A systematic analysis of protein interaction networks leading to the drug discovery

— Development of ultra sensitive mass spectrometry analytical platform —

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Inside each cell that constitutes organism, over 100 thousand different types of proteins function for vital biological processes. The proteins form groups and organizations that function as networks. We were able to achieve large-scale protein network analysis with high sensitivity, high reproducibility, and high efficiency, by newly developing liquid chromatography system with extremely low flow rate of 100 nL/min. The large scale data set obtained using this analysis contributed not only to the discovery of new cellular systems, but also to the understanding of development mechanism of diseases at molecular level. Our research developed into Full Research that led to the development of new diagnostic and treatment methods as well as new drug discovery targets.

Keywords : Proteomics, mass spectrometry, protein network, drug discovery, protein microquantative analysis

1 Background of research

Human body consists of about 30 trillion cells, and each cell contains over 100 thousand different types of proteins to sustain life activity. These proteins do not function separately, but form groups and organizations and function as networks. The work of mapping the network of proteins in the cell is called protein network analysis.

Network analysis is important not only for the clarification of biological processes, but also for the understanding of development mechanism of diseases at molecular level, and contributes to the development of new diagnosis and treatment methods and to new drug discovery targets (Figure 1). However, protein network analysis is technically not easy. There was no established methodology, because the demand in actual protein network analysis was to analyze several hundreds or several thousands of proteins at once.

It was practically impossible to meet this demand with technology of the 1990s. However, the technology came to a turning point in the 21st Century, with the maturity of ionization mass spectrometry for protein invented by Koichi Tanaka of the Shimadzu Corporation. Analysis work that previously required dozens of hours to identify just one protein could be accomplished in a few minutes or even a few seconds using the new mass spectrometry method. Also in theory, the sensitivity increased several hundred times, and it seemed that the researchers were released from the limiting requirement of purifying large amount of samples. However, even after protein researchers obtained their high-tech mass spectrometers, high-sensitivity analysis did not necessarily become instantly available. That was because each of the hundred thousand types of protein possessed different form and size, varied in chemical properties, and were unstable. Microquantity protein degraded and denatured in the test tubes, and became undetectable as they became adsorbed into the test tube wall. Although mass spectrometer was an ultra sensitive “detector,” evanescence of samples determined the real limit of analysis sensitivity and throughput. Therefore, unless this problem was solved, it was impossible to conduct microquantative protein analysis by fully employing the performance of the state of art mass spectrometer. Even if the performance of mass spectrometer increased in the future, there is concern that its advantage would not be utilized fully.

2 Real problem that must be solved (liquid chromatography technology)

The most important method when treat microquantity protein is to maintain the sample in micro-space at concentrated condition as much as possible. However, this is not easy since desalt and washing processes are necessary to analyze protein from organic samples by mass spectrometry. The conventional practice was the on-line reverse phase high-performance liquid chromatograph (HPLC) and the mass spectrometer. A sample was concentrated and desalted in the HPLC column, an elution fraction of liquid chromatograph was ionized, and introduced into the mass spectrometer. However, commercially available HPLC devices had low sensitivity and throughput that did not satisfy our objective of protein network analysis. Particularly, the minimum limit of the pump of conventional HPLC was a flow rate of several microliters per minute and had poor analytical reproducibility, and it was impossible to conduct large-scale, repeated and stable analyses. One of the greatest reasons was because homogenous mixing of solvents was difficult at a low flow rate of several microliters per minute.

To conduct liquid chromatography (LC), proteins and

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peptides are adsorbed and concentrated in the column carrier from the initial solvent. After desalt, elution solvent is discarded and the eluted sample is analyzed. Normally, the elution solvent is mixed gradually with initial solvent, and the liquid is delivered by generating a concentration gradient. To accomplish this, flow path for mixed delivery is necessary by connecting the pumps of two systems for initial and elution solvents. Check valve and dead volume of mixer (space to thoroughly mix the solvents) are always present in the flow path. Therefore, single analysis takes a long time, and solvents cannot be mixed evenly at a slow flow rate. In the 1990s, much research was done to decrease the flow rate by splitting the liquid delivery without slowing down the flow speed of the pump. A branch was formed in the flow path, most of the solvent was discarded, and part of the solvent was sent to the analytical column. If the split was 10 against 1, 9 parts of the solvent could be discarded and the flow rate was reduced to 1/10 (upper part of Figure 2). In this method, it was not possible to conduct analysis at a set flow rate unless the backpressure of analytical column and resistance at the split part were always constant. However, in actual practice, the backpressure of analytical column was not necessarily constant depending on load and volume of the sample. Also, the split resistance tended to increase as the frequency of analysis increased. Therefore, it was almost impossible to conduct reproducible microanalysis. This was an issue that must be definitely solved.

3 New scenario and development of elemental technology (issue of LC environment)

The scenario we employed to solve this issue was based on increasing the performance of LC. The first task was to increase the performance of LC, and then individual elemental issues would be solved as they arose, and finally we could achieve efficient high-precision protein analysis.

To increase performance of LC, we created a totally new method in which a single-system pump was used instead of a dual-system to generate concentration gradient in the elution solvent. By using a single-system pump, the flow channel could be dramatically simplified, and the dead volume, which was the greatest challenge of LC at low flow, could be minimized. Also, if this were realized, low speed liquid transfer would be possible without splitting. Here, we devised the novel split-less nano-flow gradient elution system. The system consists of several channel solvent reservoirs connected by a ten-port electrical switching valve and a manifold. Each reservoir was filled with step elution solvent for LC, which was supplied from two separate reservoirs for initial and final solvents by an

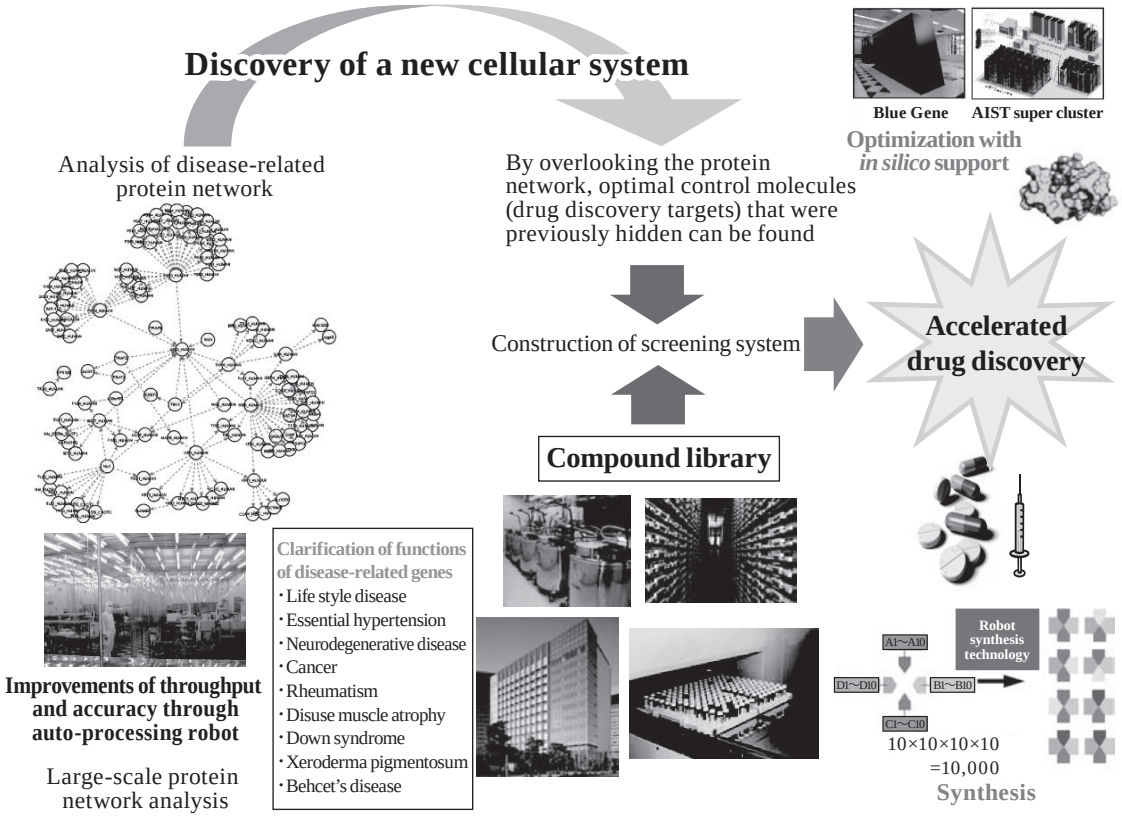

Fig. 1 Drug discovery from protein network analysis.
Proteins interact with each other and form networks. By understanding the protein network, we can discern the function of individual protein. Also, by overlooking these networks, we can find pathogenic mechanisms and new drug discovery targets. Drug discovery screening is conducted based on these informations.

automated high flow-rate mixing module.

Describing this idea in one sentence, it is "mixture is made homogenously in a larger world, and then delivered to a smaller world," much like "Columbus' egg." Although it was important to make sure that the solvents in each step would not mix with each other, we took advantage of the disadvantage of a dual-system pump where the solvents in a microflow channel do not mix (lower part of Figure 2). This method enabled extremely high reproducibility as well as continuous operation and automation of LC. Using this method, HPLC at lower than 100 nL/min with direct flow without split was installed online with mass spectrometer for the first time in the world[1]. As a result, a high sensitivity of 20~50 times the conventional method was achieved.

We also improved the analytical environment to maximize the high sensitive analysis. Even if we achieved sensitivity that allowed analysis of microquantity samples, since massive amounts of human keratin existed in general experimental environment, the signal of microquantity sample would be overpowered by contamination of air-borne keratins. Also, since the analytical channel consisted of a thin capillary with internal diameter of 10 micrometer to eliminate dead volume as much as possible, the channel became clogged readily by dust particles in the atmosphere. Therefore, continuous operation in a general environment where people walk in and out was impossible. Although elimination of dust particles and keratin were not easy, we started to use the prototype system for routine anayses.

In 2000, we started analysis using actual samples at Tokyo Metropolitan University where the technological development was carried out. Since there was no clean room at that time, we evaded the issue of experimental environment by limiting entry and exit of people to the analysis lab. The lab was thoroughly organized to eliminate dust source and covered with antistatic sheet as much as possible to prevent adsorption of dust by static electricity. Entry to the room wearing raised fabric clothes was prohibited. Also, after opening and closing the door of the lab, we waited until the dust settled, and only after then did we start the analysis.

The excitement we felt when over 100 proteins were identified at once for first time using this system was unforgettable. We gradually obtained cooperation of people who appreciated the

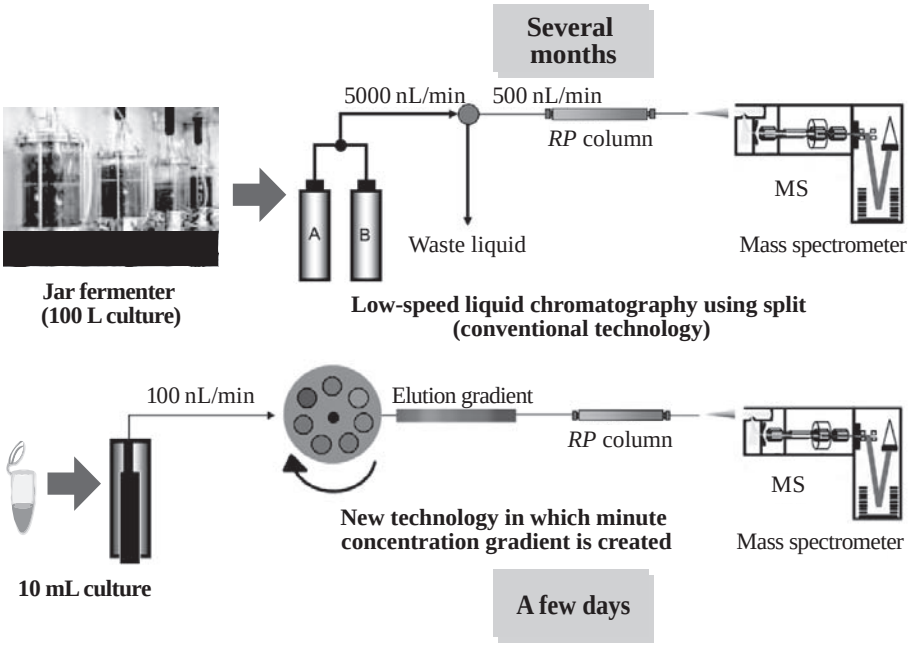

Fig. 2 Comparison of new and conventional technologies.

In conventional technology, elution gradient is created by a dual-system pump. Concentration gradient is created by shifting the transfer rate of pump A that delivers initial solvent and pump B that delivers elution solvent. However in this method, low speed mixing is not possible since the dead volume is large. Therefore, low speed is achieved by installing a splitter between the analytical columns and by discarding most of the liquid. The figure shows that the flow rate is decreased from 5000 nL to 500 nL/min by discarding 1/10 of the liquid (upper part).

In the new technology, reservoirs with multiple branches are filled with elution solvents in steps using a separate pump system. Elution gradient is created by rotating the port valve and pushing out each step with a single-system low-speed pump. There is no dead volume and it does not require a splitter (lower part).

In conventional technology, sample preparation by mass culture was necessary for a single analysis. It was common to conduct 100 L culture using jar fermenter. Several months were required to prepare for an analysis. Using the technology we developed, we achieved sensitivity where several analyses would be possible with samples from 10 mL culture. In this scale, only a few days are necessary for sample preparation, and several different samples can be prepared simultaneously.

superiority of the result, and was able to install a simple clean booth in the analysis lab. However, as a practical problem, heat produced by the mass spectrometer was too large, temperature inside the booth quickly rose to over 35 °C, and it could not be used for a long time, as the device would get damaged. We strongly felt the necessity for a fully equipped clean room to conduct stable and continuous analysis.

In the spring of 2001, the AIST Tokyo Waterfront was newly opened and we obtained the opportunity to install a clean room. We visited several semiconductor plants to learn about clean rooms from basics. However, not until the opening of Tokyo Waterfront annex in 2005 and the construction of second-generation super clean room did we solve the dust issue.

4 Execution of Type 2 Basic Research (issue of sample preparation)

After successfully developing the elemental technology for liquid chromatography, we thought it was important to move on to product realization research in order to make the hardware available commercially. However, as mentioned in the previous section, we discovered that the developed technology was useless unless the analytical environment was constructed properly. My real objective was to construct a mass spectrometry system with the highest sensitivity in the world, to conduct large-scale and high-precision protein network analysis, and to efficiently find disease mechanisms and discovery of new drug targets.

In fact, the effectiveness of developed LC system and mass spec facility was extremely significant. There was no necessity for steps of separating the sample by electrophoresis, and in about one hour, interaction complex composed of over 200 different types of proteins could be completely identified at sub-femto level. Before, sample had to be prepared from several tens to hundreds of liter volume and the analysis took several months, while now it can be done in 2~3 days. Also, since multiple analyses can be conducted, 10~20 analyses can be done simultaneously. Conducting large-scale analysis at high throughput became a reality.

By increasing throughput, it became possible to consider in details the conditions of sample preparation for high-precision analysis. There are various parameters such as affinity refinement of proteins, incubation time, and lysis of cells. To combine multiple parameters, several thousand analyses were necessary, and such analyses could not be accomplished by one researcher in his/her lifetime. Therefore, optimization of conditions for sample preparation was never done at comprehensive and thorough manner, and normally sample preparation designed on trial-and-error based on a researcher's experience and intuition. In fact, we sought sample preparation optimization through several thousand analyses, and developed an extremely precise method eliminating foul positive data maximally.

We investigated diverse parameters, and the conclusion obtained was simple: "work fast." Since sensitivity of conventional analysis methods was low, it was common knowledge to maximize yield of a sample as high as possible. However, more time was required to recover higher yield, and unstable protein denatured and aggregated to produce "dirty" data. As high-sensitivity analysis became possible, it was no longer necessary to concern ourselves with "yield" of a sample. Rather, the most important concern was to increase "quality" or to prepare the samples as fast as possible before the proteins denatured and aggregated. However, it was not easy to do this in practice, and the technician in charge developed thorough protocol for sample preparation. This involved everything from ways to hold the test tube to arrangement of reagents on the bench, and emphasis was placed on the efficiency of the operator. Also, movement of the operator was filmed on video and studied to remove any unnecessary movements. Finally, we created and executed protocol where work, which previously took several hours or overnight, could be completed within one hour.

5 Research results and commercialization

Through these technological developments, we conducted large-scale protein network analyses using 2,200 human cDNA in about 5 years. There were over 16,000 analysis sessions. We were able to discover new cellular mechanisms functioned by proteins. The results were published in nearly 30 papers including twice in Nature and 6 times in Nature affiliated journals. We succeeded in analyzing protein networks of several disease-related genes, more than initially expected. We obtained findings that may lead to discovery and understanding of molecular disease mechanism as well

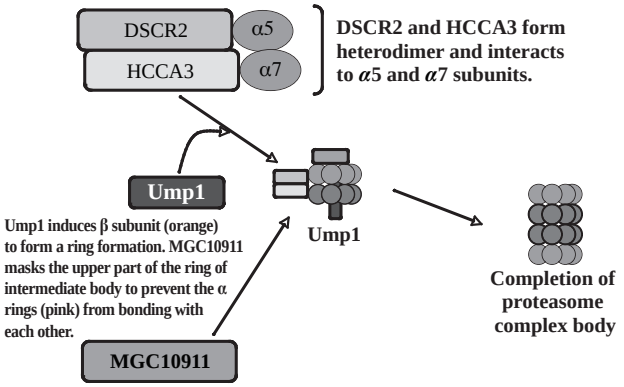

Fig. 3 Discovery of assembly factor of proteasome.
We discovered 4 assembly factors that composed the proteasome. Rather than inhibiting the proteasome itself, there is less side effect and higher possibility of being effective against wide range of cancer cells by inhibiting these assembly factors. They are better drug discovery targets.

as functional analysis of causal and related genes for cancer, life style disease, neurodegenerative disease, xeroderma pigmentosum, Down syndrome, Behcet's disease, and essential hypertension. We also discovered several cases in which proteins that were considered to have absolutely no relationship to disease may become totally new drug discovery targets[2]-[14]. I shall describe a representative example of network analysis in the following section.

There are huge protein complexes called proteasome in the cell, functioning as factories that degrade unnecessary proteins. The complexes consist of over 60 protein components, and it was long unknown how they were assembled. We discovered the assembly factors that assembled the proteasome. This was academically a significant discovery, where huge protein complexes are constructed with collaborative support of other proteins[4][11]. As shown in Figure 3, an assembly factor called DSCR2 and HCCA3 cooperated and arranged the α subunits in α ring form. Next, Ump1 and MGC10911 created the β ring structure, and α and β rings joined in a correct alignment. At the same time, this was a new drug discovery target.

Proteasome not only has a role of "quality control" or breaking down old and denatured protein, but it also has an important function of controlling diverse vital protein reactions. In cells, when new proteins become necessary for some vital reactions, it may be too late if they are synthesized as need arises. The cell continuously makes proteins that are expected to become necessary until the situation arises, and proteasome continuously degrades them. When the moment arrives when proteins become necessary, the degradation is halted and the necessary proteins can appear immediately.

For example, when cells divide, several proteins must cooperate in one direction and work closely together. This is lead by proteasome. Cancer cells that continue to divide indefinitely are thought to require more proteasomes than normal cells. It has been known that drugs that inhibit the action of proteasome possess powerful anticancer effect. However, severe side effects appear when such inhibitory drug is used, since proteasomes are necessary for a normal cell function. Therefore, this inhibitor is used only with special cancers with no other treatment. However, when the function of assembly factor of proteasome that we discovered was inhibited, the amount of newly created proteasomes decreased, and there was hardly any effect on normal cells although it was fatal for cancer cells. Since normal cells do not require as much proteasomes as cancer cells, they are less susceptible to some decrease in proteasome level. Also, we expected that that there will be fewer side effects, unlike complete inhibition of proteasome function. This assembly factor would be a new and more suitable drug discovery target.

Since these results could be directly applicable to drug discovery research, we suggested corroboration research with pharmaceutical companies in 2006. As a result, we started a drug discovery research project in which almost all Japanese major and medium pharmaceutical companies participated. The initial grand design of this project was based on protein network analysis of disease related or causative genes or proteins in which each pharmaceutical company was interested, for drug target discovery. However, the project was taken further, and aimed at establishing drug screening platforms under corroboration of pharmaceutical companies based on the information of protein network analysis. When the project finds 'hit' compounds, AIST provides the hit information for corroborative companies for developing therapeutic drugs. To do this efficiently, Computational Biology Research Center[Note] in AIST Tokyo Waterfront also got involved in the project to bridge hit compounds and combinatorial chemistry by in silico simulation using the hyper parallel computer system. Eventually, to enhance activity of the industry, the project was designed[15] to provide research resource and facility which can not be equipped in each single private sector, such as mass spec facility, large-scale natural compound library and computatonal resources like Blue Gene[Term 1].

6 Discussion: strategy toward Full Research

The most basic strategy that we implemented when we planned and started the protein network analysis project was: "we shall not aim for eccentric and extraordinary innovation." Even if we created a wonderful technology or technique, it normally takes at least 10 years before it is standardized as a analysis method and begins to generate data. In fact, it was in the early 1980s that Mr. Koichi Tanaka ionized peptide protein using matrix and conducted mass spectrometry for the first time in the world. This discovery led to the development of MALDI mass spectrometry, which the protein chemists and biologists around the world started to use in the late 1990s to 2000.

At the time, we thought spending 10 years developing analytical technology was unrealistic. We adhered to the most realistic, most down-to-earth, and "straightforward" way of doing things, that is, to minimize the greatest bottleneck in current mass spectrometry. The "straightforward" method was to "transfer microquantity of sample to mass spectrometer without loss," and we focused thoroughly on this issue. We decided not to dip our hands into new innovations such as improvement of mass spectrometer or increasing efficiency of ionization. The mass spectrometer itself was already highly sensitive, and we placed our bet on a hope that if ionization could be accomplished without loss of sample, we could obtain the target sensitivity.

If we could achieve ultra high sensitivity, large-scale analysis at high throughput would become possible. The

greatest bottleneck of protein experiment was, needless to say, the preparation of samples. Therefore, we decided to challenge the big projects that required 50~100 people in Europe and US with just a few people and limited time by improving throughput of analysis through "attainment of ultra high sensitivity." In fact, we achieved high sensitivity "that surpassed our expectation" by creating new elemental technology called gradient method, but it was not useful in practice. That was because the S/N ratio worsened due to noise from the environment. We painfully realized that improvement of S/N ratio is necessary to implement 'real' high-sensitive analysis by MS, or battle against noise, and we also understood the reason why development of micro liquid chromatography was not undertaken elsewhere in the world.
The developed prototype lacked durability, was damaged easily by dust particles, and required much time for maintenance. One success was the beginning of the next suffering. However, we have been using them, rather than improving ease of maintenance of the LC system. This was only possible since we designed the entire device all the way to its screw. Although our device and system was full of defects, we believed it was more important to "use it and get data," and set that as priority. The subject of analysis was initially narrowed down to known molecules that were very well characterized, in spite of analyzing unknown molecules. We had two reasons to this. First, if the analysis system we developed had truly high sensitivity and high throughput, there must be a new discovery even in an area that was already thoroughly characterized. Second, if there was a new discovery, we could validate and publish the data, because there is plenty of information and knowledge for well known molecules. These were our aims.

7 Conclusion

To claim a new system to be "high sensitive" or "high throughput," the system has to generate large-scale and highly accurate data. And the only way to demonstrate this is by publishing such data in as many high quality journals as possible. We thought there was no other way of objectively demonstrating the superiority of our newly developed system. This was particularly true because our strategies were steady improvements and gradual accumulation of know-how. We were unable to demonstrate our results as intellectual property by publishing papers that claimed novelty or innovativeness of methodology. In fact, the only thing we can call innovation in our development was a single-pump gradient method, and all other technologies were adaptation of existing elemental technology of other fields (semiconductors and industrial robots). We simply utilized them and thoroughly optimized the classic biochemical experiment method. Fortunately, these strategies and tactics turned out successful, and we realized the "flow" of microquantity of 100 nanoliters or less per minute. We hope this flow initiate new mainstream of drug discovery.

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Note Participation of Takatsugu Hirokawa, Research Team Leader, Drug Discovery Molecular Design Team, Computational Biology Research Center.

Terminology

Term 1. Famous episode in which massively parallel computer with 8,000 CPU beat a chess master.

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Discussion with reviewers

1 On integration of elemental technology

Question (Noboru Yumoto)

The originality of this paper is the success in integrating the elemental technologies including 1) construction of new system of liquid transfer, 2) improvement of analytical environment, 3) adjustment of analytical flow passage with minimum dead volume, and 4) optimization of sample preparation, toward the objective "to construct ultra high-sensitivity mass spectrometry system for protein network analysis." Please explain 2), 3), and 4) as you did for 1), and how you achieved the objective by integrating the selected elemental technology.

Answer (Tohru Natsume)

The technologies for 2), 3), and 4) were basically accumulation of know-how and introduction of other technologies, and they were optimizations and combinations of existing technologies. I abbreviated them because they may sound like side stories. However, they may be important, as you indicated, so I added some stories taking care not to distract from the main theme of the paper.

2 On relationship between research objective and society

Question (Hisao Ichijo)

Research for problem solving is very detailed and convincing, but I feel there is some lack of "relationship between research objective and society" and "evaluation of result and future development."

I think it will be easier to understand if you add some description that the objective "to find pathogenic mechanisms and new drug discovery targets by conducting large-scale high-precision protein network analysis" has been partially achieved.

Answer (Tohru Natsume)

The specific story of new dug discovery target was described taking the example of proteasome assembly factor that was published in *Nature*.