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species; for example, jack fruit (*Artocarpus integliforia*) contains Man-specific artocarpin and Gal-specific jacalin.[26] On the other hand, monocot-derived, GNA-related lectins[27] and hevein-type lectins[28] are basically Man-specific and GlcNAc (chitin oligomer)-specific, respectively. Many lectin families have shown much wider biological occurrence than had ever been thought; *i.e.*, in bacteria, animals and fungi. Ca^{2+}-dependent type lectins (C-type lectins[29]) are representative animal lectins, which comprise an extremely large family, though they are rarely used for glycan profiling, partly because their production in bacteria is generally difficult. Another representative family of animal lectins is the galectins, which are defined as lectins having common specificity to β-galactopyranoside and evolutionarily conserved amino acid sequences,[30] though their individual specificities are substantially diverged within the Galβ-eq. or Galβ (*syn*)*gauche* consensus rule for disaccharide recognition.[31,32] Some other lectins have also been attracting attention either for their useful specificities or unique structures (for a list of representative lectins used for glycan profiling, see Table 2; ref. 33–79).

Most of these lectins have basic preferences to a certain set of carbohydrate structures or epitopes, in an analogous manner to antigen–antibody interaction. Therefore, each shows a significant level of 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. 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 europaeus* isolectin-I (UEA-I), neither of which is a specific probe for these Lewis antigens.[80] This unexpected result typifies the characteristics of lectin-based glycan profiling.

Historically, lectin is merely a name tentatively assigned to a protein with a single aspect, *i.e.*, ability to bind to a set of glycans, while antibodies and enzymes were excluded in the old definition.[8] However, with advances in genome technology as well as glycotechnology,[7] lectin families are also found to be increasing in size: some are homogeneous families of lectins, while others are a mixture of lectins and non-lectins. The number of the known lectin families in this context probably exceeds ~40, which include siglec, a family of the immunoglobulin superfamily of sialic acid specific lectins. Therefore, there are no longer clear criteria to distinguish antibodies from lectins. However, the distinction is made that anti-carbohydrate antibodies are raised only against antigenicity in the host-defense context, while many lectins occur without this restriction. Various properties of lectins and anti-carbohydrate antibodies are compared in Table 3.

5. Precedent technologies to the lectin microarray

As precedent technologies to the lectin microarray, it is worth mentioning two related methods, *i.e.*, serial lectin affinity chromatography and frontal affinity chromatography (FAC). The former is a semi-quantitative procedure to profile glycan structures targeting *N*-linked glycans in either forms of radio-labeled *N*-glycans or glycopeptides. The method is based on the initial observation that glycopeptides with tri- and tetraantennary complex-type oligosaccharides pass through a concanavalin A (ConA)-agarose column, whereas those with biantennary complex-type, high mannose-type, and hybrid-type oligosaccharides bind to the lectin and can be differentially eluted from the column.[81,82] The use of pea (PSA) or lentil lectins (LCA) further fractionated biantennary complex-type oligosaccharides into species with and without core fucose residues.[83] Moreover, either wheat germ agglutinin (WGA) or PHA-E enabled fractionation of glycopeptides according to the presence of bisecting GlcNAc.[84,85] As a representative case, a pool of glycopeptides was prepared by pronase digestion of $[^3H]$-labeled mouse lymphoma cells (BW5147), and was subjected to lectin affinity chromatography in a consecutive manner, *i.e.*, on ConA, PHA-E (WGA), PSA and PHA-L columns.[86] As a result, from a pass-through fraction of ConA-agarose, peptides with complex-type tri- and tetraantennary *N*-linked oligosaccharides as well as biantennary oligosaccharides with bisecting GlcNAc were isolated. The last species with bisecting GlcNAc was separated from the others by PHA-E-agarose, while the remaining was further fractionated by PSA and PHA-L-agarose. On the other hand, from the retarded fraction of ConA-agarose, glycopeptides with biantennary complex-type glycans with or without core fucose were identified, while from the adsorbed fraction those with high mannose-type and hybrid-type oligosaccharides were fractionated by using WGA-agarose.[86] The method is systematic but lacks throughput, and is rather useful for fractionation of *N*-linked oligosaccharides.

The other method, FAC was developed as a quantitative affinity chromatography to determine relatively weak biomolecular interactions between immobilized ligands (*e.g.*, lectins) and labeled analytes (*e.g.*, oligosaccharides[87]). As described previously,[7] FAC has a range of detection methods, *i.e.*, radioisotopes (RI; 50), MS[88] and fluorescence (FD[89]). For FAC-RI, however, *N*-glycans must be radio-labeled, *e.g.*, with $NaB[^3H]_4$. Similarly, for FAC-MS, modification of glycans with an appropriate alkyl reagent is necessary to increase ionization efficiency in MS. This technology is more effectively applied for high-throughput screening of chemical compound libraries. On the other hand, FAC-FD enables precise determination of a series of interactions in terms of K_d between lectins and glycans in a systematic manner. In fact, numerous lectin–oligosaccharide interactions have been determined in terms of K_d.[31,32,38,45,49,52–54,67,68,71,72,77,79] Unfortunately, FAC is a chromatographic procedure that uses a single or a pair of columns and a series of purified (*i.e.* standard) glycans. On the other hand, the lectin microarray provides a novel platform enabling multiple (>40) lectin interaction analyses by a simple procedure. Moreover, all kinds of glycans (even a mixture and crude samples) can be applied to the lectin array. The method is required to provide an extremely high-throughput means for glycan profiling targeting a number of (>100) clinical samples as well as cell lines, the properties of

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Table 2 Representative lectins used for glycan profiling[a]

Nomenclature (origin) another name	Monosaccharide specificity	Preferred glycan structure (terminal epitope)	Ref. for detailed specificity
1. Legume (L-type)			
MAL (*Maackia amurensis*)	Sia	Siaα3Galβ4GlcNAc	33–35
MAH (*Maackia amurensis*)	Sia	Siaα3Galβ3(Siaα6)GalNAc	36
PHA-E (*Phaseolus vulgaris*) Phytohemagglutinin, erythroagglutinin	Gal	*N*-glycans with outer Gal and bisecting GlcNAc	37
ECA (*Erythrina cristagalli*)	Gal	Galβ4GlcNAc	38
BPL (*Bauhinia purpurea*)	Gal	Galβ3GalNAc, GalNAc	32
EEL (*Euonymus europaeus*)	Gal	Galα3Galβ4GlcNAc, Fucα2(Galα3)Galβ4GlcNAc	39
PNA (peanut, *Arachis hypogaea*)	Gal	Galβ3GalNAc	32,40
GSL-I B4 (*Griffonia simplicifolia*)	Gal	α Gal	32
GSL-I A4 (*Griffonia simplicifolia*)	GalNAc	α GalNAc	41
VVA (*Vicia villosa*)	GalNAc	α GalNAc, GalNAcα3Gal	42,43
DBA (*Dolichos biflorus*)	GalNAc	GalNAcα3GalNAc (Blood group A), GalNAcα3GalNAc	34
SBA (soybean, *Dolichos biflorus*)	GalNAc	GalNAc, GalNAcα3Gal	32
WFA (*Wisteria floribunda*)	GalNAc	GalNAcβ4GlcNAc, Galβ3(-6)GalNAc	32,44,45
PTL-I (*Psophocarpus tetragonolobus*)	GalNAc	α GalNAc	46
LTL (*Lotus tetragonolobus*)	Fuc	Fucα3(Galβ4)GlcNAc (Le^x), Fuca2Galβ4GlcNAc (H-type 2)	47
UEA-I (*Ulex europaeus*)	Fuc	Fucα2Galβ4GlcNAc (H-type 2)	48
PSA (pea, *Pisum sativum*)	Fuc/Man	Fucα6GlcNAc, High-Man	49
LCA (lentil, *Lens culinaris*)	Fuc/Man	Fucα6GlcNAc, High -Man	49
ConA (*Canavalia ensiformis*) Concanavalin A	Man	High-Man including Manα6(Manα3)Man	50,51
GSL-II (*Griffonia simplicifolia*)	GlcNAc	Agalactosylated tri/tetra antennary glycans, GlcNAc	52
PHA-L (*Phaseolus vulgaris*) Phytohemagglutinin, leukoagglutinin	Complex	Tri/tetra-antennary complex-type *N*-glycan	37
2. Ricin B-cahin-like (R-type)			
RCA120 caster bean, (*Ricinus communis*)	Gal	Galβ4GlcNAc	38
EW29 (earthworm, *Lumbricus terrestris*)	Gal	non-sibstituted Gal	53
MOA (*Marasmius oreades*)	Gal	Galα3Galβ4GlcNAc, Fucα2(Galα3)Galβ4GlcNAc	39
SNA (*Sambucus nigra*)	Sia	Siaα2-6Gal/GalNAc	34
SSA (*Sambucus sieboldiana*)	Sia	Siaα2-6Gal/GalNAc	54
TJA-I (*Tanthes japonica*)	Sia	Siaα2-6Gal/GalNAc	55
ACA (*Amaranthus caudatus*)	Gal	Galβ3GalNAc	40,56
3. Monocot (GNA-related)			
GNA (*Galanthus nivalis*)	Man	High-Man including Manα3Man	57
NPA (*Narcissus pseudonarcissus*)	Man	High-Man including Manα6Man	58
HHL (*Hippeastrum hybrid*)	Man	High-Man including Manα3Man or Manα6Man	58
TxLCI (*Tulipa gesneriana*)	Man/GalNAc	Man$_3$ core, bi- and tri-antennary *N*-glycans, GalNAc	59
4. Hevein (Chitin-type)			
LEL (tomato, *Lycopersicon esculentum*)	GlcNAc	(GlcNAcβ4)$_n$, (Galβ4GlcNAc)n (polylactosamine)	60
STL (potato, *Solanum tuberosum*)	GlcNAc	(GlcNAcβ)$_n$, (GlcNAcβ4MurNAc)n (peptidoglycan backbone)	61
UDA (*Urtica dioica*)	GlcNAc	GlcNAcβ4GlcNAc, Man5 ∼ Man9	62
PWM (pokeweed, *Phytolacca americana*)	GlcNAc	(GlcNAcβ4)$_n$	63
DSA (*Datura stramonium*)	GlcNAc	(GlcNAcβ4)$_n$, triantennary, tetraantennary *N*-glycans	64
WGA (wheat germ, *Triticum aestivum*)	GlcNAc	(GlcNAcβ4)$_n$, NeuAc	35
5. Jacalin			
MPA (*Maclura pomifera*)	Gal	Galβ3GalNAc, GalNAc	65,66
Jacalin (*Artocarpus integrifolia*)	Gal	Galβ3GalNAc, αGalNAc (6O-unsubstituted)	67,68
Artocarpin (*Artocarpus integrifolia*)	Man	High-Man (Man$_{2-6}$), *N*-glycans devoid of bisecting GlcNAc	68
Calsepa (*Calystegia sepium*)	Man	High-Man (Man$_{2-6}$), *N*-glycans including bisecting GlcNAc	68
BanLec (banana, *Musa acuminata*)	Man	High-Man (Man$_{7-9}$)	68
6. Galectin			
Galectin-1 (*Homo sapiens*)	Gal	Galβ4GlcNAc (LacNAc), Branched *N*-glycans	31,32,69
Galectin-2 (*Homo sapiens*)	Gal	Galβ4GlcNAc (LacNAc), Branched *N*-glycans	32,69
Galectin-3 (*Homo sapiens*)	Gal	LacNAc$_n$ (polylactosamine), A-tetra, A-hexa	31,32,69
Galectin-4 (*Homo sapiens*)	Gal	(Galβ3)$_n$ B-tetra/N-CRD, A-hexa/C-CRD	31,32
Galectin-7 (*Homo sapiens*)	Gal	Galβ3GlcNAc (type 1 LacNAc)	31,32
Galectin-8 (*Homo sapiens*)	Gal	Siaα3Galβ4Glc (GM3)/N-CRD, A-hexa/C-CRD	31,32
Galectin-9 (*Homo sapiens*)	Gal	Forssman penta, A-hexa, LacNAc$_n$/N-CRD, LacNAc$_n$/C-CRD	31,32
GCA (sponge, *Geodia cydonium*)	Gal	Forssman penta, A-hexa	31,70
ACG (mushroom, *Agrocybe cylindracea*)	Gal	Siaα3Galβ4GlcNAc	31,71
7. Fucose lectin (six-bladed β-propeller)			
AOL (fungus, *Aspergillus oryzae*)	Fuc	Fucα6GlcNAc (core Fuc), Fuca2Galβ4GlcNAc (H-type 2)	72

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Table 2 ***(continued)***

Nomenclature (origin) another name	Monosaccharide specificity	Preferred glycan structure (terminal epitope)	Ref. for detailed specificity
AAL (fungus, *Aleuria aurantia*)	Fuc	Fucα6GlcNAc (core Fuc), Fucα3(Galβ4)GlcNAc (Le^x)	72
RSL (bacterium, *Ralstonia solanacearum*)	Fuc	Fucα2Gal, Fucα6GlcNAc	73
8. Discoidin			
Discoidin 1 (slime mold, *Dictyostelium discoideum*)	Gal/GalNAc	Gal/GalNAc3Gal/GalNAc	74
Discoidin 2 (*Dictyostelium discoideum*)	Gal/GalNAc	Gal/GalNAc4GlcNAc6Gal/GalNAc	74
HPA (snail, *Helix pomatia*)	GalNAc	αGalNAc	75
9. Others			
RSL (fungus, *Rhizopus stolonifer*)	Fuc	Fucα6GlcNAc (core Fuc)	76
PhoSL (fungus, *Pholiota squarrosa*)	Fuc	Fucα6GlcNAc (core Fuc)	77
TJA-II (*Tanthes japonica*)	Gal	Fucα2Galβ1, GalNAcβ1	78
ABA (fungus, *Agaricus bisporus*)	Gal, GlcNAc	Galβ3GalNAc, GlcNAc	79

[a] Appropriate publications are not necessarily available as regards those reporting dissociation constants (K_ds) toward a common set of oligosaccharides for systematic comparison, whereas comprehensive data are published through internet in LfDB (Lectin frontier Database; http://riodb.ibase.aist.go.jp/rcmg/glycodb/LectinSearch) obtained by quantitative frontal affinity chromatography (FAC) and CFG (Consortium for Functional Glycomics; http://www.functionalglycomics.org/glycomics/publicdata/primaryscreen.jsp) obtained by the high-throughput glycan microarray.

Table 3 Comparison of properties of lectins and anti-carbohydrate antibodies

Properties	Lectin	Anti-carbohydrate antibody
Biological distribution	Ubiquitous	Vertebrate
Architecture	Relatively simple, but sometimes linked to other domain(s)	Complex: $(H-L)_2$ (IgG) $(H-L)_5$ (IgM)
Molecular size	Variable but relatively small: 10–100 kDa	~150 kDa (IgG) ~900 kDa (IgM)
Scaffold (protein family)	Variable (>40)	Unique
Specificity range	Wide	Restricted to antigenic carbohydrate antigenicity
Selectivity	Relatively wide	Supposed to be narrow, but not rigorously examined
Discrimination of difference:		
Monosaccharides	Possible	Probably possible (no sufficient data)
Anomers (α/β)	Possible	Probably possible (no sufficient data)
Linkages (1–2/3/4/6)	Possible	Probably possible (no sufficient data)
Position isomers	Depends on the case	Probably possible (no sufficient data)
Density	Depends on the case	Probably possible (no sufficient data)
Affinity	Relatively low, but often enhanced by the cluster effect	Supposed to be high, but not rigorously examined
Production in bacteria	Possible in some lectins, but difficult in other lectins	Difficult

which are significantly different. High reproducibility and sensitivity are necessary to realize this.

6. Principle and practice of the lectin microarray

As described, the precedent technologies do not meet a requisite for current glycomics, which inevitably created a keen need for a new technology that is capable of detecting glycan-lectin interactions in a comprehensive and high-throughput manner. As shown in Fig. 1, a general procedure for practice of the lectin microarray is simple. A panel of lectins (>20), for which specificity has been well documented, are immobilized on an appropriate glass slide. Then, an appropriately fluorescent-labeled probe molecule (*e.g.* Cy3-labelled glycoprotein) is allowed for simultaneous interactions with the lectins. Usually, extensive washing is performed to remove the unbound probe, and the resulting fluorescence intensity on each lectin spot is measured using a confocal-type fluorescence scanner. Most of the lectin microarray systems utilize this type of scanner.[10–12]

On the other hand, the authors' group adopted a unique detection system based on an evanescent-field activated fluorescence detection principle: briefly, excitation light is injected from both sides of the glass slide at a certain angle to achieve total reflection at both interfaces between the slide glass and liquid phase and between the slide glass and air. This excitation geometry is ideal to generate an evanescent field in a liquid phase (*i.e.*, on the lower refractive index side compared to the higher refractive index side made of glass) over an area as large as that of the slide glass. The evanescent wave thus generated is only within a limited space from the surface (so-called 'near optic field'). When Cy3-labelled glycoprotein is used as a probe, the near optic field should be within 120 nm in terms of evanescent field depth (defined as a distance from the surface that the electric field intensity becomes $1/e$, where e is Napier's constant) for an excitation wavelength of 530 nm. Roughly speaking, since the intensity of the evanescent field is exponentially reduced from the surface,[90] it is considered that a

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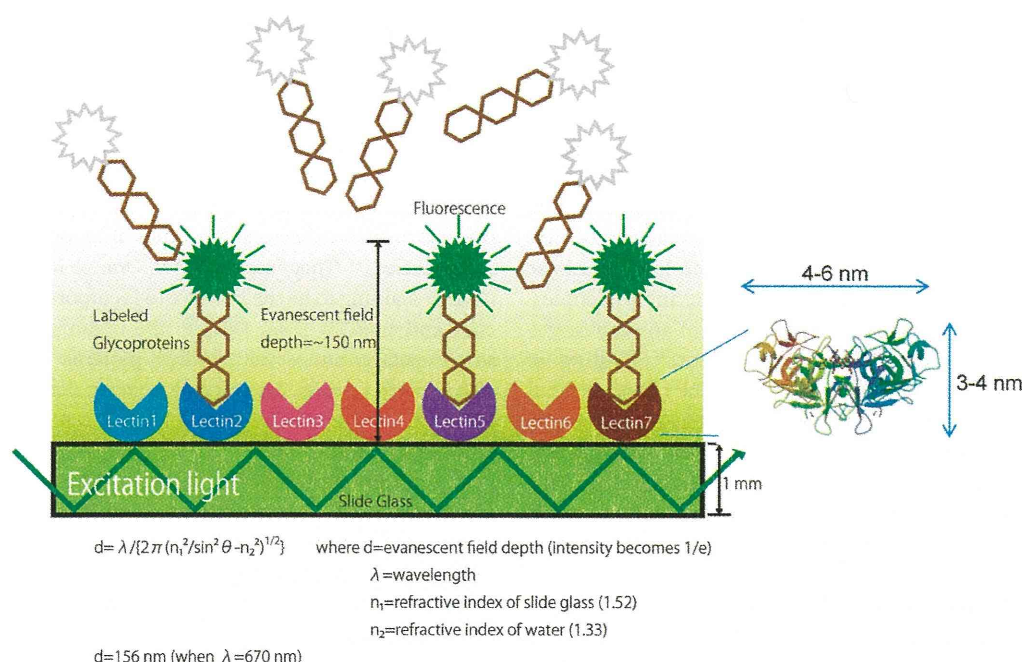

Fig. 3 Features of the lectin microarray platform based on the evanescent-field activated fluorescence detection procedure.

substantially effective near optic field is 200 nm or shorter. The evanescent field fluorescence detection system thus enables liquid phase observation of an interaction under equilibrium conditions, unlike the confocal detection principle, which most of the other microarray techniques could also adopt. Fig. 3 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^{-1} for glycoprotein (asialofetuin) and 100 pM. In short, we believe that the lectin microarray methodology described here is potentially very powerful from the following viewpoints: (i) it is quick and easy to use (*i.e.*, structural profiling can be performed without cleavage of glycans from proteins and directly from the liquid phase without washing), (ii) it is highly sensitive (*i.e.*, 1–100 ng order of glycoproteins), and (iii) it is high-throughput.

7. State-of-the-arts of lectin microarray technology

Although emphasis has been made on the technical merit of the evanescent-field activated fluorescent detection principle, it is also important to note that it is substantially possible to carry out our lectin microarray analysis using a conventional confocal-type scanner. This is particular because actual natural samples containing multivalent glycoconjugates, such as glycoproteins and those expressed on cell surface, are highly clustered in terms of glycan density and presentation efficacy.[91–93] Thus, substantial avidity has been provided with natural glycoproteins, with which interaction in many cases can be observed even with successive washings as has been pointed.[94,95] This is also true when analyzing glycan-binding proteins using the glycan microarray.[96]

Notably, most of the early reports of the lectin microarray concerned basic fabrication procedures,[9–12,97,98] while recent publications are more and more about applications of this technology. They are represented by glycan-related biomarker investigations, stem cell profiling toward regeneration medicine, microbial infections as well as glycoprotein profiling in the light of functional glycomics (described later). Thus, target samples that contain glycans have been greatly expanded from simple, model compounds to more complex glycoconjugates including cell and tissue extracts as well as bacteria and viruses.

In this sense, the current "state-of-the-art" of the lectin microarray technology can be well understood by following the brief history of its application to different types of samples. In the following sub-sections, relevant technologies developed for various orders of hierarchy (*i.e.*, molecules, cells, tissues and body fluids) are described.

7-1. Samples of a homogeneous glycome

(1) **Oligosaccharides.** Oligosaccharides are the simplest form of glycans. Therefore, one may feel curious about the fact that there are only few reports describing the application of the

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lectin microarray to free oligosaccharides.[15,80] This is partly because a substantial merit of this microarray is its direct applicability to glycoproteins without liberation of oligosaccharides and partly because it requires a high-sensitivity detection technique. From a practical viewpoint, it is not easy to prepare a series of purified oligosaccharides, which are labelled with a long-wave-activated fluorescent reagent. Uchiyama *et al.* used tetramethylrhodamine-labelled *N*-linked oligosaccharides belonging to different structural types, *i.e.*, M6 high-mannose type, α2-6-disialobiantennary, and asialobiantennary complex-type glycans, to demonstrate the performance of an evanescent-field activated fluorescence detection system.[80] Binding of these labelled oligosaccharides was found to be stable without a washing procedure, while some of them were lost immediately after single buffer replacement. It is also important to note that when sequential glycosidase digestion was performed for the disialobiantennary oligosaccharide, the resultant shifts in signal patterns were quite clear compared with those obtained for more complex samples, such as cell extracts.

(2) **Purified glycoproteins.** Glycoproteins are major targets for direct analysis by the lectin microarray either in their purified (*e.g.*, glycoprotein drugs) or crude (*e.g.*, cell supernatant and body fluids) forms. The analysis enables profiling of both *N*- and *O*-glycans. Since the glycan profiles are unique to individual glycoproteins and are possibly affected by many other factors (types of cells, pH, temperature, cell density, *etc.*), the method will contribute to rapid evaluation of glycoprotein drugs, such as immunoglobulins.[98]

(3) **Eukaryotic cells.** As mentioned previously, glycan structures differ significantly between different types of cells (species and states). Accordingly, the lectin microarray technique should work efficiently for differential profiling between different types of cells. The first demonstration was made by Ebe *et al.*[99] using CHO and its LEC mutant strains, and the work in this context was further extended in a more systematic manner by Tao *et al.*[100] These studies used detergent-solubilised cell membrane fractions as a glycoprotein source. However, significant care is necessary for the preparation of such glycoprotein fractions, which may contain interfering materials, such as non-glycosylated cytoplasmic proteins and immature glycoproteins included in the endoplasmic reticulum and Golgi apparatus.

Mahal's group developed a unique procedure for differential profiling named the ratiometric two-colour lectin microarray method,[101,102] which rapidly evaluates differences in the glycosylation of mammalian cells in analogy to two-dimensional differential gel electrophoresis (2-D-DIGE) using a Cy3/Cy5 dual colour system.[103] Tateno *et al.* reported a convenient method to profile a cell-surface glycome using a commercial CellTracker dye.[104] This series of reagents passes freely through the cell membrane, but once inside the cell, they are transformed into cell-impermeable forms (Fig. 2). One of the commercial products, OrangeTM CMRA (Invitrogen) can fluoresce only after it is metabolized by endogenous esterase. The procedure is simple and proved to be applicable to an extensive range of eukaryotic cells including fungi.[105]

(4) **Bacterial cells.** Hsu *et al.*[106] successfully differentiated commensal and pathogenic glycosylation patterns of closely related *Escherichia coli* strains. Since the bacterial glycans are largely different from those of eukaryotes and there is no established methodology for their structural analysis, the emergence of a novel technique for this purpose is quite valuable. More recently, Yasuda *et al.* reported an alternative method to analyse bacterial glycomes of 16 strains of *Lactobacillus casei* species.[107] They found CYTOX Orange used for intracellular nuclear staining to be the best dye to incorporate into the bacterial cells and bind to DNA. Despite the organisms being from the same species, almost all binding patterns obtained for these strains were unique. These results clearly demonstrated that the principle of lectin-based glycomics technology actually worked even for bacteria.

(5) **Virus and related particles.** Krishnamoorthy *et al.* reported a quite interesting result of glycan profiling of the HIV virus.[108] Lectin microarray analysis using 68 lectin probes revealed that glycome signatures of HIV and host cell microvesicles were almost identical, which explains well the origin of the virus particles. This observation supports the "exosome" hypothesis that HIV largely relies on the biosynthetic machineries of host cells. For this analysis, the authors utilized a sensitive ratiometric two-color detection method described above.[101,102] When focusing on differential analysis, this approach seems to be useful to enhance subtle differences in the glycome. However, it should be mentioned that the proteomic procedure (2-D-DIGE) and the glycomic procedure (dual colour method) are critically different: the latter includes substantial competition for immobilized lectins between two sets of differentially coloured glycoproteins. Therefore, the obtained dual colour profiles may differ from those combined by two independent results of mono colour experiments.

7-2. Samples of heterogeneous glycome

(1) **Tissue sections.** Matsuda *et al.* developed a skilful technique for differential glycan profiling targeting small areas (*i.e.* 1.5 mm in diameter, 5 μm in thickness) of paraffin-embedded and formalin-fixed tissue sections.[109] Considering that tissue samples to be compared are of the same date and same individual, obtained data will be more reliable than those obtained from sera. The method is innovative from the viewpoint of translational medicine, because it provides many clinicians with the most practical approach to differential glycan analysis. In fact, some researchers successfully applied the technique to the investigation of disease-related glycoprotein markers under the concept of glycoproteomics.[110,111] In the future, combination with a laser micro-dissection technology will also be promising for a more systematic analysis.

(2) **Body fluids (bile, sera) in biomarker investigations.** Body fluids, such as patient sera and urine, are primary targets for biomarker diagnosis. An increasing number of clinical researchers have found the importance of glycoproteomics in discovering really useful diagnostic markers. For this realization, however, one should have in mind that serum is a highly heterogeneous bio-resource consisting of diverse glycoproteins

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originating from multiple organs. Therefore, it is quite difficult to detect a particular glycosylation change of a particular glycoprotein present in patient sera, because the proportion of such a specific target is extremely minor. Fortunately, lectin microarray techniques can be adequately utilized in two steps of the discovery phase. One is the tissue-section targeted analysis,[109] and the other is an antibody-overlay method.[16]

8. Applications of the lectin microarray to disease-related glycoprotein marker development

Since the first report of the lectin microarray in 2005, the technology has now been introduced in various laboratories. As far as the commercialized evanescent-field fluorescence scanner (http://www.gpbio.jp/) is concerned, the number of publications using this system is >10 excluding those by the authors of ref. 112–122. Apparently, the technology has been most intensively applied in medical fields for development of disease-related biomarkers, where the key concept "glycoproteomics" is essential[110,111]: *i.e.*, drastic glycosylation change should occur along with various biological phenomena, which include development, differentiation (undifferentiation), tumorigenesis, metastasis (Fig. 4). In the following subsections, two examples of the development of glycoprotein markers are described: one is liver fibrosis and the other is cholangiocarcinoma (CC).

8-1. The case of liver fibrosis

On the basis of the glycoproteomics concept, extensive glycosylation changes should occur on protein molecules during tumorigenesis. If this is the case, selection of the appropriate glycoproteins is most important for sensitive and precise detection of such changes. As regards liver fibrosis, people infected with hepatitis B virus (HBV) or hepatitis C virus (HCV) have an increased risk of developing chronic hepatitis, cirrhosis, and finally hepatocellular carcinoma (HCC). By a recent estimation, as many as 350 million and 170 million people are chronically infected with HBV and HCV, respectively. Even though HCV infection itself is asymptomatic for a long period (*ca.* 10 years) before chronic hepatitis develops, it gradually causes irreversible fibrosis. Staging of fibrosis (F0–F4) is significantly correlated with carcinogenesis; while only 0.5% of patients with the F0/F1 stage develop HCC, as high as 7.9% of the F4 stage patients in Japan develop HCC each year.[123] Therefore, from a clinical viewpoint, it is very important to accurately monitor the progression of fibrosis to identify F4 stage patients as a "high-risk" group of developing HCC.

Recently, Kuno *et al.* developed a compatible multiple lectin–antibody sandwich immunoassay on the basis of the results obtained by the lectin microarray analysis for monitoring fibrosis.[124] They targeted α1-acid glycoprotein (AGP), a well-known acute-phase protein secreted from the liver, glycosylation change of which has been reported to be closely associated with liver cirrhosis.[125]

AGP-enriched fractions derived from 0.5 μl sera of 125 patients with staging-determined fibrosis (26.4% F0–F1, 25.6% F2, 24% F3, and 23.2% F4) were subjected to systematic analysis by the antibody-overlay lectin microarray described above (Fig. 1). As a result, signal spots for 15 lectins were observed and signal patterns of the 12 selected lectins reflected fibrosis-associated glycosylation change alteration of AGP. After normalization of each binding signal by a max-normalization procedure,[126] we found that half of them (DSA, SNA, SSA, TJA-I, WGA, and RCA120) served as internal standards (*i.e.*, as a "pattern formatter"), whereas the remaining half (AOL, AAL, PHA-E4, LEL, STL, and MAL) served as "fibrosis indicators". Kuno *et al.* found a specific lectin at each stage of fibrosis (*i.e.*, significant fibrosis, severe fibrosis, and cirrhosis) with a statistically significant score ($P < 0.0001$). Especially, the combinational use of 3 lectins, *i.e.*, AOL (positive fibrosis indicator), MAL (negative fibrosis indicator) and DSA (internal standard), was found to show the best scores for diagnosis of liver cirrhosis. Then, they conducted a blind test using sera from 45 patients with cirrhosis and from 43 patients with chronic hepatitis. As a result, the combinational use of the 3 lectin signals allowed identification of F4 patients suffering from liver cirrhosis with 95% diagnostic sensitivity, 91% diagnostic specificity, and 93% accuracy, which were greatly

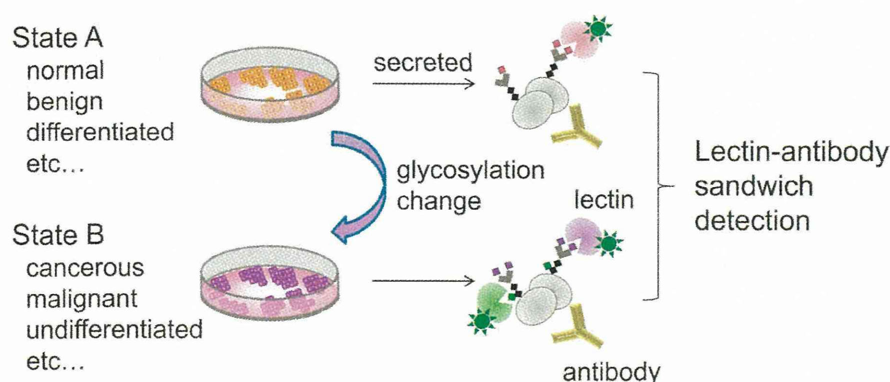

Fig. 4 A basic concept of glycoproteomics. In different states of cells (*i.e.* states A and B), glycosylation patterns substantially change, though protein moieties are largely the same. To develop useful glycoprotein markers in various biological scenarios, *e.g.*, tumorigenesis and cell differentiation, it is necessary to detect precisely glycosylation change of a fixed glycoprotein. For this purpose, a lectin-antibody sandwich detection system is desired for clinical use.

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improved compared with a previous report.[127] It is important to note that the triplex-sandwich immunoassay employing DSA/MAL/AOL can be simplified to fit for the existing bedside clinical chemistry analyzers. In fact, Kuno *et al.* recently successfully reconstructed the detection system from the lectin microarray to a fully automated immunoassay analyzer (Sysmex HISCL-2000i) to achieve rapid measurement as short as 20 min,[128] and further validated the results.[129,130] Thus, the developed method of multiple lectin–antibody sandwich immunoassay targeting AGP should be a good example for rapid and sensitive monitoring of disease progression in chronic hepatitis patients at risk of developing HCC.

8-2. The case of cholangiocarcinoma (CC)

CC is a representative malignant cancer, for which useful markers for early diagnosis are not presently available. Although it contributes to only 15% of the total incidence of primary liver cancer, according to recent epidemiological reports the CC incidence has increased significantly in the past decades. Lack of a reliable biomarker results in CC being in most cases fatal by the time it is diagnosed. Prognosis of this cancer is also poor, with a 5-year survival rate of less than 5%. In general, CC can be cured only if a surgical resection is performed at a relatively early stage. Development of a good diagnostic marker is therefore necessary. A special focus on glycosylation changes of appropriate glycoproteins appears likely to provide a solution.

Matsuda *et al.* found that WFA was the best probe lectin to differentiate intrahepatic cholangiocarcinoma (ICC) lesions from normal bile duct epithelia ($P < 0.0001$) using an advanced technique of lectin microarray targeting paraffin-embedded formaldehyde-fixed tissue sections.[109] The subsequent histochemical study confirmed ICC-specific WFA staining on 165 tissue specimens. Moreover, the WFA staining was closely associated with that of a monoclonal antibody, MY.1E12, which was previously established against sialylated mucin 1 (MUC1). Alteration of glycosylation in MUC1 was also verified by western blot analysis of WFA-captured bile samples from CC patients.[131] For more convenient CC diagnosis, the authors constructed an enzyme-linked immunosorbent assay system, in which WFA-coated ELISA plates were treated with the bile specimens from CC, including ICC ($n = 30$) and benign diseases ($n = 38$), and were overlaid with the MY.1E12 antibody for specific detection. As a result, CC was clearly distinguished from the benign diseases with good statistical scores (sensitivity = 90.0%, specificity = 76.3%, and area under the ROC (Receiver Operating Characteristic) curve = 0.85). In particular, the sensitivity is the highest reported so far. In conclusion, the approach of focusing on a significant glycosylation change of a particular glycoprotein (WFA-MUC1) yielded a novel diagnostic system for CC with satisfactory clinical scores.

9. Application of the lectin microarray to evaluation of stem cells and investigation of pluripotent cell markers

Another example of the successful application of the lectin microarray is glycome diagnosis of a series of stem cells (Fig. 5). As described, cell surface glycans are considered to be ideal targets for analyzing or diagnosing the state of each cell: first, glycans are located at the outermost cell surface. Second, the total repertoire of such cell surface glycans is actually known to vary at various levels of biological organization. Third, a global change in the cellular glycome also occurs during development. Therefore, cell surface glycans are regarded as the "cell signature" closely reflecting cellular backgrounds and conditions. Induced pluripotent stem cells (iPSCs) can now be produced from various somatic cell lines by ectopic expression of the four transcription factors Sox2, Oct4, Klf4 and c-Myc. Although this procedure has been fully demonstrated to induce global changes in gene and microRNA expressions, it remains largely unknown how this reprogramming procedure affects the total glycan repertoire expressed on the cells.

A few precedent studies including ours have shown substantial changes in glycan structures upon undifferentiation.[118,132,133] Subsequently, Tateno *et al.* performed a more comprehensive analysis using 114 types of human iPSCs generated from five different somatic cells (SCs) and compared their glycome profiles with those of human embryonic stem cells (ESCs) using a high-density lectin microarray.[134] In unsupervised cluster analysis of the results obtained using the lectin microarray, both undifferentiated iPSCs and ESCs were clustered as one large group, while they were clearly separated from the group of differentiated SCs, whereas all of the four SCs had apparently distinct glycome profiles from one another. This result demonstrates that SCs with originally distinct glycan profiles have acquired those similar to ESCs upon induction of pluripotency. Thirty-eight lectins discriminating between SCs and iPSCs/ESCs were statistically selected, and characteristic features of the pluripotent state were speculated. That is, the expression of α2-6sialylation, α1-2fucosylation, and type1 LacNAc is increased, whereas that of α2-3sialylation and tetra-antennary *N*-glycans is decreased upon induction of pluripotency. These glycan alterations were also confirmed by the expression profiles of relevant glycosyltransferase genes (Fig. 5). Among the 38 lectins, rBC2LCN was found to detect only undifferentiated iPSCs/ESCs but never differentiated SCs. More recently, as a responsible glycan, H type3 *O*-glycan was identified from iPSCs by conventional LC combined with glycosidase and MS analyses.[135] Interestingly, the depicted structure is closely related to a recently reported novel undifferentiation marker named SSEA-5[136] and also type 1 *N*-acetyllactosamine structure identified as a glycan epitope to Tra1-60.[137] Hence, the high-density lectin microarray has proved to be valid for not only comprehensive analysis of glycans, but also for diagnosis of stem cells with investigation of a novel lectin probe.

10. Future engineering of lectins

The lectin microarray has been developed in recent years essentially by utilizing naturally-available lectins, most of which are derived from plants and are commercialized. However, it is also true that these natural lectins cannot provide a fully comprehensive repertoire to profile glycome complexity. Moreover, natural lectins are not further modified with molecular biological technology.

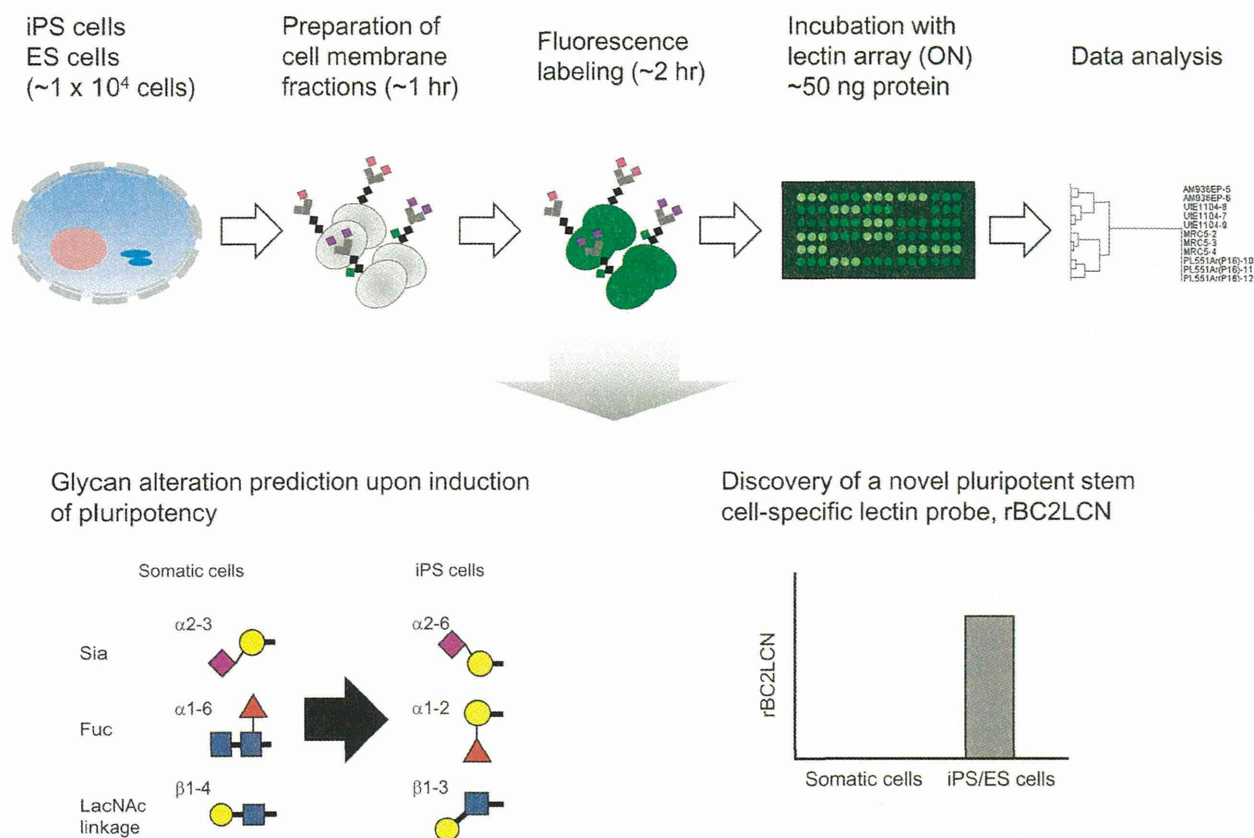

Fig. 5 Schematic representation of glycan profiling of iPSCs and ESCs using the lectin microarray.

In this context, oriented immobilization using recombinant lectins has been attempted by Mahal's group for more efficient glycan binding.[138,139] Tateno *et al.* also included several dozens of recombinant lectins to develop a high-density lectin microarray for stem cell profiling.[134] Once the system of recombinant lectins is introduced, a novel possibility is pioneered for future research. In this context, Yabe *et al.* attempted to tailor a novel specificity using as a scaffold EW29Ch (earthworm 29 kDa lectin, C-terminal half domain), a ricin-B chain-like galactose-binding protein,[53] and succeeded in creating an α2-6Sia specificity, which the parental EW29Ch lacks.[54] More recently, Hu *et al.* successfully engineered a novel 6′sulfo-Gal-specific lectin by using the same EW29Ch template.[140] Notably, however, individual lectin scaffolds have some structural constraints in relation to potential carbohydrate-binding specificity. For example, the above protein structures of the R-type lectins, which are originally Gal-specific, have room for the accommodation of 6-OH modified Gal, while they never afford 3-OH modification. Interestingly the case is opposite for galectins, which require an unsubstituted 6-OH of Gal, while modification of 3-OH is in most cases permitted, and in some cases greatly enhances the affinity.[31,32] For future lectin engineering, it is essential to understand the potential capabilities of individual lectins. In this context, a basic question arises: which protein scaffolds are entitled to be lectins, and *vice versa*? The search for novel glycan-binding affinity has been enhanced by the development of a powerful tool, the glycan microarray. It makes quite possible the targeting of both natural and engineered lectin proteins as well as other bio-molecules, such as aptamers[141] and boronate-containing synthetic molecules.[142]

11. Conclusions

The lectin microarray is an emerging technology, and as such offers many opportunities for further innovations to take place, and for its range of applications to the life sciences to be greatly expanded. It should also make a significant contribution to the wider acceptance of the basic glycome concept; that all kinds of cells constituting all living organisms, after their origin on earth some 3.5 billion years ago, are covered with a dense layer of complex glycans, the structures of which are closely associated with biological phenomena as a necessary result of biological evolution. The technique is likely to be highly beneficial for life scientists in the near future, while lectin engineering has just begun to help us understand the fundamentals of lectin- and higher-order recognition of glycans, in particular those expressed on the cell surface.

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