**Table 1** Representative research papers reporting applications of the lectin microarray

| Application fields and paper titles (year) | Reference |
|---|---|
| **1. Glycan synthesis and glycoprotein production** | |
| *Pichia pastoris*-produced mucin-type fusion proteins with multivalent *O*-glycan substitution as targeting molecules for mannose-specific receptors of the immune system (2011) | Gustafsson et al. [50] |
| Chemoenzymatic synthesis and lectin array characterization of a class of *N*-glycan clusters (2009) | Huang et al. [51] |
| Engineering of mucin-type human glycoproteins in yeast cells (2008) | Amano et al. [52] |
| A lectin array-based methodology for the analysis of protein glycosylation (2007) | Rosenfeld et al. [12] |
| **2. Glycoprotein profiling relevant to functional glycomics** | |
| Survey of glycoantigens in cells from α-1,3-galactosyltransferase knockout pig using a lectin microarray (2010) | Miyagawa et al. [53] |
| Transient expression of an IL-23R extracellular domain Fc fusion protein in CHO vs HEK cells results in improved plasma exposure (2010) | Suen et al. [54] |
| Glycomic analyses of glycoproteins in bile and serum during rat hepatocarcinogenesis (2010) | Nakagawa et al. [55] |
| Testicular angiotensin-converting enzyme with different glycan modification: characterization on glycosylphosphatidylinositol-anchored protein releasing and dipeptidase activities (2009) | Kondoh et al. [56] |
| Polylactosamine on glycoproteins influences basal levels of lymphocyte and macrophage activation (2007) | Togayachi et al. [57] |
| Functional glycosylation of human podoplanin: glycan structure of platelet aggregation-inducing factor (2007) | Kaneko et al. [58] |
| **3. Development of disease-related glycoprotein markers** | |
| A unique *N*-glycan on human transferrin in CSF: a possible biomarker for iNPH (2011) | Futakawa et al. [59] |
| Multilectin assay for detecting fibrosis-specific glyco-alteration by means of the lectin microarray (2011) | Kuno et al. [16] |
| Lectin microarray profiling of metastatic breast cancers (2011) | Fry et al. [60] |
| Identification of various types of α2-HS glycoprotein in sera of patients with pancreatic cancer: possible implication in resistance to protease treatment (2010) | Kuwamoto et al. [61] |
| *Wisteria floribunda* agglutinin-positive mucin 1 is a sensitive biliary marker for human cholangiocarcinoma (2010) | Matsuda et al. [48] |
| High levels of E4-PHA-reactive oligosaccharides: potential as marker for cells with characteristics of hepatic progenitor cells (2009) | Sasaki et al. [62] |
| **4. Glycan profiling of stem cells relevant to regenerative medicine** | |
| Possible linkages between the inner and outer cellular states of human induced pluripotent stem cells (2011) | Saito et al. [47] |
| Glycome diagnosis of human induced pluripotent stem cells using the lectin microarray (2011) | Tateno et al. [46] |
| Lectin microarray analysis of pluripotent and multipotent stem cells (2011) | Toyoda et al. [45] |
| **5. Glycan profiling relevant to pathogen infection** | |
| HIV-1 and microvesicles from T cells share a common glycome, arguing for a common origin (2009) | Krishnamoorthy et al. [63] |
| Analyzing the dynamic bacterial glycome with a lectin microarray approach (2006) | Hsu et al. [64] |

3. *Eukaryotic cells*

As mentioned previously, glycan structures differ significantly between different types of cells (species and states). According to this concept, i.e., "cellular glycomics," the lectin microarray should work ideally for differentiation of different types of cells. The first demonstration was made by Ebe et al. [65] using CHO and its LEC mutant strains, and work in this context was further extended in a more systematic manner by Tao et al. [13]. These studies used detergent-solubilized 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.

Tateno et al. reported a convenient method to profile a cell-surface glycome using a commercial CellTracker dye (Invitrogen) [66]. This series of reagents passes freely through the cell membrane, but once inside the cell, they are transformed into cell-impermeant forms. One of the commercial products, Orange™ CMRA, can fluoresce only after it is metabolized by endogenous esterase. The procedure is simple and applicable to an extensive range of eukaryotic cells [44].

4. *Bacterial cells*

Hsu et al. [64] successfully profiled glycosylation patterns of closely related *Escherichia coli* strains, including pathogenic ones. Those authors differentiated commensal and pathogenic strains in a manner of facile fingerprinting. More recently, Yasuda et al. reported an alternative method to analyze bacterial glycomes with emphasis on differential profiling of 16 strains of *Lactobacilli casei* species [67]. They found CYTOX Orange, usually used for intracellular nuclear staining, to be the best dye to be incorporated into the bacterial cells and bound to DNA. Despite the organisms being from the same species, almost all binding patterns obtained for these 16 strains were unique. It should also be noted that the current systems of the lectin microarray, largely composed of plant lectins, can work for bacterial glycome profiling.

5. *Virus and related particles*

Krishnamoorthy et al. reported an interesting analysis of glycan profiles of the HIV virus which focused on comparison with virus-resembling microvesicles, which are secreted from host cells [63]. Lectin microarray analysis using 68 lectin probes revealed that glycome signatures of HIV and host cell microvesicles were almost identical. This observation provides important support for the "exosome" hypothesis that HIV largely relies on the biosynthetic machineries of host cells, and thus they should give totally the same glycan profiles. For this analysis, the authors utilized a sensitive ratiometric two-color detection method (described later).

### 4.2 Samples of Nearly Homogeneous Glycome

1. *Tissue sections*

   Matsuda et al. developed a skilful technique for differential glycan profiling targeting small areas (i.e. 1.5 mm diameter, 5 μm thickness) of paraffin-embedded and formalin-fixed tissue sections [48]. The method provides clinicians with a very useful approach to differential glycan analysis. Considering that tissue samples to be compared are of the same date and same individual, the data obtained are more reliable than those obtained from sera. In fact, the authors applied the technique to an investigation of glycoprotein markers for diagnosis of cholangiocarcinoma, a representative malignant tumor, for which no useful markers are presently available. As a result, they found that *Wisteria floribunda* agglutinin specific for GalNAc was the best probe to differentiate cholangiocarcinoma lesions from normal bile duct epithelia ($p < 0.0001$) [49]. Though not yet performed, combination with laser micro-dissection followed by high-throughput differential glycan profiling should become a powerful means for future biomarker discovery.

### 4.3 Samples of Highly Heterogeneous or Complex Glycomes

1. *Body fluids (bile, sera) in biomarker investigations*

   Body fluids, such as patient sera and urine, are primary targets for biomarker diagnosis. Under the concept of glycoproteomics, an increasing number of researchers of both glycomics and proteomics have been involved in the discovery phase of glycoprotein markers. For this realization, however, one should consider the fact that serum is a highly heterogeneous mixture of glycoproteins originating from many different organs. Therefore, their individual cellular glycomes are also heterogeneous. A well-organized strategy is described, which relies on the high-sensitivity technique of the lectin microarray at two different phases [6, 7]. One is the tissue-section targeted analysis described above, and the other an antibody-overlay method described below.

## 5 Modified Technologies of the Lectin Microarray

1. *Antibody-overlay method in biomarker qualification/verification*

   Kuno et al. reported a highly practical approach to the differential glycan profiling of an antibody-targeted glycoprotein in the course of biomarker development [28]. As the discovery phase of biomarker development proceeds, several biomarker candidates (i.e., glycoproteins) are nominated, whereas it is not yet certain that they really work as robust diagnostic markers, e.g., for hepatocellular carcinoma. For the purpose of pre-validation (i.e., qualification

or verification), it is important to carry out the analysis on several dozen clinical samples (e.g., usually body fluids such as sera). Therefore, establishment of a high-throughput procedure such as the lectin microarray is particularly important in glycoprotein biomarker development. The developed method makes maximum use of an antibody, which is raised against the protein moiety of the target glycoprotein: the antibody is used for enrichment (immunoprecipitation), semi-quantification (Western blotting), and overlay to the lectin microarray (*lower* scheme in Fig. 1). A target glycoprotein included in each clinical sample need not be fluorescence-labeled, and thus high-throughput (>100 samples) analysis is easily performed. Therefore, the lectin microarray is used in two steps in the strategy developed for glycoprotein biomarker investigation. The approach was applied to the investigation of an hepatic fibrosis marker, α-1-acid glycoprotein probed with *Maackia amurensis* lectin (MAL) and *Aspergillus oryzae* lectin (AOL) [16].

2. *Dual color measurement*

   Pilobello et al. developed a method for the rapid evaluation of glycosylation changes of heterogeneous mammalian samples using a ratiometric two-color lectin microarray approach [68]. This approach is reminiscent of a proteomic procedure called two-dimensional differential in-gel electrophoresis (2-D DIGE), which uses two differentially labeled (i.e., Cy3/Cy5) protein samples [69]. When focusing on differential analysis, this approach can be extremely useful to enhance substantial differences in the glycome. However, it should be mentioned that these two methods, i.e., proteomic 2-D DIGE and the glycomic dual color method, are essentially different in that the latter procedure includes "competition" between immobilized lectins toward a set of various glycans. In other words, quantitative comparison of lectin signals needs careful consideration.

## 6 Perspective

The technology of the lectin microarray described here is expanding its application fields rapidly to broad areas of life sciences, which include both basic and applied sciences. Because the method is still new, relatively few researchers appreciate its innovative features, which previous technologies have lacked, i.e., discrimination based on biological affinity. However, considering that every biological phenomenon comprises cellular communications, it is quite natural to assume that carbohydrate-protein (e.g., lectins) interactions are fundamental for carcinogenesis, embryogenesis, morphogenesis, pathogenesis, etc. Thus it should not be surprising that the lectin microarray platform works very well to analyze, differentiate, and elucidate these complex cellular processes under the basic concept of cellular glycomics/glycoproteomics. For further development of the system, however, production of a series of recombinant lectins is necessary, considering the history of restriction enzymes [18]. In this context, several groups have already shifted to

using recombinant lectins for improved sensitivity [70] and resolution [46]. If a recombinant lectin is established with a good bacterial expression system, its propagation to produce new lectins with a wider glycome coverage and a better cost effectiveness is feasible. An alternative approach is to synthesize "artificial" lectins equipped with boronic acid functionalized peptidyl ligands to carbohydrates [71]. In this regard, the lectin microarray enters its really creative phase, and is poised for further development.

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