal affinity chromatography-mass spectrometry detection method. [d]Isothermal calorimetry. [e]Surface plasmon resonance. [f]Fluorescence polarization.

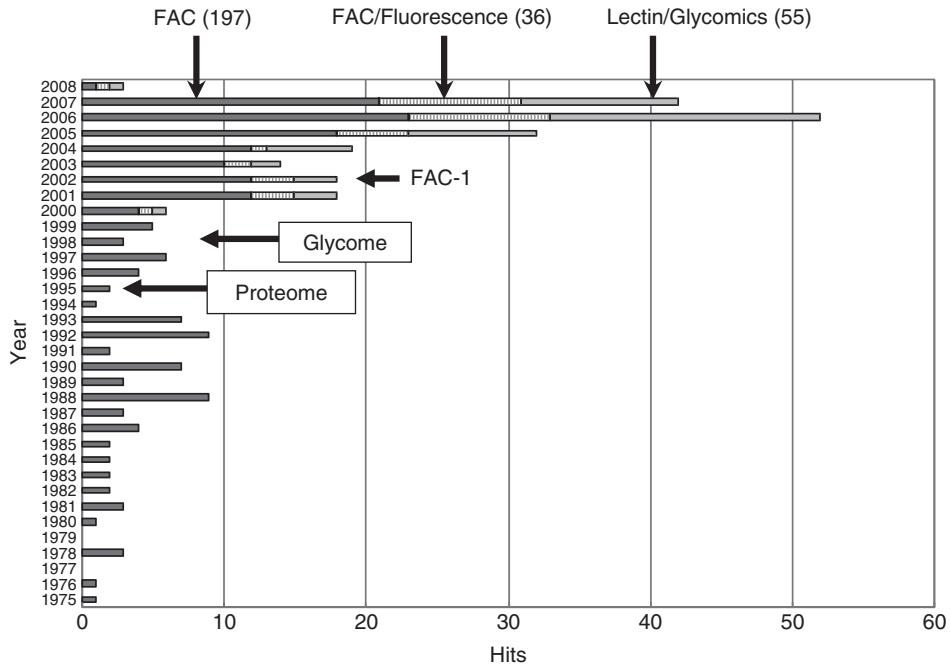

Fig. 2. **Results of a PubMed search for papers dealing with lectin, glycomics and frontal affinity chromatography (FAC).** Note that after the glycome concept was presented in 1998 the number of lectin/glycomics papers published shows a rapid increase in association with the increase in FAC/fluorescence papers.

satisfactory throughput and speed. For the latter two requirements, equilibrium dialysis and isothermal calorimetry (ITC) may not be adequate, though these methods may be used to determine reliable affinity constants (K_a) or dissociation constants (K_d). Among the remaining methods in Table 1, FAC has a range of methods for detection; *i.e*. radioisotope (RI; *59*), MS (*60*) and fluorescence (FD). For FAC-RI, however, *N*-glycans must be radio-labelled, *e.g*. with NaB[^{3}H]$_4$. Similarly, for FAC-MS, modification of glycans with an appropriate alkyl reagent is necessary to increase ionization efficiency in MS. On the other hand, FAC-FD with pyridylaminated (PA)-glycans enables both efficient separation and high-sensitivity detection in LC. In this context, PA-glycans are particularly suited for FAC-FD in that they afford not only sufficient sensitivity (<1 pmol/analysis) but also high reliability and reproducibility, because the labelled glycans show no detectable non-specific adsorption on the resin on which lectins are immobilized.

FAC-FD enables precise determination of interactions in terms of K_d between lectins and glycans in a highly systematic manner. Thus, the method provides us with extensive data regarding lectin specificity. Since K_d (or K_a) values are inherent to individual lectin–glycan combinations at any given temperature, they are fundamental knowledge essential for development of a lectin-based glycan profiling system (to be described subsequently) as well as for understanding lectins from a global viewpoint. At present, we are convinced that the use of lectins is advantageous over antibodies for the purpose of glycan profiling for the following reason: glycan structures are extremely diverse. Though it is difficult to determine an actual size of the glycome for each biological species, it would certainly exceed the order of 10^3–10^4. Therefore, it is almost impossible to prepare a set of antibodies strong and specific enough to detect the glycome from a practical viewpoint. Anti-carbohydrate antibodies, if any, do not seem to cover a full spectrum of the glycome. On the other hand, legume lectins, for example, are well-known to have a wide range of binding specificities for *N*-glycans (both high-mannose-type and complex-types with and without sialic acid), *O*-glycans and glycolipid-type glycans. In contrast to antibodies, lectins show generally much weaker binding affinities (*i.e*. in terms of K_d, 10^{-3} to 10^{-7} M) compared with the known affinities of antibodies (10^{-6} to 10^{-9} M). This is a consistent feature of bio-molecules (Fig. 3): the higher the specificity of a bio-molecule, the higher its affinity. On the other hand, lower specificity is suitable for the sake of glycan profiling, because it requires for the first place 'coverage'. Because most lectins show cross-affinity to structurally related glycans to different extents, a set of lectins would be expected to cover a much wider range of structures than a set of antibodies (Fig. 4). It is not necessarily true that some specific lectins discriminate closely related structures; *e.g*. Lewis x (Galβ1-4 (Fucα1-3) GlcNAc) and Lewis a antigens (Galβ1-3 (Fucα1-4) GlcNAc). In fact, these structural isomers were successfully differentiated by the combination of *Bauhinia purpurea* lectin known as BPA and *Ulex europaeu*s isolectin-1 (UEA-1), neither of which is a specific probe for these Lewis antigens (*68*).

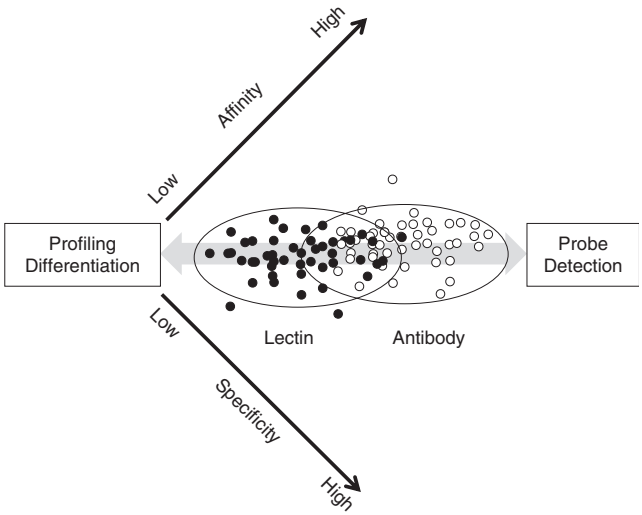

Fig. 3. **A schematic diagram to demonstrate lectin and antibody performance.** In general, antibodies show a high specificity and affinity toward their antigen, while lectins show much less specificity and affinity toward their counterpart (carbohydrate) ligand. The former provides the best characteristics for specific detection and probing in different biochemical scenarios, whereas the latter is more suitable for glycan profiling and differentiation purposes in glycomics.

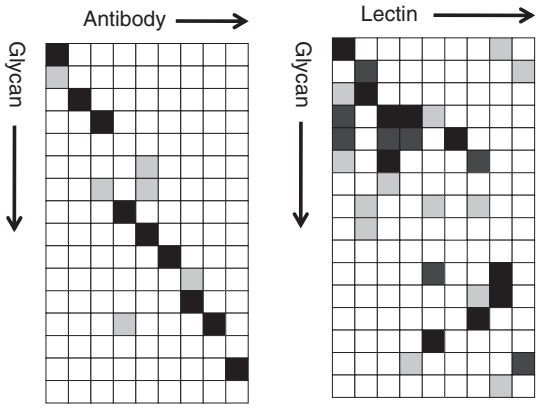

Fig. 4. **Schematic diagram to show the concept of glycan profiling in particular comparison with specific detection means using antibodies.** In general, lectins show a range of affinities to a wide variety of glycans, and thus, have wide epitope coverage to glycans. This unique property, however, is superior for the sake of glycan profiling aiming at comprehensive characterization of glycosylation features of glycoproteins, cells and tissue extracts as well as body fluids. Also note that lectins are considered to function *in vivo* in various biological situations as glycan decipherers.

This unexpected result typifies the characteristics of lectin-based glycan profiling.

In the following two sections, the author describes two profiling methodologies which have been investigated and improved in our laboratory.

Frontal Affinity Chromatography—The first method to be addressed is FAC-FD. Since the original theory of FAC (*38*) as well as recent improvements using

PA-glycans (*32–37*) and an automated machine (*40–42*) have already been described, only the essence is mentioned in this mini-review. The basic equation of FAC is:

$$K_d = \frac{B_t}{(V - V_0)} - [A]_0 \quad (1)$$

where B_t is an effective ligand content (expressed in mol) of a lectin-immobilized column, V and V_0 are elution front volumes of analyte and a control substance, respectively, and $[A]_0$ is the initial concentration of the analyte (PA-glycans in this case).

The recently developed automated machine (FAC-1) is equipped with a pair of capsule-type miniature columns (each 2.0 mm in diameter and 10 mm in length, 31.4 μl bed volume) in line with a fluorescence detector (Shimadzu RF10AXL: for more details, see *40–42*). Typical conditions are as follows: analytical speed, 5 min/analysis; sample requirement, 0.3–1.0 ml of PA-glycan solution (5–10 nM); resolution (experimental error), 3–5 μl in V–V_0. In most cases, K_d values for lectins and glycans (10^{-3} to 10^{-7} M) are much larger than $[A]_0$ ($5–10 \times 10^{-9}$ M). Hence, Eq. 1 can be simplified to Eq. 2:

$$K_d = \frac{B_t}{(V - V_0)} \quad (2)$$

As Eq. 2 does not include $[A]_0$, interaction experiments avoid experimental errors attributed to PA-glycan concentration. This is a basic reason why FAC is able to provide precise data with sufficient reproducibility and reliability.

To summarize the merits of FAC-FD:

1. Clarity of the principle essentially based on Langmuir's adsorption principle.
2. Applicability to even weak interactions such as those between lectins and glycans.
3. Requirement for a low analyte (*e.g.* PA-glycan) concentration (5–10 nM).
4. Suitability to systematic and high-throughput analysis (a series of K_d's can be obtained once B_t is fixed).
5. Accuracy and reproducibility due to simple isocratic elution as well as independence from $[A]_0$ by Eq. 2.
6. Simplicity of the apparatus basically consisting of an isocratic elution system.

To our knowledge, FAC-FD is the only available method for the quantitative determination of lectin specificity (*i.e.* not in terms of relative values such as I_{50} in the haemagglutination assay and enzyme-linked assay, but of absolute values, *i.e.* K_d or K_a) with a full panel (>100) of oligosaccharides. Lectin-glycan interactions determined so far number as many as 20,000. Approximately 200 lectins have been analysed for interaction with 100 glycans, among which 12,000 interactions were determined in terms of K_d. A global view of the interaction analyses so far achieved is given in Fig. 5.

On the other hand, the method has the following potential drawbacks:

1. Immobilization of lectins to the matrix (gel) might result in modification (reduction) of their binding properties. This is particularly the case for sialic acid-binding lectins which have lysine residue(s) in their active site. Appropriate immobilization methods other than the amino-coupling method usually used for immobilization can improve this problem.
2. Even using a miniature column (2×10 mm, 31.4 μl), a relatively large amount of lectins—(*e.g.* approximately 500 μg) is required for completion of the analysis.
3. Crude samples (glycans) cannot be analysed. Unlike FAC-MS, mixed samples cannot directly be analysed.
4. For the determination of B_t, only a limited repertoire of saccharide derivatives having a UV-sensitive group (*e.g.* *p*-nitrophenyl, *p*-methoxyphenyl, Methotrexate) is available.

As a result of comprehensive interaction analysis using approximately 200 lectins and 100 glycans, systematic elucidation is made for most lectins in terms of K_d, whereas it is not possible, at the moment, to determine K_d of some lectins, because B_t values cannot easily be obtained due to the lack of appropriate sugar derivatives (*e.g.* *p*-nitrophenyl derivative) required for concentration-dependency analysis to calculate B_t. Even in the latter case, however, relative affinity can be discussed according to Eq. 2 (K_a proportional to V–V_0). Another impact of the FAC analysis is re-investigation of lectin specificity: this is represented by a GlcNAc-binding plant lectin from *Grifornia simplicifonia*, GSL-II, for which the detailed sugar-binding specificity has not been elucidated. Recent FAC analysis revealed that GSL-II recognizes, in a highly specific manner, a GlcNAc residue transferred by the action of GlcNAc transferase IV (*47*). Other examples of re-investigation of lectin specificity include galectins (*36*, *51*), *Agaricus bisporus* lectin (known as ABA) (*48*), mannose-binding-type Jacalin-related lectins (*49*) and animal C-type lectins (*50*). Thus, new information can be gained by re-examination of older work using FAC. FAC is also a powerful tool for the investigation of the sugar-binding specificity of novel lectins.

Lectin Microarray—The second approach to glycan profiling in the particular context of differential profiling is lectin microarray. Basically, FAC is performed by a chromatographic procedure using a single or a pair of columns and a series of purified (*i.e.* standard) glycans. On the other hand, the lectin microarray is a novel platform enabling multiple lectin interaction analyses simultaneously. Moreover, all kinds of glycans (even a mixture and crude samples) can be applied to the lectin array. The method is therefore expected to provide an extremely high-throughput means for glycan profiling. To realize this, however, there is the basic issue that usual microarray techniques (*e.g.* for DNA and antibody) require extensive washing procedures before scanning of the bound probes on the microarray. As emphasized previously, lectin–glycan interactions are weak (K_d = 10^{-3} to 10^{-7} M) compared with those of antigen-antibody and DNA-RNA hybrids. Such washing procedures would eliminate weakly bound glycans on the lectin microarray, resulting in loss of significant information on glycan (probe) affinity, which provides important clues to glycan structures.

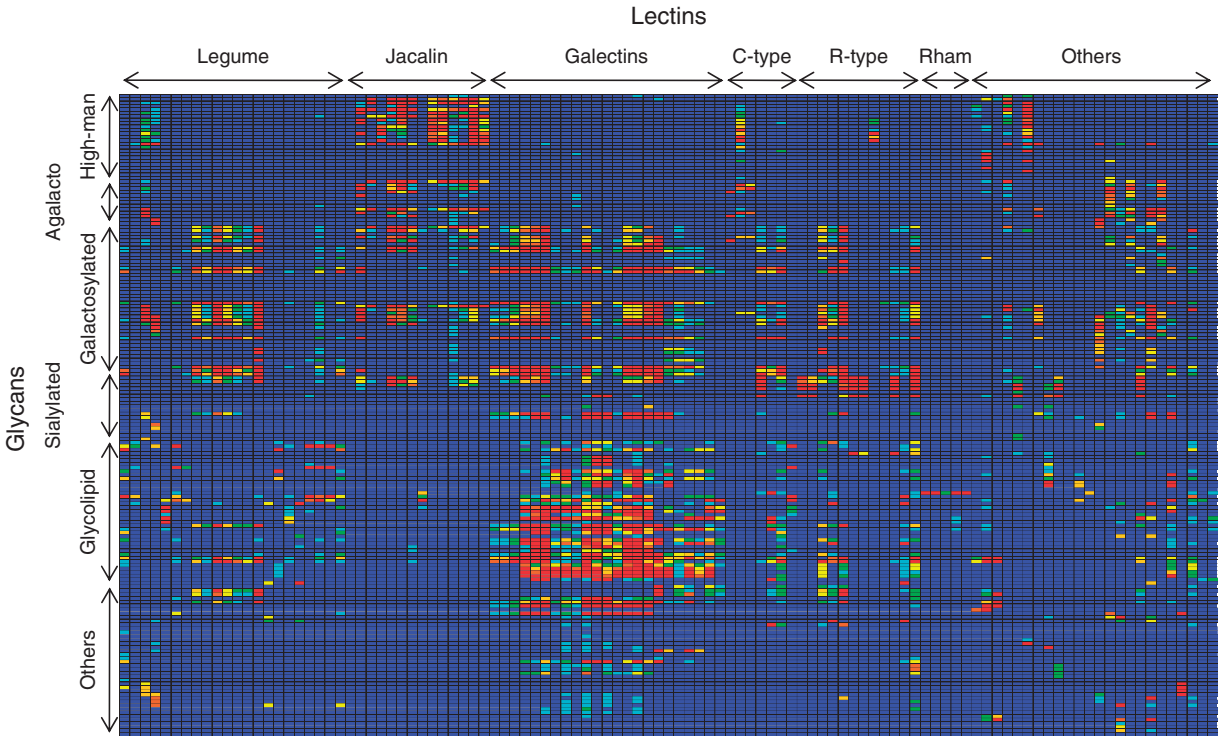

Fig. 5. **A matrix summarizing the present state of comprehensive interaction analysis by FAC, designated the 'Hect-by-Hect' Project.** Here, only interactions of 120 lectins, for which K_d (or K_a) values have already bean determined, are listed. The strength of each interaction is expressed according to six different colours; *i.e. red* ['strongest' to show >30 μl in terms of $V–V_0$ (see text)], *orange* ('second strongest' to show 25–30 μl of $V–V_0$), *dark yellow* ('third strongest' to show 20–25 μl) *yellow* ('medium' to show 15–20 μl of $V–V_0$), *green* ('second weakest' to show 10–15 μl of $V–V_0$), *sky blue* ('weakest but significant' to show 5–10 μl of $V–V_0$) and *blue* ('no interaction' showing <5 μl of $V–V_0$).

Recently, we developed a unique infrastructure for the lectin microarray, which is based on an evanescent-field activated fluorescence detection principle (*69*, *70*). In the procedure, about 40 lectins, for which specificity has been elucidated by FAC, are immobilized on a glass slide. After probe (*e.g.* Cy3-labelled glycoprotein) incubation, excitation light is injected from both sides of the slide glass at a certain angle to make a total reflection between the solid phase (slide glass) and liquid phase (reaction chamber formed by a rubber sheet). Here, an evanescent wave is generated within a limited space from the surface (so-called 'near optic field'), which is approximately a half distance of the wave length used for excitation. When Cy3-labelled glycoprotein is used as a probe, the near optic field should be within ~250 nm, for an excitation wavelength of 488 nm. Since the intensity of the evanescent wave is exponentially reduced from the surface, it is considered that a substantially effective near optic field is 200 nm or shorter. The system enables specific detection of fluorescently labelled (*e.g.* Cy3) glycans without any washing process. The evanescent wave fluorescence detection system thus enables liquid-phase observation in an equilibrium state, unlike the confocal detection principle, which most of other microarray techniques adopt. Figure 6 illustrates the geometry of the lectin microarray platform, and shows the total dimensions of this unique detection system, and how the evanescent principle works. In our standard protocol, 0.1 ml of probing solution (Cy3-labelled glycan solution) of 1 mm depth is used in each reaction chamber. Since the evanescent field is generated within 200 nm, in theory the effective activation field occupies only 0.02% of the bottom of the reaction chamber. This means 0.02% of the applied probe solution is exposed to the evanescent wave activation. Thus the background level is extremely low. In fact, the evanescent-field activated fluorescence detection system shows the highest sensitivity among the lectin microarray systems reported so far: *i.e.* limit of detection (LOD) values are 100 pg/ml of glycoprotein (asialofetuin) and 100 pM glycan (asialo-biantennary *N*-glycan) probes, respectively (*68*).

Unlike other profiling methods, the lectin microarray has the extremely useful property of direct applicability to crude samples. These are typically clinical samples, such as sera and tissue extracts. Moreover, combination of the lectin microarray with an antibody specific for a target glycoprotein is highly feasible for high-throughput analysis of clinical samples for bio-marker investigation, because individual labelling (*e.g.* with Cy3-SE reagent) and removal procedures can be omitted with the aid of Cy3-labelled antibody (or appropriate second antibody). Alternatively, the biotin-avidin system can be used. Such applications of the lectin microarray will be described elsewhere, as well as its practical application to glycoprotein-biomarker investigations in the context of the

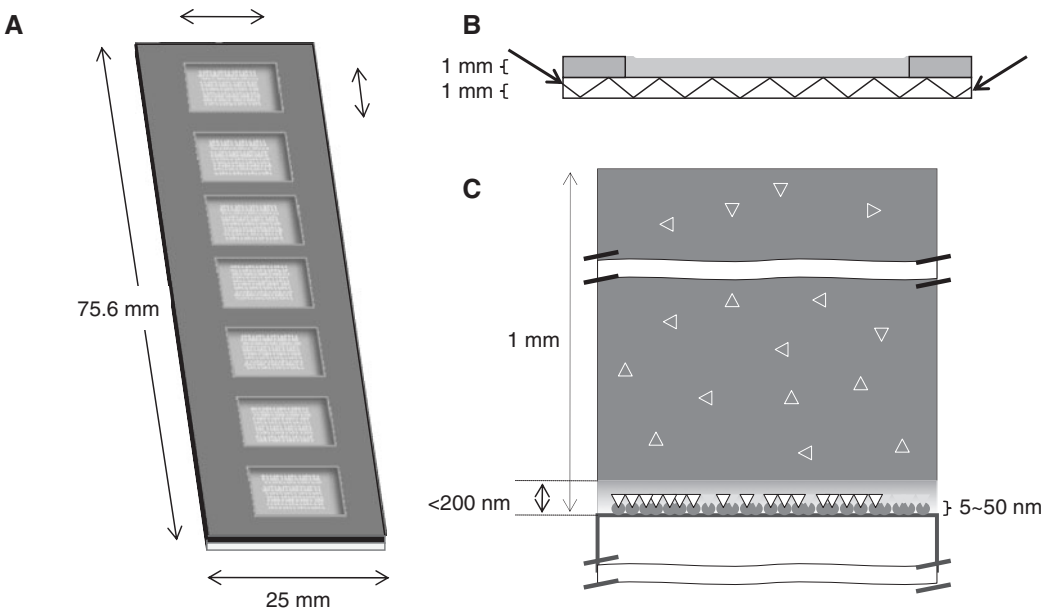

Fig. 6. **Diagram showing total dimensions of lectin microarray, which is based on an evanescent-field activated fluorescence detection principle.** (A) Overall view of the commercial lectin microarray with a rubber sheet to make seven reaction chambers. (B) Front view of the array with a rubber sheet. To generate an evanescent wave, an excitation light is injected from both sides of the slide glass (shown by *arrows*). (C) Rough estimates of the inside dimensions of each reaction chamber. Triangles represent fluorescently labelled glycomaterials (Cy3-labelled glycoprotein). Note that they are concentrated in the extremely restricted space almost within the generated evanescent field.

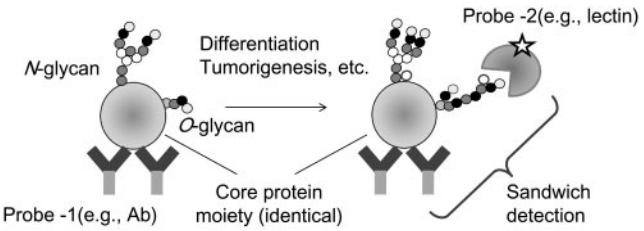

Fig. 7. **Developmental principle of glycoprotein biomarker.** The scheme shows that significant glycosylation change occurs in a manner associated with various changes in cell states, *e.g.* in development, differentiation and tumourigenesis.

Medical Glycomics project of NEDO (New Energy and Industrial Technology Development Organization), the basic concept of which is summarized in Fig. 7.

CONCLUDING REMARKS

In this mini-review, recent advances in lectin-based glycotechnologies are described. The concept of glycan profiling is realized by means of the lectin microarray, but this emerging technique is extensively supported by fundamental information provided by FAC. Probably, both lectin microarray and FAC are necessary tools for structural studies of glycans (*i.e.* for structural glycomics) as well as other analytical methods. All of these are necessary for elucidation of glycan biological functions (*i.e.* functional glycomics). With these tools, we have been able to approach even evolutionary issues involving glycans (*i.e.* comparative glycomics). Overall, why do we study glycans? The author would answer: because they are old (for comparative glycomics), important (for functional glycomics) and difficult (for structural glycomics). By any measure, glycobiology occupies a most fundamental position in life science, because we now know that all scientific roads lead to the Glycome.[1]

REFERENCES

1. Mahal, L.K. (2008) Glycomics: towards bioinformatic approaches to understanding glycosylation. *Anticancer Agents Med. Chem.* **8**, 37–51
2. Packer, N.H., von der Lieth, C.W., Aoki-Kinoshita, K.F., Lebrilla, C.B., Paulson, J.C., Raman, R., Rudd, P., Sasisekharan, R. Taniguchi, N., and York, W.S. (2006) Frontiers in glycomics: bioinformatics and biomarkers in disease. An NIH White Paper prepared from discussions by the focus groups at a workshop on the NIH campus, Bethesda MD (September 11–13, 2006). *Proteomics* **8**, 8–20
3. Seeberger, P.H. and Werz, D.B. (2007) Synthesis and medical applications of oligosaccharides. *Nature* **446**, 1046–1051
4. Turnbull, J.E. and Field, R.A. (2007) Emerging glycomics technologies. *Nat. Chem. Biol.* **3**, 74–77
5. Wada, Y., Azadi, P., Costello, C.E., Dell, A., Dwek, R.A., Geyer, H., Geyer, R., Kakehi, K., Karlsson, N.G., Kato, K., Kawasaki, N., Khoo, K.H., Kim, S., Kondo, A., Lattova, E., Mechref, Y., Miyoshi, E., Nakamura, K., Narimatsu, H., Novotny, M.V., Packer, N.H., Perreault, H., Peter-Katalinic, J., Pohlentz, G., Reinhold, V.N., Rudd, P.M., Suzuki, A., and Taniguchi, N. (2007) Comparison of the methods for profiling glycoprotein glycans–HUPO Human Disease Glycomics/Proteome Initiative multi-institutional study. *Glycobiology* **17**, 411–422

[1] In analogy to the proverb 'All roads lead to Rome'.

6. Valki, A., Cummings, R., Esko, J., Freeze, H., Hart, G., and Marth, J. (eds.) (1999) *Essentials of Glycobiology*. Cold Springer Harbor Laboratory Press, New York
7. Apweiler, R., Hermjakob, H., and Sharon, N. (1999) On the frequency of protein glycosylation, as deduced from analysis of the SWISS-PROT database. *Biochim. Biophys. Acta.* **1473**, 4–8
8. Kang, P., Mechref, Y., Kyselova, Z., Goetz, J.A., and Novotny, M.V. (2007) Comparative glycomic mapping through quantitative permethylation and stable-isotope labeling. *Anal. Chem.* **79**, 6064–6073
9. Liu, X., McNally, D.J., Nothaft, H., Szymanski, C.M, Brisson, J.R., and Li, J. Mass (2006) Spectrometry-based glycomics strategy for exploring N-linked glycosylation in eukaryotes and bacteria. *Anal. Chem.* **78**, 6081–6087
10. Lapadula, A.J., Hatcher, P.J., Hanneman, A.J., Ashline, D.J., Zhang, H., and Reinhold, V.N. (2000) Congruent strategies for carbohydrate sequencing. 3. OSCAR: an algorithm for assigning oligosaccharide topology from MS^n data. *Anal. Chem.* **77**, 6271–6279
11. Zhang, H., Singh, S., and Reinhold, V.N. (2005) Congruent strategies for carbohydrate sequencing. 2. FragLib: an MS^n spectral library. *Anal. Chem.* **77**, 6263–6270
12. Ashline, D., Singh, S., Hanneman, A., and Reinhold, V. (2005) Congruent strategies for carbohydrate sequencing. 1. Mining structural details by MS^n. *Anal. Chem.* **77**, 6250–6262
13. Hase, S., Ikenaka, T., and Matsushima, Y. (1978) Structure analyses of oligosaccharides by tagging of the reducing end sugars with a fluorescent compound. *Biochem. Biophys. Res. Commun.* **85**, 257–263
14. Tomiya, N., Awaya, J., Kurono, M., Endo, S., Arata, Y., and Takahashi, N. (1988) Analyses of N-linked oligosaccharides using a two-dimensional mapping technique. *Anal. Biochem.* **171**, 73–90
15. Takahashi, N., Nakagawa, H., Fujikawa, K., Kawamura, Y., and Tomiya, N. (1995) Three-dimensional elution mapping of pyridylaminated N-linked neutral and sialyl oligosaccharides. *Anal. Biochem.* **226**, 139–146
16. Kameyama, A., Kikuchi, N., Nakaya, S., Ito, H., Sato, T., Shikanai, T., Takahashi, Y., Takahashi, K., and Narimatsu, H. (2005) A strategy for identification of oligosaccharide structures using observational multistage mass spectral library. *Anal. Chem.* **77**, 4719–4725
17. Kameyama, A., Nakaya, S., Ito, H., Kikuchi, N., Angata, T., Nakamura, M., Ishida, H.K., and Narimatsu, H. (2006) Strategy for simulation of CID spectra of N-linked oligosaccharides toward glycomics. *J. Proteome Res.* **5**, 808–814
18. Sekiya, S., Yamaguchi, Y., Kato, K., and Tanaka, K. (2005) Mechanistic elucidation of the formation of reduced 2-aminopyridine-derivatized oligosaccharides and their application in matrix-assisted laser desorption/ionization mass spectrometry. *Rapid Commun. Mass Spectrom.* **19**, 3607–3611
19. Haslam, S.M., North, S.J., and Dell, A. (2006) Mass spectrometric analysis of N- and O-glycosylation of tissues and cells. *Curr. Opin. Struct. Biol.* **16**, 584–591
20. Tissot, B., Gasiunas, N., Powell, A.K., Ahmed, Y., Zhi, Z.L., Haslam, S.M., Morris, H.R., Turnbull, J.E., Gallagher, J.T., and Dell, A. (2007) Towards GAG glycomics: analysis of highly sulfated heparins by MALDI-TOF mass spectrometry. *Glycobiology* **17**, 972–982
21. Hitchcock, A.M., Costello, C.E., and Zaia, J. (2006) Glycoform quantification of chondroitin/dermatan sulfate using a liquid chromatography-tandem mass spectrometry platform. *Biochemistry* **45**, 2350–2361
22. Minamisawa, T., Suzuki, K., and Hirabayashi, J. Multistage mass spectrometric sequencing of keratan sulfate-related oligosaccharides. *Anal. Chem.* **78**, 891–900.
23. Kamoda, S., Nakanishi, Y., Kinoshita, M., Ishikawa, R., and Kakehi, K. (2006) Analysis of glycoprotein-derived oligosaccharides in glycoproteins detected on two-dimensional gel by capillary electrophoresis using on-line concentration method. *J. Chromatogr. A.* **1106**, 67–74
24. Dang, F., Kakehi, K., Nakajima, K., Shinohara, Y., Ishikawa, M., Kaji, N., Tokeshi, M., and Baba, Y. (2006) Rapid analysis of oligosaccharides derived from glycoproteins by microchip electrophoresis. *J. Chromatogr. A* **1109**, 138–143
25. Mechref, Y. and Novotny, M.V. (2006) Miniaturized separation techniques in glycomic investigations. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* **841**, 65–78
26. Balaguer, E. and Neusüss, C. (2006) Glycoprotein characterization combining intact protein and glycan analysis by capillary electrophoresis-electrospray ionization-mass spectrometry. *Anal. Chem.* **78**, 5384–5393
27. Hirabayashi, J. (2004) Lectin-based structural glycomics: glycoproteomics and glycan profiling. *Glycoconj. J.* **21**, 35–40
28. Sharon, N. (2007) Lectins: carbohydrate-specific reagents and biological recognition molecules. *J. Biol. Chem.* **282**, 2753–2764
29. Cummings, R.D. and Kornfeld, S. (1982) Fractionation of asparagine-linked oligosaccharides by serial lectin-Agarose affinity chromatography. A rapid, sensitive, and specific technique. *J. Biol. Chem.* **257**, 11235–11240
30. Harada, H., Kamei, M., Tokumoto, Y., Yui, S., Koyama, F., Kochibe, N., Endo, T., and Kobata, A. (1987) Systematic fractionation of oligosaccharides of human immunoglobulin G by serial affinity chromatography on immobilized lectin columns. *Anal. Biochem.* **164**, 374–381
31. Bierhuizen, M.F., Hansson, M., Odin, P., Debray, H., Obrink, B., van, Dijk, and W., (1989) Structural assessment of the N-linked oligosaccharides of cell-CAM 105 by lectin-agarose affinity chromatography. *Glycoconj. J.* **6**, 195–208
32. Hirabayashi, J., Arata, Y., and Kasai, K. (2000) Reinforcement of frontal affinity chromatography for effective analysis of lectin-oligosaccharide interactions. *J. Chromatogr. A.* **890**, 261–271
33. Hirabayashi, J. and Kasai, K. (2002) Separation technologies for glycomics. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* **771**, 67–87
34. Hirabayashi, J., Arata, Y., and Kasai, K. (2003) Frontal affinity chromatography as a tool for elucidation of sugar recognition properties of lectins. *Methods Enzymol.* **362**, 353–368
35. Arata, Y., Hirabayashi, J., and Kasai, K. (2001) Application of reinforced frontal affinity chromatography and advanced processing procedure to the study of the binding property of a *Caenorhabditis elegans* galectin. *J. Chromatogr. A.* **905**, 337–343
36. Hirabayashi, J., Hashidate, T., Arata, Y., Nishi, N., Nakamura, T., Hirashima, M., Urashima, T., Oka, T., Futai, M., Muller, W.E., Yagi, F., and Kasai, K. Oligosaccharide specificity of galectins: a search by frontal affinity chromatography. Biochim. Biophys, Acta. 1572, 232–254
37. Arata, Y., Hirabayashi, J., and Kasai, K. (2001) Sugar binding properties of the two lectin domains of the tandem repeat-type galectin LEC-1 (N32) of Caenorhabditis elegans. Detailed analysis by an improved frontal affinity chromatography method. *J. Biol. Chem.* **276**, 3068–3077
38. Kasai, K. and Ishii, S. (1975) Quantitative analysis of affinity chromatography of trypsin. A new technique for investigation of protein-ligand interaction. *J. Biochem.* **77**, 261–264
39. Nakamura, S., Yagi, F., Totani, K., Ito, Y., and Hirabayashi, J. (2005) Comparative analysis of carbohydrate-binding properties of two tandem repeat-type Jacalin-related lectins, *Castanea crenata* agglutinin and *Cycas revoluta* leaf lectin. *FEBS J.* **272**, 2784–2799
40. Nakamura-Tsuruta, S., Uchiyama, N., and Hirabayashi, J. (2006) High-throughput analysis of lectin-oligosaccharide interactions by automated frontal affinity chromatography. *Methods Enzymol.* **415**, 311–325

41. Tateno, H., Nakamura-Tsuruta, S., and Hirabayashi, J. (2007) Frontal affinity chromatography: sugar-protein interactions. *Nat. Protoc.* **2**, 2529–2537
42. Nakamura-Tsuruta, S., Uchiyama, N., Kominami, J., and Hirabayashi, J. (2007) Frontal affinity chromatography: systematization for quantitative interaction analysis between lectins and glycans in *Lectins: Analytical Technologies* (Nilsson, C.L., ed.) Elsevier, Amsterdam.
43. Itakura, Y., Nakamura-Tsuruta, S., Kominami, J., Sharon, N., Kasai, K., and Hirabayashi, J. Systematic comparison of oligosaccharide specificity of *Ricinus communis* agglutinin I and *Erythrina* lectins: a search by frontal affinity chromatography. *J. Biochem.* 142, 459–469
44. Van Damme, E.J., Nakamura-Tsuruta, S., Hirabayashi, J., Rougé, P., and Peumans, W.J. (2007) The *Sclerotinia sclerotiorum* agglutinin represents a novel family of fungal lectins remotely related to the Clostridium botulinum non-toxin haemagglutinin HA33/A. *Glycoconj. J.* **24**, 143–456
45. Van Damme, E.J., Nakamura-Tsuruta, S., Smith, D.F., Ongenaert, M., Winter, H.C., Rougé, P., Goldstein, I.J., Mo, H., Kominami, J., Culerrier, R., Barre, A., Hirabayashi, J., and Peumans, W.J. (2007) Phylogenetic and specificity studies of two-domain GNA-related lectins: generation of multispecificity through domain duplication and divergent evolution. *Biochem. J.* **404**, 51–61
46. Huang, X., Tsuji, N., Miyoshi, T., Nakamura-Tsuruta, S., Hirabayashi, J., and Fujisaki, K. (2007) Molecular characterization and oligosaccharide-binding properties of a galectin from the argasid tick *Ornithodoros moubata*. *Glycobiology.* **17**, 313–323
47. Nakamura-Tsuruta, S., Kominami, J., Kamei, M., Koyama, Y., Suzuki, T., Isemura, M., and Hirabayashi, J. (2006) Comparative analysis by frontal affinity chromatography of oligosaccharide specificity of GlcNAc-binding lectins, *Griffonia simplicifolia* lectin-II (GSL-II) and *Boletopsis leucomelas* lectin (BLL). *J. Biochem.* **140**, 285–291
48. Nakamura-Tsuruta, S., Kominami, J., Kuno, A., and Hirabayashi, J. (2006) Evidence that Agaricus bisporus agglutinin (ABA) has dual sugar-binding specificity. *Biochem. Biophys. Res. Commun.* **347**, 215–220
49. Nakamura-Tsuruta, S., Uchiyama, N., Peumans, W.J., Van Damme, E.J.M., Totani, K., Ito, Y., and Hirabayashi, J. (2008) Analysis of the sugar-binding specificity of mannose-binding-type Jacalin-related lectins by frontal affinity chromatography: an approach to functional classification. *FEBS J.* (in press)
50. Oo-Puthinan, S., Maenuma, K., Sakakura, M., Denda-Nagai, K., Tsuiji, M., Shimada, I., Nakamura-Tsuruta, S., Hirabayashi, J., Bovin, N.V., and Irimura, T. (2008) The amino acids involved in the distinct carbohydrate specificities between macrophage galactose-type C-type lectins 1 and 2 (CD301a and b) of mice. *Biochim. Biophys. Acta.* **1780**, 89–100
51. Nagae, M., Nishi, N., Nakamura-Tsuruta, S., Hirabayashi, J., Wakatsuki, S., and Kato, R. (2007) Structural analysis of the human galectin-9 N-terminal carbohydrate recognition domain reveals unexpected properties that differ from the mouse orthologue. *J. Mol. Biol.* **375**, 119–135
52. Watanabe, Y., Shiina, N., Shinozaki, F., Yokoyama, H., Kominami, J., Nakamura-Tsuruta, S., Hirabayashi, J., Sugahara, K., Kamiya, H., Matsubara, H., Ogawa, T., and Muramoto, K. (2007) Isolation and characterization of l-rhamnose-binding lectin, which binds to microsporidian Glugea plecoglossi, from ayu (*Plecoglossus altivelis*) eggs. *Dev. Comp. Immunol.* **32**, 487–499
53. Kobata, A. (2000) A journey to the world of glycobiology. *Glycoconj. J.* **17**, 443–464
54. Feizi, T. Progress in deciphering the information content of the 'glycome'–a crescendo in the closing years of the millennium. Glycoconj. J. 17, 553–565
55. Taniguchi, N., Ekuni, A., Ko, J.H., Miyoshi, E., Ikeda, Y., Ihara, Y., Nishikawa, A., Honke, K., and Takahashi, M. (2001) A glycomic approach to the identification and characterization of glycoprotein function in cells transfected with glycosyltransferase genes. *Proteomics.* **1**, 239–247
56. Hirabayashi, J., Arata, Y., and Kasai, K. Glycome project: concept, strategy and preliminary application to *Caenorhabditis elegans*. *Proteomics* 1, 295–303
57. Wasinger, V.C., Cordwell, S.J., Cerpa-Poljak, A., Yan, J.X., Gooley, A.A., Wilkins, M.R., Duncan, M.W., Harris, R., Williams, K.L., and Humphery-Smith, I. (1995) Progress with gene-product mapping of the Mollicutes: Mycoplasma genitalium. *Electrophoresis* **16**, 1090–1094
58. Mega, T. and Hase, S. (1991) Determination of lectin-sugar binding constants by microequilibrium dialysis coupled with high performance liquid chromatography. *J. Biochem.* **109**, 600–603
59. Ohyama, Y., Kasai, K., Nomoto, H., and Inoue, Y. (1985) Frontal affinity chromatography of ovalbumin glycoasparagines on a concanavalin A-sepharose column. A quantitative study of the binding specificity of the lectin. *J. Biol. Chem.* **260**, 6882–6887
60. Schriemer, D.C., Bundle, D.R., Li, L., and Hindsgaul, O. (1998) Micro-scale frontal affinity chromatography with mass spectrometric detection: a new method for the screening of compound libraries. *Angew. Chem. Int. Ed.* **37**, 3383–3387
61. Fort, S., Kim, H.S., and Hindsgaul, O. (2006) Screening for galectin-3 inhibitors from synthetic lacto-N-biose libraries using microscale affinity chromatography coupled to mass spectrometry. *J. Org. Chem.* **71**, 7146–7154
62. Dam, T.K. and Brewer, C.F. (2004) Multivalent protein-carbohydrate interactions: isothermal titration microcalorimetry studies. *Methods Enzymol.* **379**, 107–128
63. Rani, P.G., Bachhawat, K., Reddy, G.B., Oscarson, S., and Surolia, A. (2000) Isothermal titration calorimetric studies on the binding of deoxytrimannoside derivatives with artocarpin: implications for a deep-seated combining site in lectins. *Biochemistry* **39**, 10755–10760
64. Yamamoto, K., Ishida, C., Shinohara, Y., Hasegawa, Y., Konami, Y., Osawa, T., and Irimura, T. (1994) Interaction of immobilized recombinant mouse C-type macrophage lectin with glycopeptides and oligosaccharides. *Biochemistry* **33**, 8159–8166
65. Shinohara, Y., Kim, F., Shimizu, M., Goto, M., Tosu, M., and Hasegawa, Y. (1994) Kinetic measurement of the interaction between an oligosaccharide and lectins by a biosensor based on surface plasmon resonance. *Eur. J. Biochem.* **223**, 189–194
66. Ideo, H., Seko, A., and Yamashita, K. (2005) Galectin-4 binds to sulfated glycosphingolipids and carcinoembryonic antigen in patches on the cell surface of human colon adenocarcinoma cells. *J. Biol. Chem.* **280**, 4730–4737
67. Sörme, P., Kahl-Knutsson, B., Wellmar, U., Magnusson, B.G., Leffler, H., and Nilsson, U.J. (2003) Design and synthesis of galectin inhibitors. *Methods Enzymol.* **363**, 157–169
68. Uchiyama, N., Kuno, A., Tateno, H., Kubo, Y., Mizuno, M., Noguchi, M., and Hirabayashi, J. (2008) Optimization of evanescent-field fluorescence-assisted lectin microarray for high-sensitive detection of monovalent oligosaccharides and glycoproteins. *Proteomics.* (in press)
69. Kuno, A., Uchiyama, N., Koseki-Kuno, S., Ebe, Y., Takashima, S., Yamada, M., and Hirabayashi, J. (2005) Evanescent-field fluorescence-assisted lectin microarray: a new strategy for glycan profiling. *Nat. Methods* **2**, 851–856
70. Uchiyama, N., Kuno, A., Koseki-Kuno, S., Ebe, Y., Horio, K., Yamada, M., and Hirabayashi, J. (2006) Development of a lectin microarray based on an evanescent-field fluorescence principle. *Methods Enzymol.* **415**, 341–351