Development of lectin microarray, an advanced system for glycan profiling

— From frontal affinity chromatography to evanescent wave excitation fluorescence detection method —

Jun HIRABAYASHI

[Translation from *Synthesiology*, Vol.7, No.2, p.105-117 (2014)]

Glycans are called the third class of biopolymers, after nucleic acids (first class) and proteins (second class). Elucidation of glycan functions has long been hampered by the difficulty in analyzing their structure. However, recent progress in proteomics technology has also accelerated progress in glycomics, which is the systematic study of glycans. As a result, glycan profiling has increasingly attracted attention as a method that enables rapid analysis of complex features of glycans. Lectin microarray provides a novel platform with a simplified experimental procedure, because it does not require glycan liberation and separation prior to the analysis. It is now being applied to tumor marker investigation, stem cell qualification, and biologics development. The author reviews the last 10 years of lectin microarray development, a period that began as a national project in which he has been actively involved.

Keywords : Glycan profiling, frontal affinity chromatography, lectin microarray, evanescent wave, GLIT (Glyco-innovation and Industrial Technology)

1 Introduction

1) Why glycomics is difficult

Glycans or sugar chains are biological information molecules with complex structures and varied forms of existence. Unlike nucleic acids and proteins, they have diverse bonding forms and branch structures, and therefore the structures cannot be predicted from genetic information. These make both the analysis and understanding of glycans very difficult. One of the structural characteristics of glycans is the existence of multiple isomers. Since these isomers usually have similar properties, it is generally very difficult to completely isolate them from each other. On the other hand, glycans are present in milk or urine in free form, but most are covalently bound to proteins or lipids, and are present in the cells or are secreted in the bodily fluids such as blood. In glycoproteins, it is known that profuse amount of glycosylation occurs in the biosynthetic process. Figure 1 shows a model of a typical glycoprotein. The glycan structure has basic common properties such as the monosaccharide composition, but this complicates the preparation of antibodies for glycans, and makes the tissue distribution determination and isomer detection difficult. On the other hand, glycan structures often differ significantly by organisms. Recently, the mainstream method is to use animal cells to produce hormones and antibodies needed for biopharmaceuticals, and many of the biopharmaceuticals are actually glycoproteins. Therefore, even if the amino acid sequence of a protein is the same as humans, the glycan structure may be heterologous depending on the production host. The technology to attain complete human glycan structures has not been perfected, and the dangers of allergies and acute rejections have been indicated in the biopharmaceuticals prepared from animal cells such as CHO (Chinese hamster ovary) cells.

Although it is extremely difficult to analyze glycans, fortunately, glycan research has been active in Japan, and many analysis technologies and glycosidase essential for glycomic research were created in Japan.[Note 1)] On the other hand, at the beginning of the 21st century, it was thought that structural glycomics would be done well by "mass spectrometry." While mass spectrometry is highly effective for detailed structural analysis, the disadvantage is that it is very difficult to handle biological samples with this technique. On this point, the glycan profiling technology, which is the subject of this paper, has the possibility of overcoming some of the disadvantages of mass spectrometry. Described in a few words, glycan profiling is to obtain structural characteristics of glycans quickly and easily, and although precise structural identification may not be possible, major characteristics (type of glycan, presence of epitope,[Note 2)] degree of branching, degree of glycosylation, etc.), as well as differences or similarities between the compared samples can be shown.

2) Looking back at year 2002 (year before the launch of the Structural Glycomics Project)

To introduce myself, the author joined the National Institute of Advanced Industrial Science and Technology (AIST) in

Research Center for Stem Cell Engineering, AIST Tsukuba Central 2, 1-1-1 Umezono, Tsukuba 305-8568, Japan
E-mail: jun-hirabayashi@aist.go.jp

Original manuscript received August 2, 2013, Revisions received October 14, 2013, Accepted October 15, 2013

November 2002, as a member participating in the Project for the Development of Glycomics Technology of the New Energy and Industrial Technology Development Organization (NEDO) that started in 2003. Invited by Hisashi Narimatsu, Deputy Director (at the time) of the Research Center for Glycoscience and the person who would become the Project Leader, I resigned from a private university where I taught for 21 years. This was the start of the Structural Glycomics (SG) Project. Narimatsu had moved from a university to AIST a few years back to lead the "Project for the Development of Glyco-gene (GG) Library" of the Ministry of Economy, Trade and Industry (FY 2001~2003) and contributed to the development of the glycogene library in Japan.[2] After this success, the development of the analysis technology for glycan structures was considered a priority issue. The matrix-assisted laser desorption/ionization (MALDI) method created by Koichi Tanaka, particularly the AXIMA-QIT-MALDI device, was expected to be the most useful method for analyzing complex glycan structures.

The actual SG Project progressed in two part development of structural analysis and synthesis technology. The details of the framework of structural analysis technology development and the research subjects in which the author was directly involved are shown in Table 1.

3) Objective and demand

Since the glycans exist as mixtures of heterogeneous structures within the protein, it is almost impossible to accurately determine their chemical structures and existing amounts. However, if the minute differences in structure

Table 1. SG Project (supplementary budget in FY 2002 ~ FY 2005): Topics for the development of glycoproteomics technology and the organizations involved

1. Development of the glycoproteomics technology
1-1. Glycoproteomics (AIST, Tokyo Metropolitan U, Kinki U, Shimadzu Corp, GL Sciences, etc.)
- Proteome strategy (identification of large glycoproteins by proteomics method)
- Glycome strategy (development of methodology to obtain both protein identification and glycomic information)
- Glycoform analysis (analysis of methods to excise glycans from glycoproteins and to analyze glycans)

1-2. Glycomics using mass spectrometry (AIST, Shimadzu Corp, Cyber Laser, Mitsui Knowledge, Tokyo U of Science, etc.)
- Glycomics by MALDI-QIT-TOF mass spectrometry, exploration of high-throughput soft ionization method suitable for glycopeptides, and exploration of fragmentation method suitable for structural analysis of glycopeptides

1-3. Glycan profiling technology (AIST, Shimadzu Corp, J-Oil Mills, U of Tokyo, etc.)
- FAC: Comprehensive interaction analysis between lectins and standard glycans
- Development of glycan profiler: Development of lectin microarrays based on evanescent wave excitation method

2. Construction of glycomic identification database (AIST, Mitsui Knowledge, Fujitsu)
- Construction of glycoprotein database
- Construction of oligosaccharide database
- Analysis of lectin profiles
- Development of glycomic software by mass spectrometry
- Development of glycan fragmentation prediction software based on molecular calculation

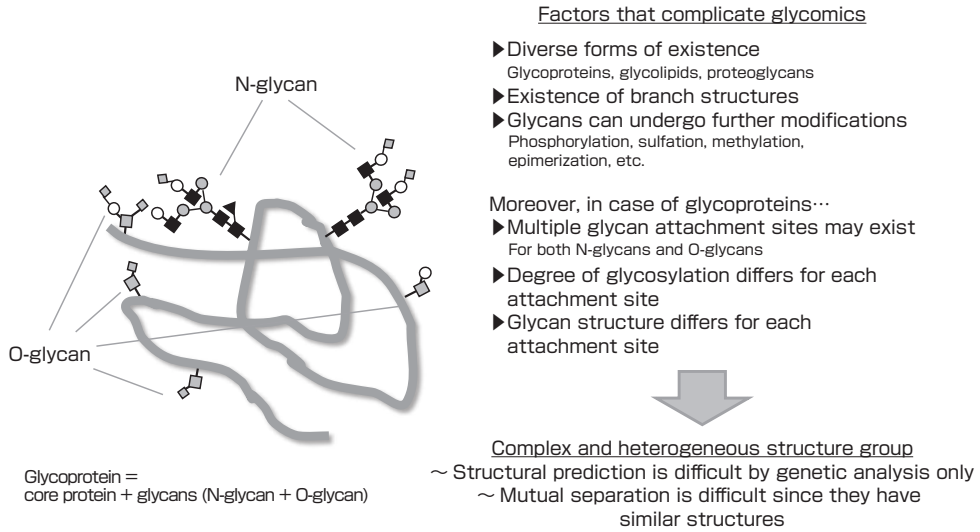

Fig. 1 Schematic diagram of a glycoprotein and factors that complicate glycomics

The peptide chain that forms the core protein is shown as the thick stringy line. Glycosylation of most of the secreted and membrane-bound proteins occurs during or after the translation process. There may be one or multiple glycan attachment sites. The glycan types are roughly divided into the N-glycans that attach to the asparagine residue (-NH_2) side chain and the O-glycans that attach to the serine-threonine residue (-OH) side chain. The rate of glycosylation at every attachment site is not necessarily 100 %, and normally 10 or more structural variations may coexist. When these factors are combined, the structural diversity of glycoproteins is enormous. The glycan structures differ as the types of cells differ. The various glycan biomarkers utilize this characteristic. This is the reason why glycans are called the "cell signatures"

and proportion of glycans can be detected quickly and easily, the extracted information will be very useful in detailed structural analyses and search for biomarkers later. In cases such as the comparison of cell-surface glycans of microorganisms for which no analysis has ever been done,[3][4] glycan profiling may provide sufficiently meaningful results. Yet, this technology focuses on its use in the introductory part of the analysis strategy in biomarker development. The main difference from the analysis methods such as mass spectrometry or LC mapping is that glycans are fluorescent labeled and analyzed in their original form, without excising them from the protein. The LC mapping method[5] is a reference identification method[Note 3)] for glycan structures developed in Japan. In general, glycans are excised by glycosidase or hydrazine, and the reducing terminals of the glycans that were bonded to the protein are labeled by a fluorescent agent to facilitate mutual separation and detection.[6] However, it is not easy to separate and identify glycans of a glycoprotein all at once, since there may be several tens to several hundred types. Therefore, in most cases, the structural analysis alone becomes the research objective and the real objective beyond this cannot be reached. If the necessary information can be obtained using the shortest route by eliminating the procedures of excision and mutual separation of glycans, the speed and quality of glycan analysis can be increased dramatically. The role of glycan profiling is to shorten this time to move on quickly to the study of glycan functions (Fig. 2). It can be said that the reason glycan research did not progress, although many researchers have realized that it was important, was because there was no efficient glycan profiling technology.

Yoshitaka Nagai, Director (at the time) of the Mitsubishi Kagaku Institute of Life Sciences and the pioneer of glycan research, stated the significance of device development when starting the SG Project in the *Nikkan Kogyo Shimbun* dated July 4th, 2003: "If there is no automated device, the research can be taken up only by specialists. If sequences can be known and the necessary glycans be produced using an automated device without spending time listening to specialists and getting their permission, anyone can do it. This will increase the number of researchers and progress will occur explosively." Glycan profiling was certainly a subject that matched this objective and demand.

2 Elemental technology and the new concept, "glycan profiling"

The author focused on lectins, proteins that specifically bind to certain moieties within the glycan structure, and devised an analyzing platform where multiple types of lectins are arranged on a slide glass (Fig. 3). Although the thought was like "wishful thinking," I remember it was accepted at various academic societies, forums, and study sessions because of its "novelty." Many people might have felt that a new image would eventually materialize from where there

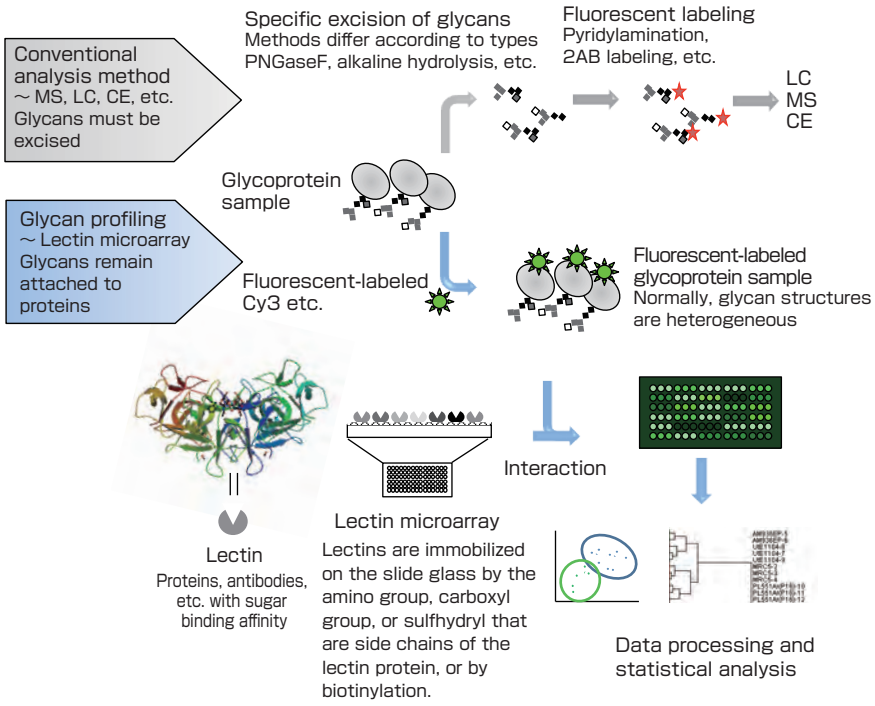

Fig. 2 Differences in the conventional glycomic methods (LC, MS, CE) and the glycan profiling by lectin microarray method
In the former, it is necessary to excise glycans from glycoproteins to compare with the standard products with known structures (memory verification method). In the MS, it is necessary to mutually separate isomers and to refine them by LC prior to analysis. In contrast, with the lectin microarray, the glycoprotein sample can be directly fluorescent-labeled, and the interaction with the lectin series can be analyzed all at once. Since the results of the interaction with lectins are reflected in the output, the analysis patterns may change due to the types or concentration of the core protein even if the glycan structures are the same.

was nothing.[7][8]

I mentioned that the objective of glycan profiling is not the strict determination of the chemical structure, but is to discern the "differences" in the structure among several samples. However, this idea and principle did not descend on me suddenly, but a prior technology provided an important clue. I shall describe the two elemental technologies that were essential in achieving the glycan profiling technology.

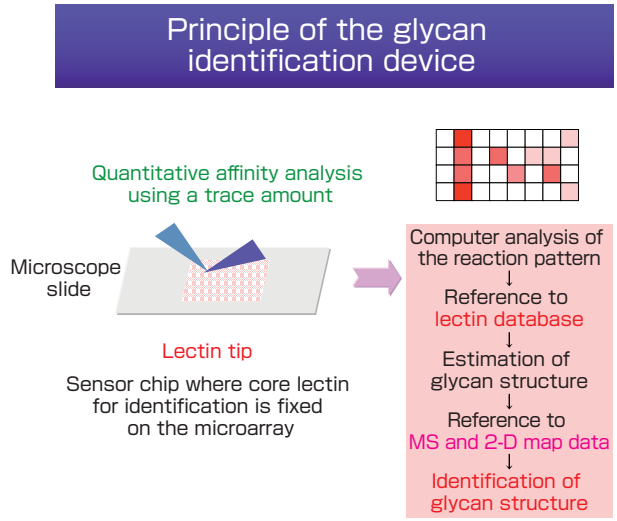

Fig. 3 Diagram showing the principle of the glycan profiler

Tens of lectin types (proteins that bind to sugars) are immobilized on the glass substrate such as the slide glass. The idea is to make glycans and glycoproteins that are glycosylated with suitable labeling groups react, and trace detection is attempted using a special detection principle. (From the author's presentation slide used at the study session organized by the Research Association for Biotechnology held in July 30, 2002).

1) Prior technologies: investigation of the principle by FAC and the Hect-by-Hect Project

By analyzing the interactions of 100 (hecto) lectins and 100 glycans by the quantitative analysis FAC, the database for interaction information was created. It was also found experimentally that each individual glycan had a unique "fingerprint" (that was left there by lectins).

It was readily predicted that the lectin microarray was appropriate for glycan profiling. However, no one had investigated whether there was really unique information like fingerprints for individual glycans, or how many lectins should be used to identify the glycan structure. Therefore, the author conducted a comprehensive analysis by FAC, a prior technology, to determine the affinity among multiple lectins and multiple glycans.

Frontal affinity chromatography (FAC), also called the frontal analysis, is a quantitative interaction analysis method developed by Ken-ichi Kasai *et al.* of the Hokkaido University (at the time) in 1975.[9] The principle was published the year before by B. M. Dunn and I. M. Chaiken, and it was essentially the same as the method that would be later called the zonal analysis method.[10] The procedures and principles of FAC are shown in Fig. 4. This technology was devised to analyze the interaction of proteases such as trypsin and substrates (inhibitors). When David Schriemer *et al.* of the University of Alberta succeeded in linking this technology to MS detection at the end of the 20th century, the road to high throughput was opened.[11] The author participated in the International Carbohydrate Symposium (San Diego) in 1998, and dropped in on a lecture for the reception of the Whistler Award by Ole Hindsgaul (currently, Carlsberg Research Center), who was

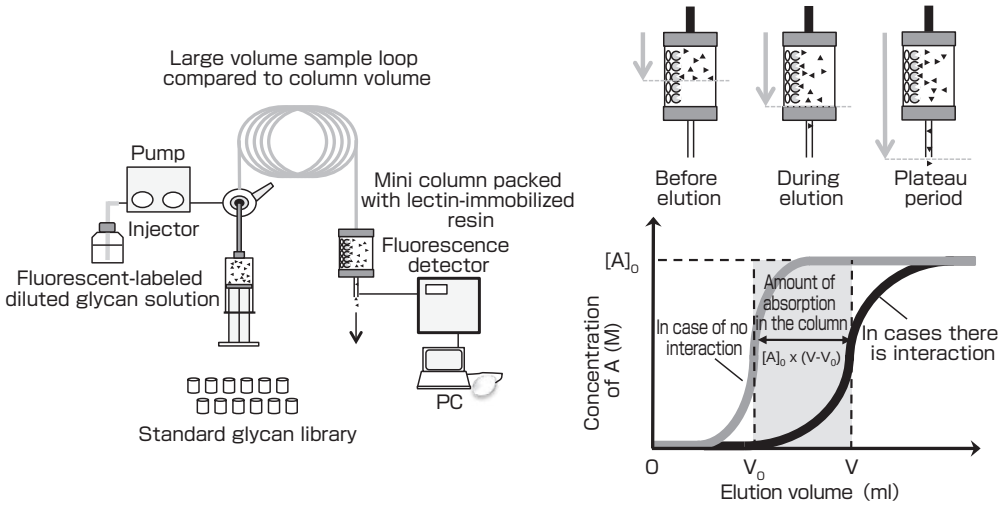

Fig. 4 Principle of FAC (right) and its procedure (left)

When glycans interact with the ligands (lectins) immobilized in the column, the elution front volume (V) that overflows from the column becomes greater compared to the control substance with no interaction (V_0). The difference ($V-V_0$) has the relationship $K_d = B_t/(V-V_0)-[A]_0$ (where B_t is the effective ligand content for the column used, and $[A]_0$ is the initial concentration of the glycan) with the dissociation constant (K_d) that is the scale that expresses the affinity between the glycan and lectin. In general, the dissociation constant of lectin against the glycan is large (dissociates easily), and since the fluorescent-labeled glycan is sufficiently diluted, $K_d >> [A]_0$ holds. Therefore, the above equation becomes $K_d = B_t/(V-V_0)$ that is not dependent on the concentration of the glycan used. This is the reason FAC is considered to be a method suitable for analyzing weak interaction.

Schriemer's superior. At the end of the lecture, Hindsgaul said, "There's wonderful news." He said he applied FAC, which Kasai developed 20 years ago, to the screening of compounds synthesized by combinatorial chemistry. They filled a PEEK tube about 10 cm long with beads to which lectins were immobilized, and cleverly linked this "column" to the ESI-MS device via an ingenious handmade device. I was amazed that my boss' name suddenly came up in an award reception lecture, but more than that, their achievement was way beyond what conventional FAC was thought to be able to do.

I was stimulated, of course. At that time, my colleagues were conducting analysis using the open glass columns and fraction collectors. I abandoned this preconception or the norm and sought the road to quick, highly sensitive, and high throughput by a method different from Schriemer. Although MS devices were not available in regional universities at that time, the laboratory had an isocratic liquid chromatography (LC) pump and a fluorescence detector that a joint research company left behind. Conveniently, we also had some standard fluorescent-labeled glycans called pyridylamino (PA) glycans that had just recently become commercially available. Thinking about what could be done with these items, one day, an "idea from a different angle" emerged. With FAC, the break-through of affinity capacity is observed as the emergence of "elution front" when excess diluted analyte floods the relatively small column. Maintaining the relationship of these two "volumes" (i.e., those of analyte and column) is essential for the analysis, and I thought, "Can't we keep the column size as small as possible, but flood the column with analyte using a relatively long sample loop with 20 times or more capacity than the column?" With usual LC, a sample loop with small capacity is used to maintain the resolution, and thus making the loop longer was unthinkable. It was found that volume ratio of 20:1 could be easily maintained if a PEEK tube with appropriate internal diameter was used.

After trial and error, we used the commercially available guard column (inner diameter of 4 mm, length of 10 mm, volume of 126 μl). A solution of PA glycans was poured into this column at a constant flow rate via the 2-ml sample loop. The fluorescence of the eluting PA glycans was tracked using an integrator connected to a fluorescence detector. First, the column was packed with agarose resin to which commercially available plant lectins were immobilized. Then the PA glycans that were thought to be interacting eluted with delay, while the glycans with no interaction eluted through (Fig. 5, refer to the chromatogram on the left).[12] One day, my colleague Yoichiro Arata (currently, Josai University), who was impressed by the beauty of the elution curve, PC-converted the digital data read by the fluorescence detector, and developed an algorithm to automatically calculate the elution frontal volume (V value in Fig. 4).[13] This was the birth of the original high-performance fluorescence detector FAC.

After that, improvements were made to FAC, and the product

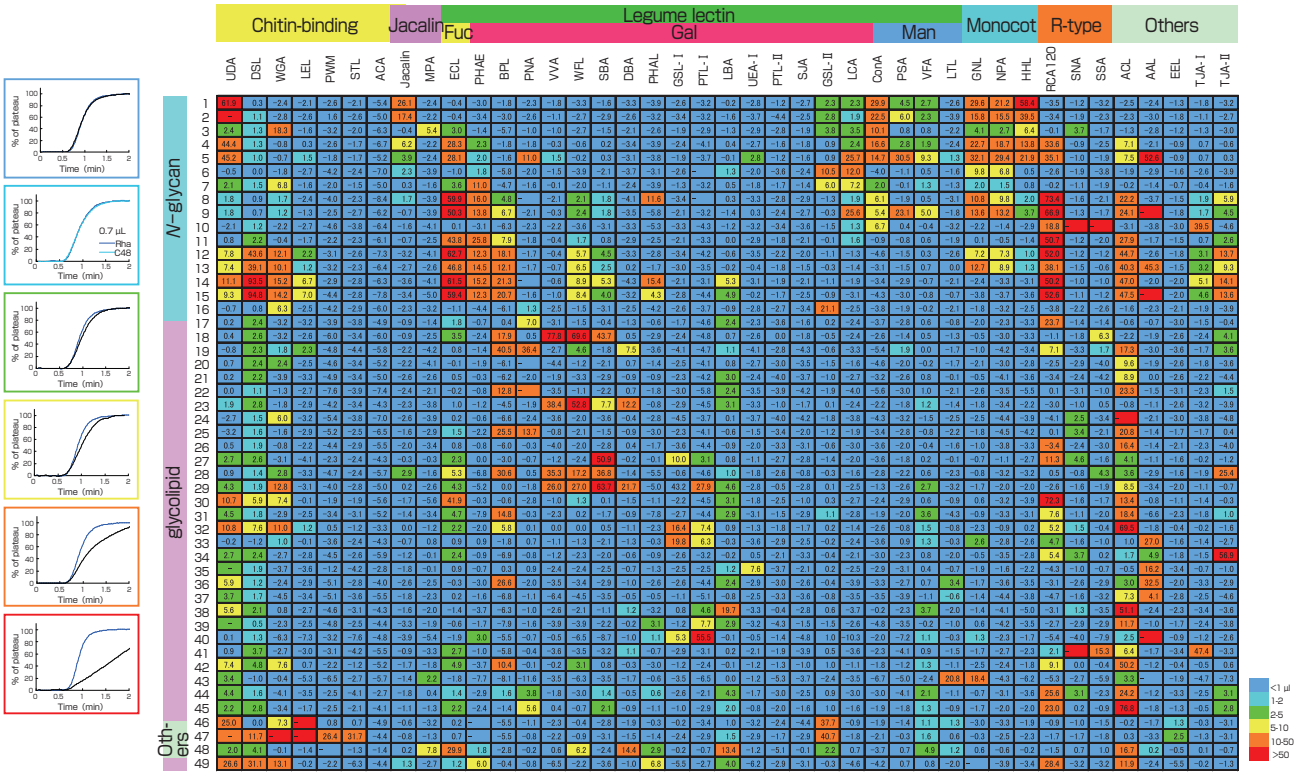

Fig. 5 Lectin-glycan interaction data (partial) produced in the Hect-by-Hect Project
The degree of interaction is observed as the delay in the elution front ($V\text{-}V_0$). The matrix shows the strongest interaction in red, and cases without interaction in blue.

was commissioned for manufacture and sales from Shimadzu Corporation as Automatic FAC.[Note 4] The FAC using the fluorescence detector developed by the author *et al.* operated and was used differently from Schriemer's FAC directly connected to MS, and it has been positioned as an almost different technology.[14][15] The high-performance FAC played a leading role in the Hect-by-Hect Project[Note 5] in NEDO's SG Project mentioned earlier. Here, the specificities of the several known and new lectins were clarified at a speed unseen before. However, the most important point that the high-performance FAC showed was that there was a unique pattern to each glycan in terms of reactivity with various lectins. I had suspected this when I was working on a manual device at the university, but the voluminous data from the Hect-by-Hect Project (Fig. 5) proved that this was correct. The matrix in Fig. 5 is composed of the vertical axis (type of glycans) and horizontal axis (type of lectins), and the grid shows the strength of the interaction. Red is the strongest and blue shows no interaction. When this matrix is cut in the "vertical direction" and taken apart, one sees that the pattern of each strip is different. This shows that there are differences in the glycan specificity of each lectin, and merely confirms the conventional way of seeing the issue. Then what about cutting the strips in the "horizontal direction"? One can see that the affinity pattern for each lectin of each glycan shown in each strip is also different. This shows that when the lectin affinity is weighed, each glycan leaves a different "fingerprint."

If the high-performance FAC had not been achieved, the voluminous data resulting from increased throughput could not have been viewed. Remember that FAC is a particularly excellent method for measuring the weak interactions (see explanation in Fig. 4). Although many methods for measuring interactions are suitable for seeking strong bindings, they do not focus on measuring the weak interactions. Since FAC could accurately calculate the affinity (expressed as the reciprocal of dissociation constant $1/K_d$) for weak interactions, this added some rich interaction information to the matrix of Fig. 5, and therefore, the fingerprints unique to glycans could be found easily. As the proof-of-principle was accomplished, the rest was how to construct the actual glycan profiler.

2) Evanescent wave excitation fluorescence detection method
This is a method to selectively detect the bound molecules in the liquid phase without the washing procedure, after interacting the fluorescent-labeled glycoprotein to the lectin array. This method is useful for glycans with weak interaction.

New ideas for research are often conceived by different groups at about the same time. In fact, when the authors succeeded in developing lectin microarrays by the principle of evanescent wave excitation fluorescence detection and published the paper in 2005, three other research groups published papers on the principle of the lectin microarray.[16]-[19] Although the paper was published later, Procognia Ltd., an Israeli venture company, was working earlier on the development of the lectin array platform (http://www.procognia.com/). Although there were several other papers on the development of the principle of lectin microarrays,[20][21] the prior technology called the serial lectin affinity chromatography was the major influence rather than FAC. In this method, several types of lectin columns are prepared, glycans that are radioactive-labeled by tritium or others are poured in order, and the degree of absorption (strong, weak, or absent) is studied to estimate the glycan structure.[22]-[24] While it made sense, many researchers must have been secretly thinking about shifting to an easier method because it took so much time and effort. On the other hand, although FAC had become higher performing, simultaneous analysis of multiple samples could not be done because it was LC, and the subject of analysis was limited to refined glycans. What was in demand at the site of R&D was the technology to directly and freely handle cellular extracts and blood serums.

Here, the author *et al.* focused on the detection principle. The technology to use the evanescent wave[Note 6] as the excitation wave of fluorescence was known for a long time, and had been applied to evanescent microscopes. To use this on the slide glass of a lectin microarray, it was necessary to apply the wave to a wide field of view rather than to the small microscope field. Before joining AIST, the author checked a venture company called the Nihon Laser Electronics (NLE) K.K. that engaged in the development of DNA microarrays using a method of illuminating the exciting incident light from the edge of the slide glass. Based on the explanation diagram for the evanescent waves shown on the company's website, I have drawn the concept for lectin microarrays using the evanescent wave excitation fluorescence detector (Fig. 6).

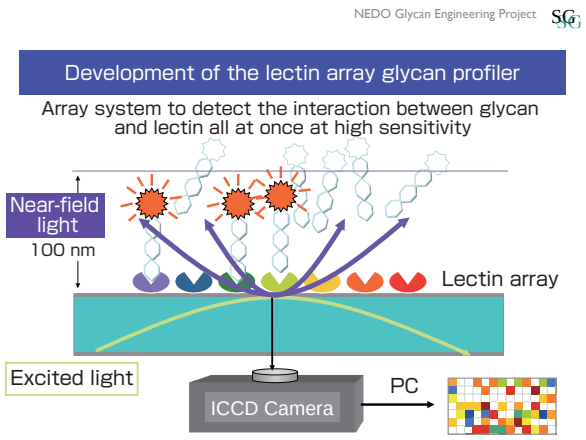

Fig. 6 Diagram showing the principle of the lectin microarray by evanescent wave excited fluorescence detection method
Created based on the slide used in the first meeting of the Japan Consortium for Glycobiology and Glycotechnology that was also the kick-off of the SG Project (Tokyo, November 3, 2003). Things have progressed remarkably compared to Fig. 3.

One of the characteristics of this technology was to excite fluorescence by illuminating evanescent waves from the edge of the glass. Since evanescent waves are light that soaks through from the glass surface to proximal range (<100 nm), the fluorescent-labeled glycans (actually glycoproteins) trapped at the surface of the slide glass to which the lectins are immobilized are selectively excited. I mentioned that the affinity between lectin-glycan is generally weaker compared to those between DNA-DNA (RNA) and antibody-antigen. That is, the glycoproteins bound weakly to lectins are easily torn off in the washing procedure after the reaction, and this binding is missed by the confocal fluorescence scanner that is normally used. The dissociation constant (K_d) between the glycan-lectin observed by FAC is at most only about 10^{-6} M (1 μM), where most are around 10^{-4} M (100 μM), and this is 100 times weaker than the antigen-antibody interactions. The most important advantage of illumination from the glass edge is that the entire surface of the glass is excited by the evanescent waves. The high-throughput analysis on the microarray platform rather than the small range of microscopes is made possible by this edge illumination. In fact, this became an important point in the later patent evaluation. There are secondary advantages in using the evanescent wave excitation method. Since the washing procedure is not necessary, the operation is facilitated, and this may lead to reduced analysis time and improved reproducibility.

To the present, only the author's group has used the evanescent wave excitation scanner, and all others use the confocal methods (washing procedure is necessary). However, they somehow function as lectin arrays because in many cases, the glycoproteins are in the multivalent state, and the affinity to the lectins that assume the oligomer structure is actually sufficiently high.[25] Measurement of the binding of excised glycans has been accomplished only by our evanescent wave excitation method.[26] However, the actual advantage of using our system, above all others, is the high detection sensitivity. In a sense, this might be expected as the washing procedure is not required, but in reality, it is not that simple. The employment of a high-output halogen lamp was essential for achieving high sensitivity, but the reduction of background was even more important. Specifically, various know-how was needed in the optically optimum filter, the selection of the slide glass, the fixation method in the biochemical procedure, the optimization of blocking method, and the image processing. While strict performance comparison with other devices is difficult, judging from the amount of samples used and users' comments reported in papers, the detection sensitivity of the evanescent wave excitation fluorescence scanner is at the top of all glycan analyzing devices. The official detection limit of this system is as follows: 100 pg/ml for the asialofetuin (the terminal sialic acid has been removed by acid treatment from the fetuin, a representative blood serum glycoprotein) against RCA120 lectin, and 100 pM for the asialo biantennary complex N-glycan against RCA120 lectin (amount used was about 0.1 ml).[26] The evanescent wave excitation fluorescent scanner (GlycoStation™ Reader 1200) was manufactured and sold by Moritex Corporation (at the time) in October 2006 along with the lectin microarray (LecChip™).

3 Up to the development of a device: corporate collaboration and IP strategy ~ the reality of bioventures

The NEDO Project for Structural Glycomics started under the supplementary budget of FY 2002, but Nihon Laser Electronics (NLE) K.K. that was the partner for the lectin array development suffered a managerial setback and went bankrupt the following year. However, AIST succeeded in the principle development of lectin arrays on its own. With the efforts of Atsushi Kuno and Noboru Uchiyama who were in charge of the development, the basic patents for the glycan profiling principle and the lectin array analysis were filed on December 25, 2003. The lectin array group was carried over by the NLE Project Group of Moritex Corporation, and with the management and technological foundations of a large corporation, the first commercial device was completed, totally refreshed from the first prototype that was reminiscent of the days of Nihon Laser Electropnics. This accomplishment received acclaim as a success case by NEDO (http://www.meti.go.jp/committee/summary/0002220/024_02_12b.pdf). However, the Lehman Shock rocked the world in 2008, and business declined rapidly and the bio section was cut off. With twists and turns of events, about a dozen members of the glycan group established the GP Biosciences Ltd. This company gained attention as a bioventure, and was taken up in NEDO's "Following up the R&D Series 1" (http://www.nedo.go.jp/hyoukabu/jyoushi_2008/gp/index.html). However, business was not easy, and the company filed for bankruptcy in April 2013.

4 Development

1) Research Center for Medical Glycoscience and GLIT

In December 2003, basic patent application was filed for the glycan profiling technology, a paper was published in December 2005, and the new NEDO project "Development of Technology for Medical Glycomics (MG: Medical Glycomics)" was started in April 2006. In December 2006, the Research Center for Medical Glycoscience (RCMG) was established for utilizing the glycans in the medical field, with Hisashi Narimatsu as the Director. One of the objectives of the new RCMG was to spread the results of the glycan research that have been accumulated over the years. At the time, basic and core research was in a noncompetitive state. To accomplish the above objective, it was thought necessary

to create a place where excellent results could be handed over quickly to the industrial world. The forum for Glyco-innovation and Industrial Technology (GLIT; http://www.glit.jp/wp/) was established with various industrial associations and interested parties by holding discussions with people inside and outside of AIST. The GLIT contributed in spreading the advanced glycan technology to industry and in promoting the exchange of human resources through various research sessions and symposiums, as a part of the joint effort with the Japan Bioindustry Association (JBA). Since these were new activities for AIST, they attracted attention from both inside and outside the institute. While it was essentially an engine for the promotion of industry-academia collaboration, it was a one-stop organization to promote such collaboration rather than a merit-based organization, and we constantly had to discuss what should be done next. Yoichi Shinma, a leader of the Collaboration Strategy Group that was newly established in the RCMG, contributed greatly to the management of GLIT.

The issues that stood before GLIT are more or less reflected in the following points taken from slides for the panel discussion at Bio Japan 2008. Although the conference was held six years ago, it serves as a reference in predicting the future of bioresearches including glycoscience.

- The positioning of the Japanese glycoscience is at the top level in the world as we possess outstanding technologies and core resources.
- However, the long time required for R&D due to various factors is making funding difficult.
- As a result, companies and universities need a "nudge" before employing the new glycan technology, and there are many issues in the use and diffusion of the advanced technology.
- It is important to clear these issues strategically to attain a breakthrough.
- In this discussion, we wish to share our thoughts by discussing the future strategy (for technology, human resources, mechanism) through the analysis of the current situation and understanding of the issues.

With the support from GLIT, 15 GlycoStation™ Reader 1200, the evanescent scanner manufactured by GP Biosciences, were sold (as of July 2013). The bio companies are realizing the importance of glycans and many businesses are interested in glycans. One company is starting to develop biopharmaceuticals, regenerative medicine, as well as biomarker diagnosis systems such as tumor markers. The GlycoStation™ Reader 1200 is gaining attention overseas as support for the development of biopharmaceuticals (particularly biosimilars).[27]

When it was first launched, the author's lab dominated the reports and papers on the usage of this system, but now the situation is reversing. It is also a fact that there are academic conflicts as a result of promoting the sales channels for the product. In that sense, perhaps GLIT has finished its role. However, the GLIT activities and the lectin microarrays were always linked, and produced many new technologies. Recently, the author and the members of the development group (Masao Yamada, Atsushi Kuno, and Hiroaki Tateno) wrote a grand review of the lectin microarray, so please refer to it for details.[28]

The following are examples of the research topics where the lectin microarray and its application went well.

2) Examples of use in the bio fields

In the aforementioned MG project, focus was placed on chronic diseases such as cancer, and excellent results were obtained for hepatic fibrosis markers and refractory intrahepatic bile duct cancer. In both cases, lectin microarrays played important roles (refer to Reference [2] by Narimatsu for details). The microarrays were partially responsible for the success of the MG Project, the main focus of the RCMG. There is no greater joy for the developer.

The application of lectin microarrays progressed first in stem cells. In the cooperative relationship between Akihiro Umezawa, Division Chief (currently Deputy Director) of the National Center for Child Health and Development (NCCHD), analyses were done for the mesenchymal stem cell lineup using the bioresources owned by the NCCHD, as well as the mouse embryonic stem (ES) cells and embryonic carcinoma (EC) cells.[29]-[32] The strategy for analysis was mostly completed in 2006, and to accomplish the technological transfer of the analysis platform to NCCHD the following year, the author *et al.* participated in the NEDO project for bridging technology and R&D (Development of Technology for Translational Research to Clinical Research / Development of Translational Research Technology / Quality Evaluation of Stem Cell Population by Glycan Profiling, R&D of Safety Evaluation System; period October 2007 to March 2009). Just when the research started, we heard the news of the "successful production of human iPS cells" by Professor Shinya Yamanaka. The world was surprised by the reality of genetic reprogramming.

Unfortunately, there were hardly any researchers studying the glycan structure of stem cells at the time. However, the SSEA-1/3/4 and Tra1-60/81 that are known as the surface markers of undifferentiated cells are certainly glycan markers. If various cells can be prepared homogenously including the undifferentiated cells, it can be shown that there are clear differences in their glycan profiles. Since the mesenchymal stem cells are heterogeneous, we thought the iPS and ES cells should be the subjects of comparative glycan profiling.

Therefore, following the above translation research project, we joined the new NEDO project (Development for the Core Technology for Applying Stem Cells such as iPS Cells to Industry, April 2009~) to fully promote glycan profiling for stem cells in collaboration with Makoto Asashima, Director (at the time) of the Organ Development Research Laboratory and his group. This project ended in two years due to various reasons, but we worked eagerly on the analysis of diverse undifferentiated cells.

Hiroaki Tateno who joined the Lectin Application Development Team from 2006 refined the system, and newly developed high-density lectin arrays composed of 96 types of lectins including recombinant lectins. The 100 ES/iPS cells were excellent subjects to try the "sharpness" of this new array. The author *et al.* collaborated with Team Leader Yuzuru Ito and Senior Researcher Yasuko Onuma of the Research Center for Stem Cell Engineering, and powerfully promoted glycan profiling for the series of stem cells. Since the results were published through press releases, many people may already know what followed. Nevertheless, the development of the research in chronological order will be explained below.

(1) Reality of glycan reprogramming
Over 100 types of human undifferentiated cells (ES/iPS) were analyzed using the newly developed high-density lectin array with 96 lectins. As a result, characteristic glycan structures common in undifferentiated cells but not present in somatic cells were extracted. In consequence, it was observed for the first time that the glycan structure is reprogrammed through the introduction of Yamanaka's four factors.[33] Particularly, the recombinant lectin called rBC2LCN that reacted commonly with all undifferentiated cells did not react with the parent cell (somatic cell) that was the base of the iPS cell production, and it was shown that rBC2LCN could be a new probe for the detection of undifferentiated markers.Note 7)

(2) Quantitative comparative analysis of the glycan structure of iPS and somatic cells
Representative iPS cell 201B7 and dermal fibroblasts that are its source were prepared in large amounts, and overall analysis for glycoprotein glycans (both N- and O-glycans) were conducted by the LC mapping method that combined MS and glycosidase digestion. The observation of the lectin microarray in (1) was actually confirmed, and the dramatic shift of the sialic acid linkage from α2-3 to α2-6 types in the N-glycan was confirmed.[34] There was interest in what was the undifferentiated glycan marker that could be recognized by the aforementioned rBC2LCN, but the H type 3 structure (Fuc α 1-2Gal β 1-3GalNAc α) was found specifically in the O-glycan.

(3) rBC2LCN can be used to stain undifferentiated cells alive
Usually, in flow cytometry and histological staining, antibody is used as the probe. The surface markers SSEA-1/3/4 or Tra1-60/81 are also detected by antibodies, but in general, the cells must be fixed. However, such cells die since the membranes are treated with formalin or glutaldehyde, however, rBC2LCN is known to possess sufficient ability to bind in the live cell condition. Moreover, when the undifferentiated cell is induced to differentiate using an agent, the staining property of rBC2LCN disappears promptly.[35] It should be noted that there are many advantages considering the future practical use, such as the lower development cost compared to antibodies.Note 8)

(4) The ligand of rBC2LCN is H type 3 structure seen in podocalyxin
Podocalyxin is a glycoprotein with large molecular weight that is expressed in kidneys or certain types of carcinoma cells. It is expected to be a sialomucin where most of the extracellular domain is heavily covered with sialylated O-glycans. The important issue in clarifying the undifferentiation mechanism is to see which glycoproteins on the undifferentiated cell are recognized by rBC2LCN that recognizes the aforementioned undifferentiation markers. By seeking out this molecule from the characteristic of the molecular structure and genetic expression, the target was identified using the antibody against podocalyxin.[36] The observations so far, including the facts that podocalyxin contains abundant O-glycans, to which rBC2LCN is specifically bound, and O-glycan with H type 3 structure has been identified from the iPS cell (201B7), now all come together in one piece.Note 8)

5 Future prospects

Other than the biomarker exploration and stem cell evaluation technology, lectin microarrays are utilized in various places. About 60 reports of the use of GlycoStation™ Reader 1200 have been published as papers, and about one-third of them are by external research institutes unrelated to the author *et al.* There are about 10 papers from overseas research institutes. While it is not addressed in this paper, the demand for this device is expected to increase rapidly in the development of biopharmaceuticals (particularly for biosimilars).

Recently, the National Academy of Science of the USA reported that the study of glycans is the core science that stretches across a wide range of life sciences. The report titled *Transforming Glycoscience* indicates that glycans are important in three major topics of "health," "materials," and "energy," and the dramatic progress in glycoscience is likely to occur at a global level.[37] The glycans are about to be released from the realm of specialists to leap into globalization.

There are many books that collect data and technology of

glycans. In contrast, the book which will be published soon from Springer Japan intends to introduce the "topics that are in the news in the periphery of glycans" for the researchers who are not glycan specialists.[38] In bioresearch, one must be facing glycan issues regardless of whether one is aware of it or not. The reason we study glycans is because it is important, universal, and difficult.[39] It must not be forgotten that glycans are widely existent. This is the other side of the fact that the origin of sugar is old. Though it may sound paradoxical, the author believes, "glycans do not exist because they are important, but became important because they have existed."[40]

There are major issues left in the synthesis technology. The creation of biopharmaceuticals by homogenous glycans is a major goal of biologics.[41] While the lectin array is evaluated highly as a tool for that purpose, lectins themselves may eventually turn out to be something major.

6 Summary: Components of the technology and the opportunities for integration

Finally, we shall investigate how the lectin microarray technology was synthesized, how the core technological components were integrated, and what were the opportunities for breakthroughs (Fig. 7). First, there was a demand for the development of glycan analysis technology. The opportunity to develop such technology came to the author in 2002. At the time, a high performance of FAC was achieved by the author at a university, and this brought about the change of thinking that it was possible to profile glycans using lectins. I joined AIST almost immediately afterwards, and was assigned to the glycomics project. The concept of glycan profiling that was merely a pie in the sky became a solid project after collaboration with companies and verifying the principle. The patent application was in 2003, the publication of the academic paper was in 2005, and the launch of the glycan profiling device was in 2006. The blooming of this technology continued. It was utilized in the biomarker development in the subsequent glycomics project, and a wider approach to its use was created through the organizational efforts of GLIT to promote the results. This strongly pushed the bridging of glycans to industry. One of the new applications was the discovery of the new undifferentiated marker detection probe called rBC2LCN in the stem cell evaluation technology. This was the impetus to new development including the detection and elimination of the undifferentiated cells in regenerative medicine, as well as the shift to recombinant lectins. Perhaps it might have opened the door to a new discipline called lectin engineering.

Looking back at the series of events, what was the most important point? The author believes it was the thorough technological pursuit of the "weak interactions" as exemplified by the glycan-lectin binding. The FAC achieved this pursuit in principle, but the achievement of high throughput was necessary before arriving at the idea of glycan profiling. However, since FAC could not handle crude extracts derived from living tissues, it was necessary to shift to a totally different platform called the lectin array. Then focus was placed on the evanescent wave excitation method that allowed capturing weak interactions. At the same time, there were many groups that proposed the lectin array, but only the author's group reached product development that produced satisfactory results. The reason the author took up, without hesitation, the evanescent wave excitation

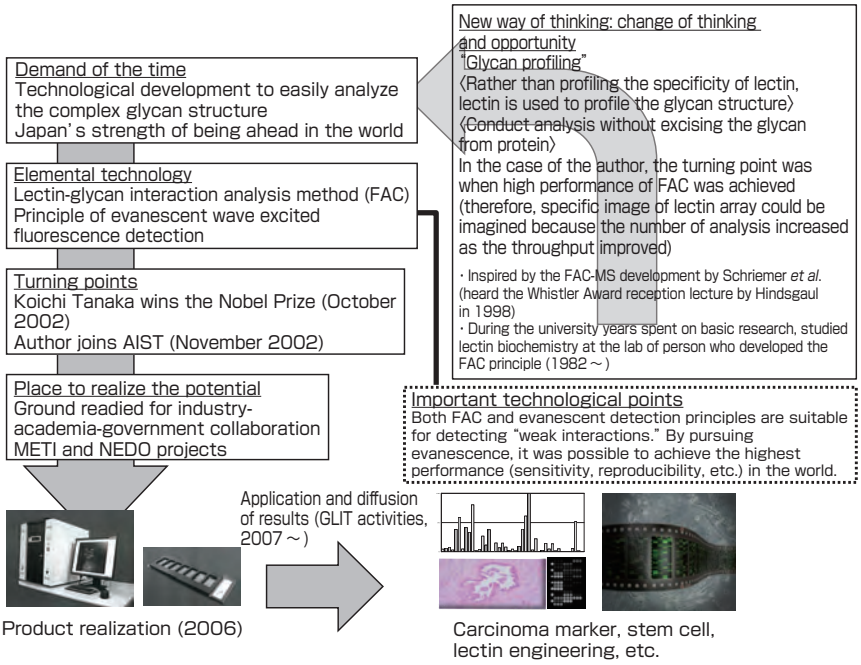

Fig. 7 Summary of the synthesis of the lectin microarray technology

fluorescence detection principle that allowed detection of weak interactions was perhaps because he was a newcomer to the optics field, and did not look aside for other technical options. In fact, theoretically "evanescence is not necessarily highly sensitive," but as of now, no device that surpasses the precision and sensitivity of the evanescent wave excitation scanner that we developed has emerged.

Notes

Note 1) Because glycans have complex structures with unknown functions, the USA and the European countries were reluctant to invest a large amount of research funds. In contrast, there was an understanding for the importance of sugars and sugar research as a national policy in Japan, and the government supported the development of glycomics technology and enzyme exploration such as glycosidase that the Japanese researchers probably started from half curiosity and half determination. Moreover, high achievement of the biochemical refinement technology strengthened the accumulation of glycan resources and know-how, and Japan was way ahead of the race for patents and papers on glycans. On the other hand, there were people overseas who focused on glycans before, and there was an interesting background in the start-up of a new discipline called glycobiology.[1]

Note 2) Antigen determinant recognized by antibodies. In a wider sense, it includes the structures involved in the recognition.

Note 3) A method to identify structures by referencing to standard products with known structures. It is also called the memory verification method. It is distinguished from the method that directly determines structures such as the methylation analysis in NMR and MS.

Note 4) I asked Koichi Tanaka of Shimadzu Corporation whom I met at a study session before the project start-up to act as the mediator for the LC section. Later, Tanaka declined participation to our project as he received the Nobel Prize.

Note 5) The project to comprehensively analyze the interactions between 100 lectins and 100 glycans (standard products with known structures) using FAC. Masugu Kamei of Honen Corporation (currently, J-Oil Mills, Inc.) traveled throughout Japan to collect lectins, and Norihiro Kikuchi and Junko Takahashi of the Mitsui Knowledge Industry Co., Ltd. was in charge of the data analysis (see Table 1). Shoko Nakamura (née), Postdoctoral Fellow (at the time), led the project at AIST.

Note 6) The meaning of this word is "light of the fleeting moment," and it is also called the evanescent field or nearoptic light. In the reflection phenomenon of the magnetic wave (light), it is the permeating magnetic wave that occurs in the reflecting medium under certain conditions. Excellent explanation is provided in the following Nikon site. http://www.nikon.co.jp/profile/technology/rd/core/optical/evanescent/index.htm

Note 7) April 2, 2012, AIST Press Release: Facile diagnosis of iPS cells using glycan profiling technology –Identification of a novel pluripotency marker by high-density lectin microarray

Note 8) May 16, 2013, AIST Press Release: A novel probe for live human iPS cell imaging–iPS cells can be cultured with monitoring of their quality

References

[1] A. Varki and N. Sharon: *Essentials of Glycobiology, 2nd edition* (edited by. A. Varki, R. D. Cummings, J. D. Esko, H. H. Freeze, P. Stanley, C. R. Bertozzi, G. W. Hart and M. E. Etzler), Cold Spring Harbor Laboratory Press (2009).

[2] H. Narimatsu: Development of basic tools for glycoscience and their application to cancer diagnosis - A 10-year strategy of the Research Center for Medical Glycoscience of AIST, *Synthesiology*, 5 (3), 190-203 (2012) (in Japanese) [*Synthesiology English edition*, 5 (3), 201-215 (2012)].

[3] E. Yasuda, H. Tateno, J. Hirabayashi, T. Iino and T. Sako: Lectin microarray reveals binding profiles of Lactobacillus casei strains in a comprehensive analysis of bacterial cell wall polysaccharides, *Appl. Environ, Microbiol*, 77 (13), 4539-4546 (2011).

[4] A. Shibazaki, H. Tateno, A. Ando, J. Hirabayashi and T. Gonoi: Profiling the cell surface glycome of five fungi using lectin microarray, *J. Carb. Chem*., 30 (3), 147-164 (2011).

[5] N. Takahashi: Three-dimensional mapping of N-linked oligosaccharides using anion-exchange, hydrophobic and hydrophilic interaction modes of high-performance liquid chromatography, *J. Chromatogr. A*, 720 (1-2), 217-225 (1996).

[6] S. Hase, S. Hara and Y. Matsushima: Tagging of sugars with a fluorescent compound, 2-aminopyridine, *J. Biochem*., 85 (1), 217-220 (1979).

[7] J. Hirabayashi and K. Kasai: Glycomics, coming of age! *Trends Glycosci. Glycotechnol*., 12 (63), 1-5 (2000).

[8] T. Feizi: Progress in deciphering the information content of the 'glycome' - a crescendo in the closing years of the millennium, *Glycoconj. J*., 17 (7-9), 553-565 (2000).

[9] J. Hirabayashi: Furontaru afiniti kuromato-bunsekiho (Frontal affinity chromatography), *Seibutsu Kogaku Kaishi*, 89 (7), 394-397 (2011) (in Japanese).

[10] K. Kasai, Y. Oda, M. Nishikata and S. Ishii: Frontal affinity chromatography: theory for its application to studies on specific interactions of biomolecules, *J. Chromatogr. B*, 376, 33-47 (1986).

[11] D. C. Schriemer, D. R. Bundle, L. Li and O. Hindsgaul: 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 (1998).

[12] J. Hirabayashi, Y. Arata and K. Kasai: Reinforcement of frontal affinity chromatography for effective analysis of lectin-oligosaccharide interactions, *J. Chromatogr. A*, 890 (2), 261-271 (2000).

[13] Y. Arata, J. Hirabayashi and K. Kasai: 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 (1-2), 337-343 (2001).

[14] E. S. Ng, N. W. Chan, D. F. Lewis, O. Hindsgaul and D. C. Schriemer: Frontal affinity chromatography-mass spectrometry, *Nat. Protoc*., 2 (8), 1907-1917 (2007).

[15] H. Tateno, S. Nakamura-Tsuruta and J. Hirabayashi: Frontal affinity chromatography: sugar-protein interactions, *Nat. Protoc*., 2 (10), 2529-2537 (2007).

[16] S. Angeloni, J. L. Ridet, N. Kusy, H. Gao, F. Crevoisier, S. Guinchard, S. Kochhar, H. Sigrist and N. Sprenger: Glycoprofiling with micro-arrays of glycoconjugates and lectins, *Glycobiology*, 15 (1), 31-41 (2005).
[17] K. T. Pilobello, L. Krishnamoorthy, D. Slawek and L. K. Mahal: Development of a lectin microarray for the rapid analysis of protein glycopatterns, *ChemBioChem*, 6 (6), 985-989 (2005).
[18] T. Zheng, D. Peelen and L. M. Smith: Lectin arrays for profiling cell surface carbohydrate expression, *J. Am. Chem.* Soc., 127 (28), 9982-9983 (2005).
[19] A. Kuno, N. Uchiyama, S. Koseki-Kuno, Y. Ebe, S. Takashima, M. Yamada and J. Hirabayashi: Evanescent-field fluorescence-assisted lectin microarray: A new strategy for glycan profiling, *Nat. Methods*, 2 (11), 851-856 (2005).
[20] R. Rosenfeld, H. Bangio, G. J. Gerwig, R. Rosenberg, R. Aloni, Y. Cohen, Y. Amor, I. Plaschkes, J. P. Kamerling and R. B. Maya: A lectin array-based methodology for the analysis of protein glycosylation, *J. Biochem. Biophys. Methods*, 70 (3), 415-426 (2007).
[21] S. C. Tao, Y. Li, J. Zhou, J. Qian, R. L. Schnaar, Y. Zhang, I. J. Goldstein, H. Zhu and J. P. Schneck: Lectin microarrays identify cell-specific and functionally significant cell surface glycan markers, *Glycobiology*, 18 (10), 761-769 (2008).
[22] K. Kornfeld, M. L. Reitman and R. Kornfeld: The carbohydrate-binding specificity of pea and lentil lectins, *J. Biol.Chem*., 256 (13), 6633-6640 (1981).
[23] K. Yamamoto, T. Tsuji, I. Matsumoto and T. Osawa: Structural requirements for the binding of oligosaccharides and glycopeptides to immobilized wheat germ agglutinin, *Biochemistry*, 20, 5894-5899 (1981).
[24] R. D. Cummings and S. Kornfeld: Fractionation of asparagine-linked oligosaccharides by serial lectin-Agarose affinity chromatography, *J. Biol. Chem*., 257 (19), 11235-11240 (1982).
[25] C. D. Rillahan and J. C. Paulson: Glycan microarrays for decoding the glycome, *Annu. Rev. Biochem*., 80, 797-823 (2011).
[26] N. Uchiyama, A. Kuno, H. Tateno, Y. Kubo, M. Mizuno, M. Noguchi and J. Hirabayashi: Optimization of evanescent-field fluorescence-assisted lectin microarray for high-sensitivity detection of monovalent oligosaccharides and glycoproteins, *Proteomics*, 8 (15), 3042-3050 (2008).
[27] A. Kuno, S. Takeishi and J. Hirabayashi: Rekuchin maikuro arei no tanpakushitu iyakuhin seisan purosesu kaihatsu eno oyo (Application of lectin microarray to the development of protein pharmaceutical production process), *Bio Iyakuhin Kaihatsu Ni Okeru Tosa Gijutsu* (Glycotechnology for Development of Biotechnology Protein Products) (T. Hayakawa, K. Kakehi, J. Hirabayashi eds.), CMC Publishing, 230-241 (2011) (in Japanese).
[28] J. Hirabayashi, M. Yamada, A. Kuno and H. Tateno: Lectin microarrays: concept, principle and applications, *Chem. Soc. Rev*., 42 (10), 4443-4458 (2013).
[29] A. Kuno, Y. Itakura, M. Toyoda, Y. Takahashi, M. Yamada, A. Umezawa and J. Hirabayashi: Development of a data-mining system for differential profiling of cell glycoproteins based on lectin microarray, *J. Proteomics Bioinformatics*, 1 (2), 68-72 (2008).
[30] M. Toyoda, M. Yamazaki-Inoue, Y. Itakura, A. Kuno, T. Ogawa, M. Yamada, H. Akutsu, Y. Takahashi, S. Kanzaki, H. Narimatsu, J. Hirabayashi and A. Umezawa: Lectin microarray analysis of pluripotent and multipotent stem cells, *Genes Cells*., 16 (1), 1-11 (2011).
[31] Y. Itakura, A. Kuno, M. Toyoda, A. Umezawa and J. Hirabayashi: Podocalyxin-targeting comparative glycan profiling reveals difference between human embryonic stem cells and embryonal carcinoma cells, *J. Glycom. Lipidom*., S5-004 (2013).
[32] S. Saito, Y. Onuma, Y. Ito, H. Tateno, M. Toyoda, A. Hidenori, K. Nishino, E. Chikazawa, Y. Fukawatase, Y. Miyagawa, H. Okita, N. Kiyokawa, Y. Shimma, A. Umezawa, J. Hirabayashi, K. Horimoto and M. Asashima: Possible linkages between the inner and outer cellular states of human induced pluripotent stem cells, *BMC Syst. Biol*., 5 Suppl 1, S17 (2011).
[33] H. Tateno, M. Toyoda, S. Saito, Y. Onuma, Y. Ito, K. Hiemori, M. Fukumura, A. Matsushima, M. Nakanishi, K. Ohnuma, H. Akutsu, A. Umezawa, K. Horimoto, J. Hirabayashi and M. Asashima: Glycome diagnosis of human induced pluripotent stem cells using lectin microarray, *J. Biol. Chem*., 286 (23), 20345-20353 (2011).
[34] K. Hasehira, H. Tateno, Y. Onuma, Y. Ito, M. Asashima and J. Hirabayashi: Structural and quantitative evidence for dynamic glycome shift on production of induced pluripotent stem cells, *Mol. Cell. Proteomics*, 11 (12), 1913-1923 (2012).
[35] Y. Onuma, H. Tateno, J. Hirabayashi, Y. Ito and M. Asashima: rBC2LCN, a new probe for live cell imaging of human pluripotent stem cells, *Biochem. Biophys. Res. Commun*., 431 (3), 524-529 (2013).
[36] H. Tateno, A. Matsushima, K. Hiemori, Y. Onuma, Y. Ito, K. Hasehira, K. Nishimura, M. Ohtaka, S. Takayasu, M. Nakanishi, Y. Ikehara, M. Nakanishi, K. Ohnuma, T. Chan, M. Toyoda, H. Akutsu, A. Umezawa, M. Asashima and J. Hirabayashi: Podocalyxin is a glycoprotein ligand of the human pluripotent stem cell-specific probe rBC2LCN, *Stem Cells Transl. Med*., 2 (4), 265-273 (2013).
[37] National Research Council: *Transforming Glycoscience*, The National Academies Press (US), (2012).
[38] N. Taniguchi, T. Suzuki and K. Ohtsubo (eds.): *Sugar Chains*, Springer, Tokyo (2014) (in press).
[39] J. Hirabayashi, A. Kuno and H. Tateno: Lectin-based structural glycomics: a practical approach to complex glycans, *Electrophoresis*, 32 (10), 1118-1128 (2011).
[40] J. Hirabayashi: *Tosa no Hanashi* (Story about Sugar Chains), Nikkan Kogyo Shimbun (2008) (in Japanese).
[41] J. Hirabayashi: Tenkanki o mukaeta baio iyakuhin – Seihi no kagi wa opun inobeshon to tosa seigyo (Biopharmaceuticals at a turning point - Key to success are open innovation and glycan control), *MEDCHEM NEWS*, 23 (2), 16-21 (2013) (in Japanese).

Author

Jun HIRABAYASHI

Graduated from the Faculty of Science, Tohoku University in 1980. Obtained the Master of Science degree from the Graduate School of Science, Tohoku University in 1982. Assistant and Lecturer, Faculty of Pharma Sciences, Teikyo University in 1982. Obtained Doctor of Science degree from the Graduate School of Science, Tohoku University in 1989. Research Leader, Research Center for Glycoscience, AIST in 2002. Deputy Director, Research Center for Medical Glycoscience, AIST in 2006. Senior Researcher, Research Center for Stem Cell Engineering, AIST in 2012 (currently, Chief Researcher).

Discussions with Reviewers

1 Overall comment

Comment (Kanji Ueda, Hyogo Prefectural Institute of Technology)

I see that there were several important leaps in the progress of this research. The research is narrated in a story form, the epochs are emphasized and the content is easy to understand. However, the leaps must have been based on some hypotheses rather than being mere coincidences. Did you hypothesize some new elemental technology, or did you set up a hypothesis to synthesize the existing technologies? Please explain clearly. I think such explanation will make the paper even more appropriate for *Synthesiology*.

Also, to enhance the readers' understanding, I think you should add an explanatory flow chart that goes along with the structure of this paper. The reader will be able to logically understand the paper along with the storytelling.

Comment (Noboru Yumoto, AIST)

The objective of this research is the "development of glycan profiling technology," and with the major breakthrough of the "proof of concept by FAC," the elemental technologies such as "lectin arrays" and "evanescent wave excitation fluorescence detection" were integrated to develop the lectin microarrays. However, the scenario is not easy to understand by people outside of the bio field. The main reason is because the description is provided in chronological order. Therefore, I think you should insert a conceptual diagram for the lectin microarray and glycan profiling, and explain why you needed the proof of concept, and how you integrated the elemental technologies for the microarray.

Answer (Jun Hirabayashi)

Except for the "application development" of the latter half, I made revisions by clarifying the technological scenario and the hypothesis, considering the point that you indicated. I added Fig. 7 that shows the correspondence between the story and the structure and reasoning of this technology, and also added a summary for the technology-centered scenario.

However, if I break the chronological (the storytelling) style, I think the characteristics will be lost (though it may depend on my writing ability) and I will end up having to rewrite the whole text. After much consideration, I left the chronological description as is. I implanted the image of how difficult glycomics is in the beginning of the paper (presentation of the issue). Then I clarified the difference between the conventional method and the lectin microarray procedure (Fig. 2), explained the flow from the principle of the lectin array (Fig. 3) to the improvement of FAC that provided the breakthrough (Fig. 4), and presented the data obtained in the Project (Fig. 5 is the image of the lectin array). Moreover, I added a summary in the end for a technology-centered scenario that can be reviewed by the readers.

2 Clarification of the difficulty of the structural analysis of glycans

Comment (Kanji Ueda)

You emphasize the facts that glycans have much structural diversity, temporary instability, and complexity, and those were the starting points of the research. I think the readers will be able to understand better if you give a numerical example of how different glycans are compared to nucleic acids and proteins.

Comment (Noboru Yumoto)

Can you describe why glycan profiling is important at the beginning for the readers outside of your field? I think you should let people know how complex it is by adding the chemical structure of a representative glycan, and by stating that there are multitudes of lectins that recognize various glycans.

Answer (Jun Hirabayashi)

I newly created Fig. 1 to explain why the structural analysis of glycans is difficult compared to other polymers, with added schematic diagram of a glycoprotein and the factors that make it complicated from the viewpoint of hierarchy. I think this makes Fig. 2 that shows the difference between the conventional method where the glycans are excised and mutually separated and the approach using lectin arrays easier to understand compared to the former text.

3 Superiority of the developed system

Comment (Noboru Yumoto)

In the section of "evanescent wave excitation fluorescence detection method," it is rather difficult to understand the superiority of the system developed by the author *et al*. You mention the superiority of "high detection sensitivity," but I cannot see from where this characteristic arises. At the end of the text, you write "theoretically 'evanescence is not necessarily highly sensitive.'" Perhaps it is not clear why high sensitivity could be achieved, but can you add some explanation?

Answer Answer (Jun Hirabayashi)

I added the point that to achieve high sensitivity, not only the employment of the principle of the evanescent wave excitation, but various measures for reducing the background including optical, biochemical, and software measures contributed.