2 Structural Biology Research Center

2-1 Overview

The Structural Biology Research Center (SBRC) was founded in May 2000, in the Photon Factory (PF), Institute of Material Structure Science (IMSS). The main tasks of the center include user support of synchrotron radiation X-ray crystallographic studies of bio-macromolecules, highly advanced technical development, and in-house structural biology research. Over 30 people now work at the center, including a professor (Dr. S. Wakatsuki), two associate professors (Drs. R. Kato and N. Igarashi), a vice associate professor (Dr. M. Hiraki), and four assistant professors (Drs. N. Matsugaki, M. Kawasaki, Y. Yamada, and L. Chavas) as the core members. Dr. N. Shimizu will join us in April 2011 as an associate professor. About half of the SBRC members are engaged in beamline operation and development, with the remainder working in biological research (Fig. 1). The in-house biological research is carried out in the Structural Biology Building, which was built in April 2001 (430 m^2) and extended twice to 765 m^2. All of the necessary steps for structural biology research can be carried out in the building, including protein expression, sample purification, crystallization, and biochemical and biophysical analyses of the targeted proteins.

Our research activities are mainly supported by scientific funds. The first high-throughput beamline, AR-NW12A, was built with funding from the PF supplemental budget during FY2000–FY2001. We were able to build a second up-to-date beamline, BL-5A, and also prepare various scientific equipment for protein expression, purification, and crystallization experiments using funding from the “Special Coordination Funds for Promoting Science and Technology” (FY2001–FY2003) from the Japan Science and Technology Agency (JST). Next, we participated in two large-scale projects—the “Protein 3000 National Project” from the Ministry of Education, Culture, Sports, Science and Technology Japan (MEXT)/JST (FY2002–FY2006) and “Development of System and Technology for Advanced Measurement and Analysis” from JST (FY2004–FY2007).

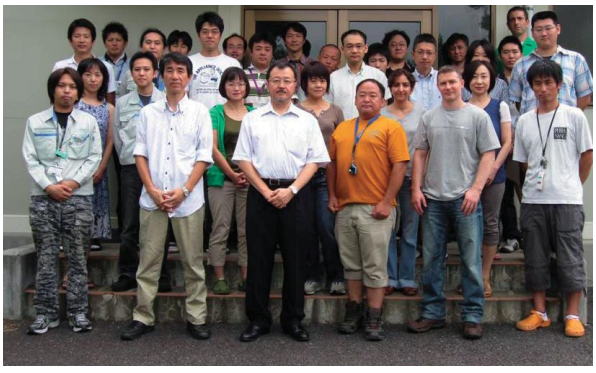

Figure 1
Members of the Structural Biology Research Center (SBRC) photographed at the entrance to the Structural Biology Building.

During the five-year Protein 3000 Project, the SBRC operated in one of the eight consortia, aimed at pursuing structural and functional analyses in the field of post-translational modification and transport. Our consortium, which consists of eleven universities and four research institutes, determined 254 protein structures and published 296 papers in this five-year period. To further assist the Protein 3000 Project, we set aside about 30% of the bio-macromolecular crystallography beam time at the PF (a total of 327 days) for users of the eight consortia. During the “Development of System and Technology for Advanced Measurement and Analysis” project, we developed a micro-beam beamline, BL-17A, and a prototype of a next-generation X-ray area detector. BL-17A is the first beamline developed at PF with a short gap undulator as a light source. The beam size can be controlled from 100 × 100 to 10 × 10 μm^2 and is useful for both modest and small protein crystal experiments. The prototype of a new generation X-ray area detector was developed in collaboration with the laboratory of Japan Broadcasting Corporation (NHK) and several other companies, and showed advantages in spatial resolution and X-ray sensitivity compared with CCD detectors.

The new five-year national project “Targeted Proteins Research Program” by MEXT/JST is now in progress; it began in FY2007 and will end in FY2011 (Fig. 2). The SBRC contributes to this project through two independent programs. The first involves structural and functional research on protein transport in cells, in close collaboration with the University of Tokyo and Kyoto University. The other involves beamline development in collaboration with SPring-8, Hokkaido University, Kyoto University, and Osaka University. The details of these programs are described later.

In addition to its own scientific research activities, the SBRC accepts many researchers from outside KEK who wish to collect diffraction data for their own macromolecular crystals under the PF Program Advisory Committee (PAC) system. Academic proposals and

Table 1 Numbers PF proposals for protein crytallography beamlines in recent five years.

Type	G	P	U	S2	Total
2006	86	0	0	1	87
2007	117	2	3	0	122
2008	101	0	0	1	102
2009	115	1	0	0	116
2010	103	2	1	0	106

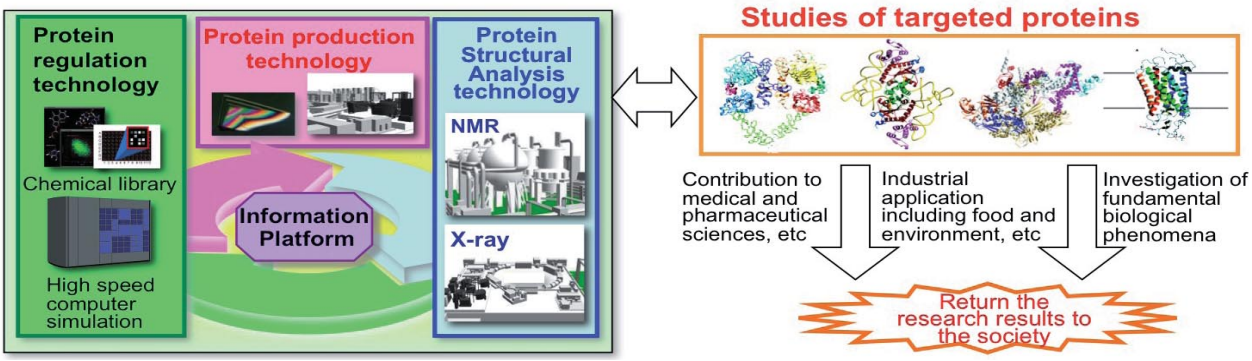

Figure 2
Scheme of the Japanese national project "Targeted Proteins Research Program" by MEXT (FY2007–2011).

users have been increasing in recent years, as summarized in Table 1. Additionally, together with advances in structure-based drug design, pharmaceutical companies require a large amount of beam time. As a direct consequence, twelve Japanese companies have been using the bio-macromolecular crystallography beamlines at KEK-PF. Among these, Astellas Pharma Inc. financed the construction of a beamline, AR-NE3A, for their research.

On March 11, 2011, a major earthquake struck the north-eastern region of Japan, with its epicenter the north-east offshore of Japan. Even though Tsukuba city is located far from the epicenter, several areas of KEK suffered serious damage. Fortunately, the SBRC suffered little damage at both the beamlines and the wet laboratories. However, because of the long shutdown and restrictions on the use of electric power, the beamline operation was canceled for first period of FY2011. Moreover, almost all of the crystallization plates were lost (Fig. 3), along with many samples and chemicals stored at low temperature.

Figure 3
Photograph of crystallization room at the SBRC building after the earthquake. The electricity supply remained cut for about two weeks after the earthquake.

2-2 Targeted Proteins Research Program (FY2007-2011)

Structure-Function Analysis of Protein Complexes that Regulate Vesicular Traffic

In the field of "Investigations of fundamental biological phenomena," we began a "Structure-function analysis of protein complexes that regulate vesicular traffic." This project is dedicated to protein targets of crucial importance and for which it is extremely difficult to determine the crystal structures such as protein complexes, including the membrane proteins involved in membrane traffic in eukaryotic cells. Cell signaling and intracellular trafficking are the means by which eukaryotic cells deliver cargo proteins to various organelles, cell membranes, and extracellular destinations. An accurate distribution of these proteins is crucial for a range of cellular functions and activities. A better understanding of the biological and biomedical functions of transport and modification proteins is indispensable for progress in the treatment of human diseases.

For this project, the SBRC is collaborating with Prof. Akihiko Nakano (University of Tokyo), who is conducting functional research on the proteins involved in Golgi formation, post-Golgi membrane traffic, and endocytosis in yeast and higher plants; Prof. Kazuhisa Nakayama (Kyoto University), who is studying the proteins involved in the regulation of cell functions such as cytokinesis, cell polarity, and cell mobility; and Prof. Ken Sato (University of Tokyo), who is researching transport vesicle formation from endoplasmic reticulum (ER) in yeast. With the close cooperation of these researchers, the SBRC is currently working on the expression, purification, crystallization, and structural determination of target proteins, which are either membrane proteins or those forming macromolecular complexes. This collaboration includes Prof. Nagano (University of Tokyo) and Prof. Sakisaka (Kobe University), who are members of the program.

Technological Development Research for Protein Structural Analysis

The phase problem is one of the most important issues when trying to determine the three-dimensional structures of bio-macromolecules using X-ray crystallography. Sulfur single wavelength anomalous dispersion (SAD) analysis is currently one of the most attractive methods to bypass this problem, especially when heavy atom (or selenomethionine) derivative crystals of the targeted macromolecules are difficult to prepare, e.g., integral membrane proteins and macromolecular complexes. We developed a new beamline dedicated to sulfur SAD experiments as a part of the national project "Targeted Proteins Research Program." This beamline, BL-1A (Fig. 4), was designed to deliver an intense lower energy beam of around 4 keV using the first harmonics of the short gap undulator as the light source, to enhance the anomalous signal from light atoms such as sulfur and phosphorous. The beamline also covers the energy range of 12–13 keV with the third harmonic, allowing multiple wavelength anomalous dispersion (MAD) measurements using Se, Hg, Au, or Pt atoms. The beamline became available to members of the program in FY2010, and a group succeeded in determining the novel structure of a protein using sulfur SAD (Fig. 4).

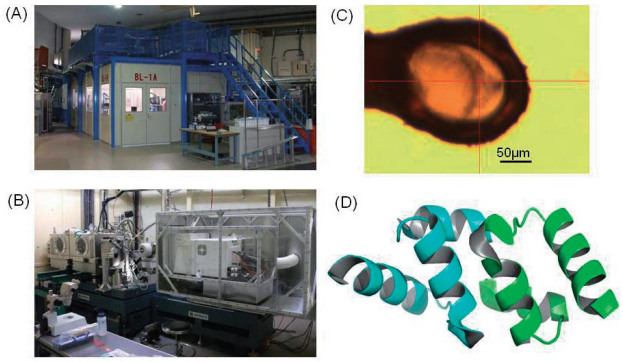

Figure 4
Outcome of the new beamline, BL-1A. Outside view (A) and inside view (B) of BL-1A. (C) Small crystals in the sample loop for measurement. (D) Structure of a globular protein, which consists of 50 amino acid residues with two sulfur atoms. The low energy sulfur SAD method was used to determine the structure. The data and structure were kindly provided by Dr. K. Arita (Kyoto University).

In addition to the construction of BL-1A at PF, this research project is involved in the development of another high-energy beamline at SPring-8, with a brilliant and small beam, as well as some environmental technology developments by other universities (Hokkaido, Kyoto, and Osaka Universities). The compatibility of the sample cassettes has been one of the most important problems, e.g., the SPACE pin developed at SPring-8 cannot be used at any other facilities, and our PAM can deal with Hampton-type general sample pins with an SSRL cassette but not the Uni-puck cassette, which is now widely used around the world. As part of this project and in collaboration with the universities, the SBRC has developed a cassette adaptor which allows PAM to handle the Uni-puck cassette, and a compatible cassette that can deal with both SPACE and Hampton pins. Furthermore, we began new collaborations with Dr. Ito (RIKEN) and Prof. Iwai (Osaka University).

2-3 Highlights on In-House Structural Biology Research

New Crystallization Method for Small Proteins Assisted by Green Fluorescent Protein

Fusion with a tag protein, in order to generate a scaffold for the crystal lattice, is an attractive approach to assist the crystallization of intractable proteins. Green fluorescent protein (GFP) has been used for monitoring the expression of soluble proteins, as well as membrane proteins, in order to identify better-expressing proteins or domains. We examined whether GFP could be used

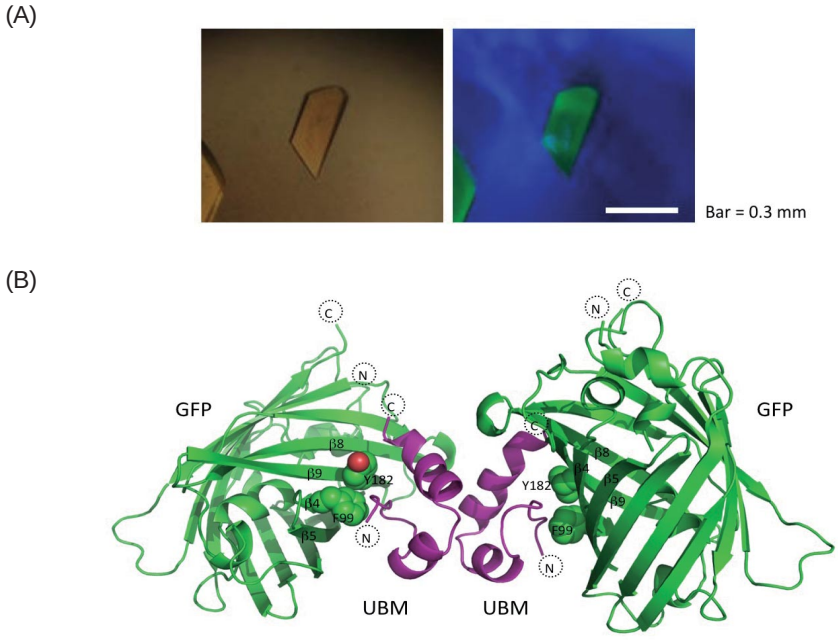

Figure 5
(A) Crystals of GFP-ubiquitin fusion protein observed under white light (left) and blue LED light (right). (B) Crystal packing of GFP-UBM2 fusion protein. The GFP and UBM2 moieties are colored green and magenta, respectively, and the N and C termini are marked by dotted circles. The side chains of Phe99 and Tyr182 of the GFP tyrosine belt involved in the interaction with UBM2 are shown as spherical models.

as a fusion tag for protein crystallization, because the visualization of proteins by GFP is advantageous, not only for expression and purification but also for crystal observation and crystal handling. As a case study, we examined the applicability of the GFP-fusion method to crystallization using small proteins or domains. We used ubiquitin as an example of a small protein and ubiquitin-binding motif (UBM) for a small domain. We noticed that tyrosine residues are arranged in a line on the surface of the β-barrel structure of GFP, a feature we termed the "tyrosine belt." The tyrosine belt of GFP is involved in protein-protein interaction in both GFP-ubiquitin and GFP-UBM fusion crystals (Fig. 5). Therefore, GFP has the potential to become a versatile tag to assist in the crystallization of small proteins or domains, as well as helping to visualize them [1].

Interaction of Human Sialidase Neu2 with Influenza Virus Targeted Drugs

Sialidases (or neuraminidases) are enzymes that catalyze the hydrolysis of sialic acids from sialoglycoconjugates. They are ubiquitously expressed in all organisms except plants, and four isoforms (Neu1–4) are observed in humans. The human cytosolic Neu2, in particular, was the first mammalian sialidase for which the crystal structure was solved and reported. Neu2 adopts the typical six-bladed β-propeller fold common to all sialidases, with 26 β-strands and 5 α-helices. This conserved three-dimensional arrangement is necessary in order for the enzyme to create the active site cleft that will recognize and hydrolyze the sialic acid substrate. As a consequence, using functionally identical amino acid residues preserves the enzymatic mechanism for all sialidases.

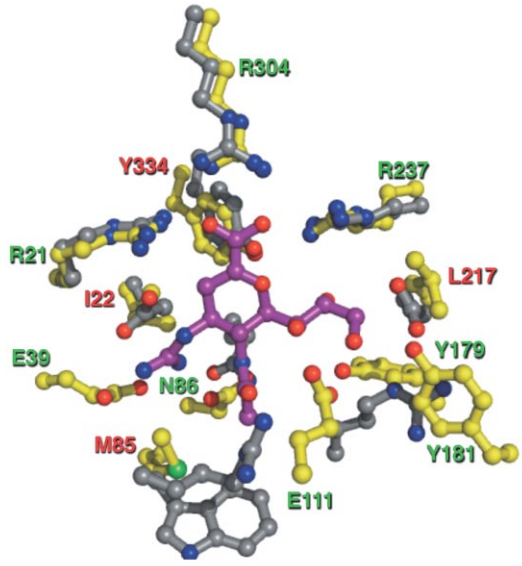

Figure 6
Coordination prints of Zmr by Neu2 and superimposed on influenza virus sialidase. The inhibitor molecule is represented as a purple ball-and-stick model. The active site residues for Neu2 (yellow) and the influenza virus sialidase (gray) show a conserved arrangement, notably for the arginine triad. The labels correspond to Neu2 residues.

A serious drawback of this conservation lies in the capacity of human sialidases to recognize inhibitors initially designed for reacting with the sialidases of other organisms such as the commercially available inhibitors Zanamivir (Zmr) and Oseltamivir, which are intensively used in the treatment of the influenza virus. To fully understand the potential differences in the binding mechanisms of these small molecules by the human sialidases, the structures of Neu2 in complex with several drugs were solved. The five different complex structures (Neu2 with inhibitors Zmr, Bcz, IEM, HEM, and DEM) show very similar recognition prints [2]. More precisely, the Neu2 arginine triad (example illustrated in Fig. 6) is responsible for the recognition of the inhibitor head group, assisted in the stabilization of the molecules by charged residues that form an extended hydrogen bond network. Interestingly, the fact that the arginine triad is completely conserved in all sialidases, while other surrounding residues show some variations in their natures, might be a starting point for developing newly designed drugs to selectively recognize either human or other sialidases. These structures not only provide a potential explanation for the secondary effects observed in infants affected by the influenza virus and treated with sialidase inhibiting drugs, but also pave the way for the possible development of new molecules that would inhibit viral sialidases with greater specificity.

Molecular Basis of Retrograde Membrane Traffic through Endosomes

Phosphatidylserine (PS) is a relatively minor constituent of biological membranes. In spite of its low abundance, PS in the plasma membrane plays key roles in various phenomena such as the coagulation cascade, clearance of apoptotic cells, and recruitment of signaling molecules. PS also localizes in endocytic organelles, but how this relates to its cellular functions remains unknown. PS was most concentrated in recycling endosomes (REs) among intracellular organelles, and Evectin-2, a protein of previously unknown function, was targeted to REs by the binding of its pleckstrin homology (PH) domain to PS.

To elucidate the molecular mechanism of how Evectin-2 interacts with a lipid membrane, especially PS, we determined the X-ray crystal structure of the human Evectin-2 PH domain in complex with O-phospho-L-serine, the head group of PS, at a resolution of 1.0 Å [3]. The overall structure was similar to the standard PH domain fold, with seven β strands forming two orthogonal antiparallel β sheets and two α helices. O-phospho-L-serine binds to the positively charged pocket made by three basic residues (Arg11, Arg18, and Lys20) (Fig. 7). Arg11 and Arg18 make two salt bridges with the L-serine oxygen atoms and the phosphate oxygen of the ligand, respectively. In addition, Lys20 makes salt bridges with both moieties of the ligand. Next, a mutational study was conducted on the amino acid residues involved in the ligand binding. The K20E mutant, in

which Lys20 was changed to Glu, lost RE localization and showed puncta around the Golgi. These puncta did not co-localize with REs, but co-localized in early endosomes and late endosomes. In addition, the PH domain mutant of Arg11, Arg18, or Lys20 (R11E, R18E, K20E) did not show any specific membrane localization, suggesting that these residues bind to the head group of PS in vivo. All of the residues involved in the direct interaction of the ligand were conserved in the Evectin-2 PH of other species, further implicating these residues in the recognition of PS.

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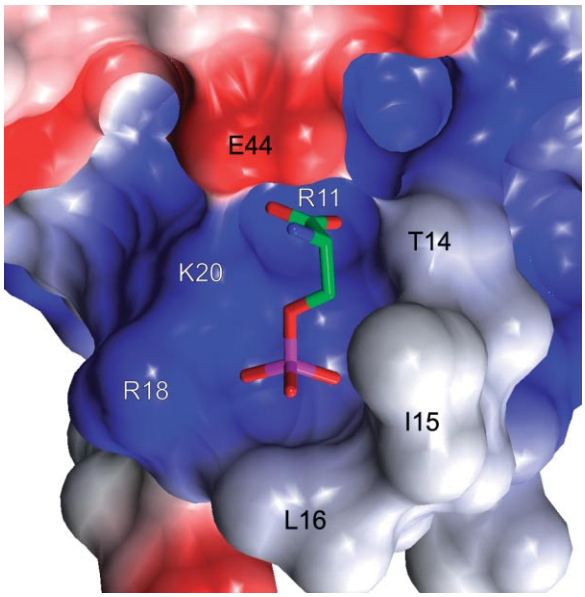

Figure 7
Charge distribution surface model of Evectin-2 PH in complex with O-phospho-L-serine. The surface is colored according to the electrostatic potential of the residues (blue, positive; red, negative). O-phospho-L-serine is shown by a stick model.

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2-4 Beamlines and Technology Developments for Macromolecular Crystallography

Beamlines

The SBRC currently operates five insertion device (ID) X-ray beamlines for bio-macromolecular crystallography (Table 2). Among these, BL-5A, AR-NW12A, and AR-NE3A are high-throughput structural biology beamlines. BL-17A is a micro-beam beamline designed for micro-crystal structure analysis, as well as SAD phasing with light atoms using a lower energy beam of around 6–8 keV. The newest beamline, BL-1A, began operation in FY2010, as described above. A rearrangement of the X-ray area detectors installed at each beamline was carried out. The ADSC Quantum 270 moved to BL-1A from BL-17A, and the ADSC Quantum 315r at BL-5A was exchanged with the ADSC Quantum 210r at BL-17A.

AR-NE3A, which began operation in FY2009, is dedicated to the high-throughput bio-macromolecular crystallography required for pharmaceutical applications. AR-NE3A can generate the most intense X-ray beam at the sample position among the bio-macromolecular crystallography beamlines at the PF [4]. The flux is about three times higher than that of another high-throughput beamline, AR-NW12A. In addition, a high-precision diffractometer, a fast-readout and high-gain CCD detector with a large active area, a sample exchange system (PAM), and an automated data collection system realize high throughput and precise data collection: a user can collect more than 150 datasets in a day. The construction of AR-NE3A was financially supported by Astellas Pharma Inc., and they have priority access to this beamline for their research. The remaining beam time will be assigned to general academic users and other industrial users.

Table 2 Summary of protein crystallography beamlines at KEK-PF.

	BL-1A	BL-5A	BL-17A	AR-NW12A	AR-NE3A
Starting year	2010	2004	2006	2003	2009
Synchrotron ring	PF (2.5 GeV, 450 mA)			PF-AR (6.5 GeV, 40-60 mA)	
Injection	continuous (top up)			twice a day (10:00, 22:00)	
X-ray source	Short Gap Undulator	Multipole Wiggler	Short Gap Undulator	Undulator	Undulator
Photon flux (photons/sec @ 1.0 Å)	2×10^{10}(1.0 Å) 2×10^{9}(2.7 Å) *1	3.0×10^{11}	6.6×10^{9}(@1.0 Å) 1.3×10^{10}(@2.0 Å) *2	2.9×10^{11}	8.0×10^{11}
Wavelength (Å)	0.9 ~ 1.1, 2.7	0.7 ~ 1.9	0.9 ~ 2.1	0.7 ~ 1.9	0.9 ~ 1.9
Beam size optimized (μm)	30 × 20	200	50	200	200
Detector	ADSC Q270	ADSC Q210r	ADSC Q315r	ADSC Q210r	ADSC Q270
Purpose	low energy micro beam	high throughput	low energy micro beam	high throughput	high throughput
Sample exchanger	PAM (288 samples / Dewar)				

*1 flux on 10 × 10 μm^2, *2 flux on 20 × 20 μm^2

Technology Developments

For further high-throughput and more convenient bio-macromolecular crystallography, we have improved the automation of the beamline control, along with the operation of the sample exchange system, automatic sample centering system, and automated data processing. We are also working extensively on the implementation of remote technologies for the beamlines and, at present, users can access our beamlines remotely in two different ways. The first is remote access to control the beamline from outside of KEK and the other is remote monitoring to monitor the experimental results at the beamlines via the Web. With a robust remote desktop technology (NX remote desktop technology, *www.nomachine.com*), users can access the machine from their homes or laboratories using beamline controlling software. To assist with remote access, the interlock group of the PF has developed an external mode of beamline shutter control. For remote monitoring, we have introduced middleware R&D Chain Management (RCM) system software, which consists of three main components, namely, a controller, Web interface, and database. The controller monitors the experiment and stores the results in the database. Users can browse their experimental results stored in the database via the Web interface. The controller also submits a data processing job, according to the experimental conditions, to a workstation cluster. This makes our development of automatic data processing more efficient. Using this monitoring system and automated data collection system, we plan to test a mail-in-service during the next fiscal year.

Because the size of protein crystals becomes small recently, it is difficult to identify the position of the crystals in the sample loop. We have been studying two independent methods to see the crystal position. One is “diffraction centering,” in which the loop containing crystal(s) is scanned using an X-ray micro-beam and diffraction images are recorded for every scanned point. The position of the crystal(s) is identified on the basis of the scanned position where diffraction patterns from the crystal are obtained. This method requires an X-ray beam of sufficiently small size and a 2-D X-ray detector with very fast readout. A test experiment was performed using a pixel array detector, Pilatus 100K (Dectris), at BL-1A, with supporting results (Fig. 8). The second

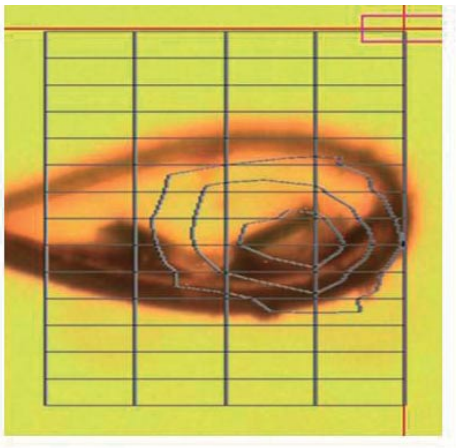

Figure 8
Diffraction centering for visualization of small crystals. The loop containing a Neu2 crystal was scanned using a 70 µm × 20 µm beam with 75 (5 × 15) grids. The contour map was drawn based on the number of diffraction spots observed for each grid. The exposure and readout time for each grid were 17 ms and 3 ms, respectively, so the total (effective) time required for the measurement was only 1.5 s.

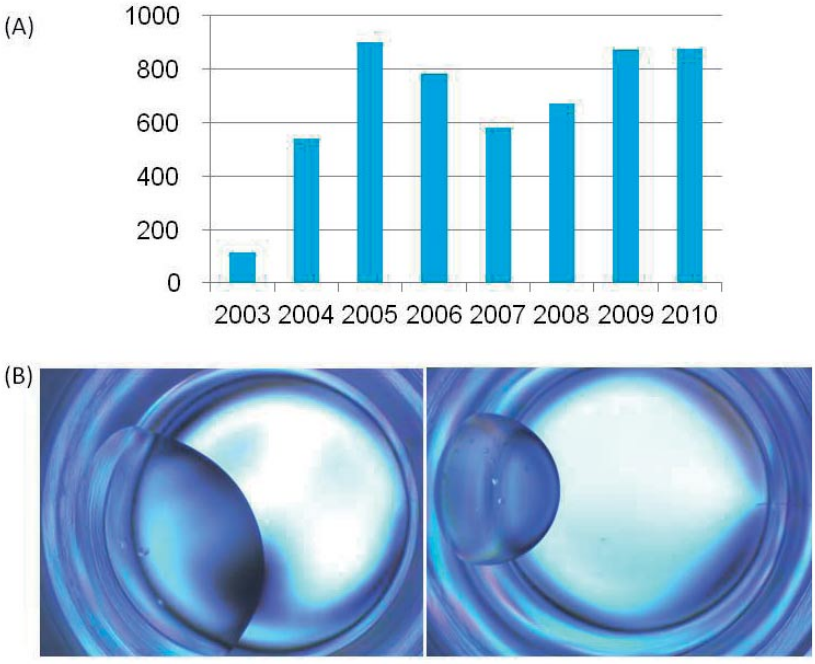

Figure 9
Status and upgrading of the fully automated crystallization robot. (A) Numbers of crystallization plates set up by the automated crystallization robot (PXS) developed by the SBRC. (B) Decreasing the dispensing volume for the crystallization drop from 0.5 µl (previous) to 0.2 µl (after the upgrade) improves the number of crystallization conditions when the same amount of sample is used.

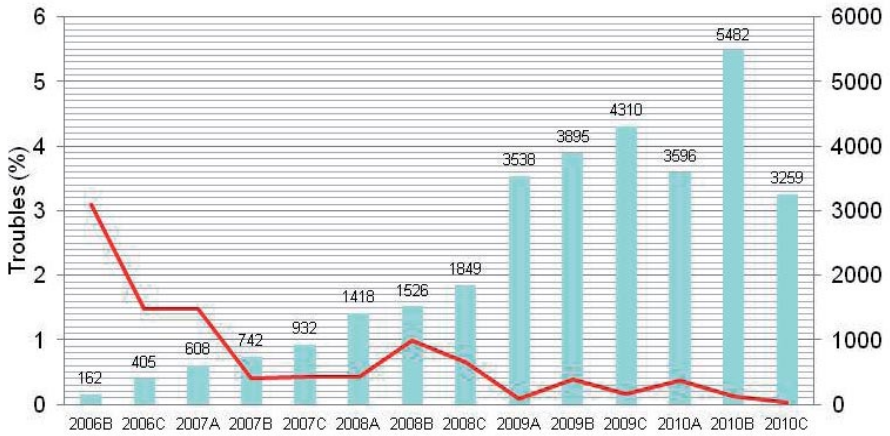

Figure 10
Status of sample exchange robot. The number of sample pins exchanged by PAM is shown by bars, and the error rate by the red line.

method involves visualizing protein crystals using UV. Proteins fluoresce when irradiated with UV light. By using the fluorescence from the protein, we can see the position of the protein crystal in the sample loop.

We have been operating two automated systems. A fully automated high-throughput crystallization and observation system was developed in 2003, and it is working without serious problem (Fig. 9A). At the end of this fiscal year, we improved the system to reduce the minimum dispensing volume to 0.2 µl from 0.5 µl (Fig. 9B). During the next fiscal year, we plan to overhaul all of the components of this crystallization and observation system. This improvement, along with routine maintenance, is important to keep the system up-to-date. The PAM system has now been installed and is in operation at all of the beamlines. The number of exchanged sample pins is increasing, and the error rate is decreasing (Fig. 10). The PAM system is essential to realize fully automated data collection [5].

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