2 Structural Biology Research Center

2-1 Overview

The Structural Biology Research Center was founded in May 2000, in the Photon Factory (PF), Institute of Materials Structure Science (IMSS). The main tasks of the center are user support of synchrotron radiation X-ray crystallographic studies of macromolecules, highly advanced technical development, and in-house structural biology research. The center now has about 30 members, including a professor (Dr. S. Wakatsuki), an associate professor (Dr. R. Kato), two vice associate professors (Drs. N. Igarashi, M. Hiraki) and three research associates (Drs. N. Matsugaki, M. Kawasaki, Y. Yamada) as the core members (Fig. 1). About half of the members are engaged in beamline operation and development, and the remaining half in biological research. After having been newly-built in April 2001 (430 m^2), the structural biology building was extended in 2003 (by 215 m^2) and in 2007 (by 120 m^2), and the total area is now 765 m^2. In the building, it is possible to conduct protein expression using bacteria, yeast, and insect-cell systems, and also to carry out purification, crystallization and biological analyses (such as surface plasmon resonance studies) of the target proteins.

Our research activity is supported by many external scientific funds. The first high-throughput protein crystallography beamline, AR-NW12A, with an undulator light source, was built under the PF FY2000-FY2001 supplemental budget. We built a second modern beamline, BL-5A, and also purchased laboratory equipment for protein expression, purification and crystallization experiments with a “Special Coordination Funds for Promoting Science and Technology” fund (FY2001-FY2003) from JST (Japan Science and Technology Agency). The next big projects were the “Protein 3000 National Project” from MEXT (Ministry of Education, Culture, Sports, Science and Technology Japan) / JST (FY2002-FY2006) and a “Development of System and Technology for Advanced Measurement and Analysis” award from JST (FY2004-FY2007).

In the five-year Protein 3000 Project, the Structural Biology Research Center serves in one of the eight consortia, pursuing structural and functional analyses in the field of post-translational modification and transport. Our consortium consists of eleven universities and four research institutes. Cell signaling and intracellular trafficking are the means by which eukaryotic cells deliver cargo proteins to various organelles, cell membranes, and extracellular destinations. During the trafficking process, more than half of the eukaryotic proteins undergo post-translational processing and modification such as glycosylation. Accurate distribution and modification of the proteins are crucial for a range of cellular functions and activities. An increased understanding of the biological and biomedical functions of transport and modification proteins is indispensable for progress in the treatment of human diseases. To facilitate the research program, the Structural Biology Research Center has taken the lead by coordinating the research network. Our initial research plan was to accomplish the structural and functional analyses of more than 70 proteins in five years, but by the end of the project 254 structures had been determined and 296 papers published [1-5]. Furthermore, we have submitted 13 domestic and two international patent applications based on our R&D and structural and functional studies of the target proteins.

Figure 1
The members of Structural Biology Research Center pictured at the entrance to the Structural Biology Building.

Since the cumulative number of structures determined by the Protein 3000 Project has exceeded 3,000, the project has been a success, to which our consortium has made a considerable contribution. To assist the Protein 3000 Project, we set aside about 30% of the beam time (total 327 days) of our synchrotron beamlines for users of the eight consortia

In the “Development of System and Technology for Advanced Measurement and Analysis” project, we developed a micro-beam beamline, BL-17A, and a prototype next-generation X-ray area detector, X-ray HARP-FEA. BL-17A is the first beamline at the PF which has a short gap undulator as its light source. We constructed the beamline with the help of other PF staff, especially from the light source division. The beam size can be varied from 10 × 10 to 100 × 100 μm^2, and is useful for both modest and small protein crystal experiments [6]. The next-generation X-ray area detector was developed in collaboration with the NHK (Japan Broadcasting Corporation) laboratory and several commercial companies. Based on HARP (High-gain Avalanche Rushing amorphous Photoconductor) and FEA (Field Emitter Array) technologies, prototype detectors were constructed which showed increased spatial resolution and X-ray sensitivity compared to CCD detectors [7].

The five-year national project “Targeted Proteins Research Program” funded by MEXT/JST began in FY2007 (Fig. 2). The Structural Biology Research Center contributes to the project in two independent programs. One program is the structural and functional research of protein transport in cells in tight collaboration with the Univ. of Tokyo and Kyoto Univ. The other is beamline development in collaboration with SPring-8, Hokkaido Univ., Kyoto Univ. and Osaka Univ. The details of the programs are described later.

Table 1 Numbers of proposals at the protein crystallography beamlines over the past five years.

Proposal type	G (general)	U (urgent)	S2 (special)	Total
2003	44	0	1	45
2004	84	3	1	88
2005	81	0	1	82
2006	86	0	1	87
2007	117	3	0	120

In addition to scientific research activities, the Structural Biology Research Center accepts many researchers from outside KEK who collect diffraction data of their own macromolecular crystals. Academic proposals and users are increasing in recent years, as summarized in Table 1.

Accompanying advances in structure-based drug design, pharmaceutical companies now require large numbers of sets of diffraction data in short periods of time. Ten Japanese companies and one pharmaceutical consortium have been using beam time at the macromolecular beamlines at KEK-PF, and a new consortium was established from them under the coordination of the Structural Biology Research Center in FY2006. One of the companies, Astellas Pharma Inc., decided to build a new beamline, AR-NE3A, for their research. The beamline will open in April 2009.

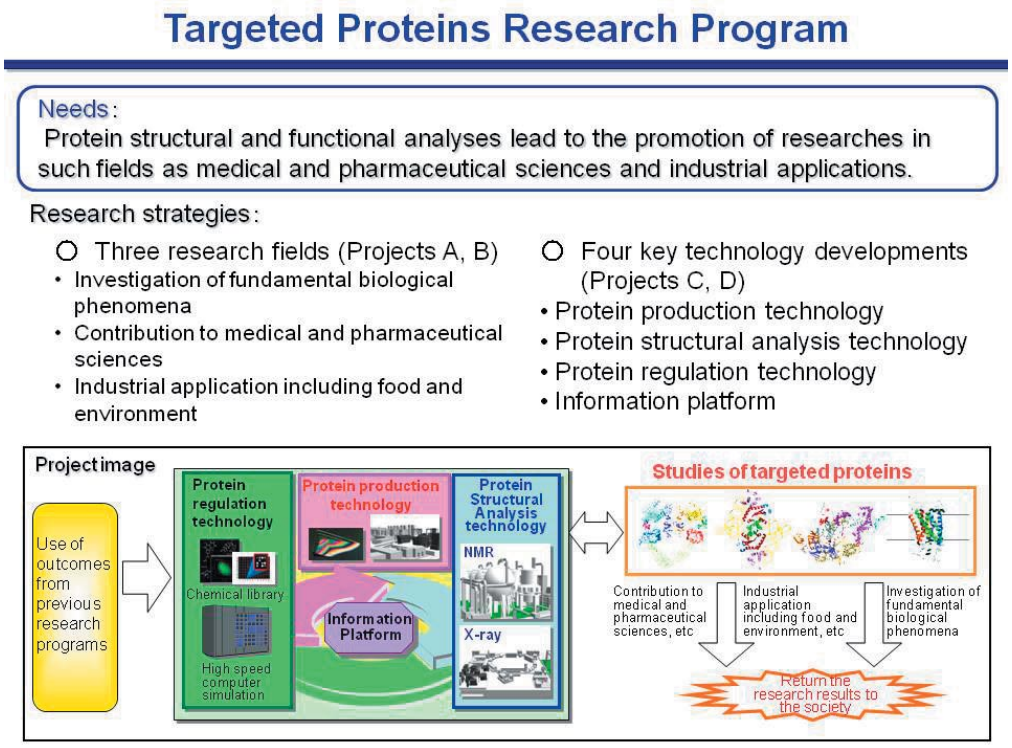

Figure 2
Schematic of the Japanese national project "Targeted Proteins Research Program" of MEXT (FY2007-FY2011).

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2-2 Targeted Proteins Research Program

Research Project A: Investigation of Fundamental Biological Phenomena

In 2007, as one of the several numbers of "Research Project A" in the field of "Investigations of fundamental biological phenomena" in the "Targeted Proteins Research Program" of MEXT, we started a project titled "Structure-function analysis of protein complexes that regulate vesicular traffic". This project is dedicated to protein targets of crucial importance but with extreme difficulty, such as protein complexes including membrane proteins involved in membrane traffic in eukaryotic cells. The project is pursued in collaboration with Prof. A. Nakano (Univ. of Tokyo), who is working on functional research into proteins involved in Golgi formation, post-Golgi membrane traffic and endocytosis in yeast and higher plants, Prof. K. Nakayama (Kyoto Univ.), working on proteins involved in the regulation of cell functions such as cytokinesis, cell polarity and cell mobility, and Associate Prof. K. Sato (Univ. of Tokyo), who is researching transport vesicle formation from endoplasmic reticulum (ER) in yeast. With tight cooperation among the above researchers, the Structural Biology Researh Center is currently working on the expression, purification, crystallization and structure determination of these target proteins, which are all protein complexes or membrane proteins, in order to elucidate the molecular mechanism of how the proteins (complexes) function.

Research Project C: Technological Development Research (Protein Structural Analysis Technology)

In determining the three-dimensional structures of macromolecules by X-ray crystallography, the phase problem is one of the most important issues. Sulfur SAD (Single wavelength Anomalous Dispersion) analysis is currently one of the most attractive methods for this, especially for the range of macromolecules for which it is difficult to prepare heavy atom (or selenomethionine) derivative crystals, e.g. integral membrane proteins and macromolecular complexes. We started to develop a new beamline dedicated to sulfur SAD experiments as a part of the national project "Targeted Proteins Research Program", which began in 2007. The beamline, BL-1A, is designed to deliver an intense low energy beam at 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 phosphorus. The expected beam intensity at the sample position is in the order of 10^{11} photons/sec in an area of 10 × 10 μm^2, suitable for diffraction measurements from small crystals. The optics and the diffractometer will be specially designed to minimize losses for the low energy beam. The beamline also covers the energy range of 12-13 keV with the 3rd harmonics, which allows MAD (Multiple wavelength Anomalous Dispersion) experiments using Se, Hg, Au and Pt atoms. The construction of the beamline is scheduled for the summer of 2009, followed by a half-year commissioning. The beamline will be opened to the members of the national project in 2010.

2-3 Highlights of In-house Structural Biology Research

Structural Basis for Ceramide Specific Recognition by CERT

Ceramide is synthesized in the endoplasmic reticulum (ER), and transferred to the Golgi apparatus for conversion to sphingomyelin, which is ubiquitous component of membrane lipids in mammalian cells. Ceramide transport occurs in a non-vesicular manner, and is mediated by CERT, a cytosolic 68-kDa protein [8]. CERT specifically recognizes natural D-erythro ceramides but not sphingosine, sphigomyelin, cholesterol, or phosphatidylcholine. The C-terminal region of CERT is homologous to the steroidogenic acute regulatory protein-related lipid transfer (START) domain. The START domain of CERT has the activity to extract ceramide from donor membranes and release the bound ceramide to acceptor membranes. CERT efficiently transfers various ceramides having C14-C20 amide-acyl chains, but not longer amide-acyl chains.

We determined the crystal structures of the CERT START domain, in the apo-form and in complex with C_6-, C_{16}- or C_{18}-ceramide [9]. The overall structure shares a helix-grip fold, with two long α-helices, α1 and α4 at the N- and C- termini, separated by nine β-strands and two shorter helices (Fig. 3A). Two Ω-loops are inserted between β5-β6 (Ω1), and β7-β8 (Ω2). The apo- and ceramide bound-CERT START domain structures reveal essentially no difference in the Cα chains. The CERT START domain has a long amphiphilic cavity in the center of the protein. This cavity is composed of curved β-sheets covered by three α-helices (α2, α3 and α4) and the Ω-loops. One ceramide molecule is buried in the amphiphilic cavity of the CERT START domain (Fig. 3A, B). The two aliphatic chains of ceramide interact with

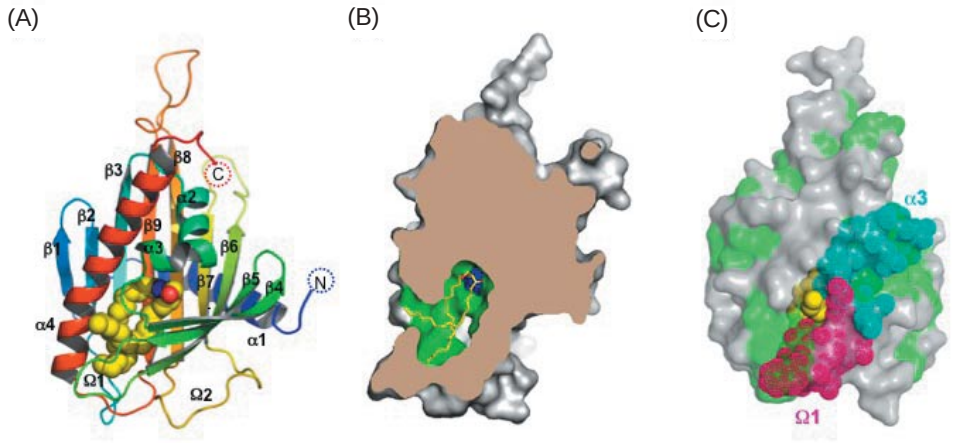

Figure 3
Ceramide trafficking protein CERT START domain in complex with ceramide.
(A) Overall structure of CERT STRAT domain (ribbon diagram) in complex with C_{16}-ceramide (yellow balls).
(B) Slice view of panel (A). The ceramide molecule is shown as a yellow wire model. Hydrophobic and charged regions in the cavity are colored green and blue, respectively.
(C) Molecular surface view of the complex. Putative gate regions are colored in red-purple (ω1) and light blue (α3).

the hydrophobic wall of the cavity. In ceramide complex structures, inspection of the space for the aliphatic group in the hydrophobic part of the cavity suggests that it might accept up to two more methylene groups, but not more. CERT may be able to distinguish the length of lipid amide-acyl chains that can be accepted in the hydrophobic cavity. In addition to the hydrophobic interaction, the binding of ceramide to the CERT START domain is achieved through recognition of the amide- and hydroxyl- group of ceramide by a hydrogen bond network involving residues Arg442, Glu446, Gln467, Asn504 and Tyr553, which are located at the far end of the amphiphilic cavity. Mutational analysis of these residues showed the reduced ceramide transfer activities by CERT. These results indicate that ceramide is recognized not only by the hydrophobic inner wall but also by a specific hydrogen bond network.

The amphiphilic cavity is almost completely closed, except for one small opening surrounded by residues of α3, α4 and the Ω1 loop, which is too small for ceramide to enter (Fig. 3C). However, higher temperature factors found in the apo-form structure might explain the mechanism. While α4 has rather low temperature factors, α3, Ω1 and Ω2 loops appear relatively mobile as compared to the rest of the apo-form CERT START domain. The right side wall of this entrance is formed by α3 and the Ω1 loop, which might act as the gate for ceramide to enter or leave the cavity. Thus, the structures demonstrate the structural basis for the mechanism by which CERT can grab a ceramide molecule and also distinguish it from other lipid types.

High Mannose Type Glycoprotein Recognition by VIP36

N-linked glycoproteins are subject to diverse modification and transport from the ER to the *trans*-Golgi network (TGN) in transport vesicles. Incorporation of the cargo glycoproteins into the transport vesicles is mediated by transmembrane transport lectins. VIP36 appears to function as transport lectin from the ER to the plasma membrane. In order to investigate the structural basis for the N-linked glycoprotein transport by VIP36, we determined the crystal structures of the exoplasmic/luminal domain of mammalian VIP36 alone and in complex with Ca^{2+} and the mannosyl ligands Man, Man_2 and $Man_3GlcNAc$ [10]. Based on the complex structures, we showed that Man-α1,2-Man-α1,2-Man, which corresponds to the D1 arm of high mannose type N-linked glycan, is recognized by eight amino acid residues of the protein through extensive hydrogen bonds in the presence of the Ca^{2+} ion (Fig. 4). The complex structures reveal the structural basis for high mannose type N-linked glycoprotein recognition by VIP36 in a Ca^{2+}-dependent and D1 arm-specific manner.

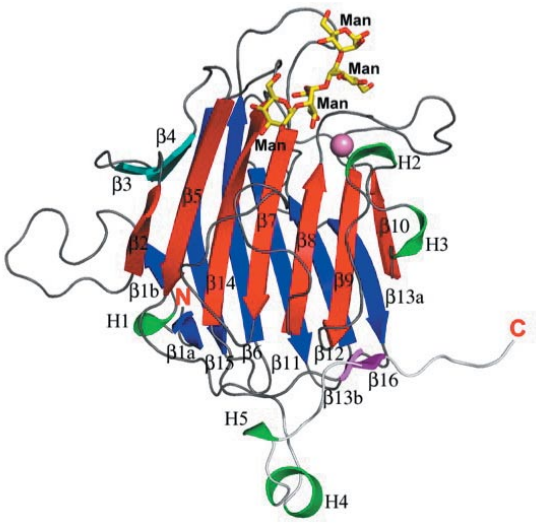

Figure 4
Glycoprotein transport lectin VIP36 in complex with high mannose type glycan.
The VIP36 protein is shown as a ribbon diagram, and the bound Ca^{2+} is shown as a pink sphere. The bound oligomannoses are shown as a yellow stick model.

Structural Basis of Carbohydrate Recognition by Animal Lectin, Galectin-9

Galectins are defined by shared consensus amino acid sequences which confer specific binding to β-galactoside-containing glycoconjugates. Galectin families are ubiquitously expressed from lower organisms, such as nematodes and sponges, to higher mammalian species such as humans. The presence of such proteins across many species coupled with the highly conserved amino acid residues which are critical for ligand recognition in the carbohydrate recognition domain (CRD) suggests that galectins are involved in critical, conserved biological processes. Galectin-9, which has two CRDs in its molecule, exhibits a variety of biological functions, including cell aggregation, eosinophils chemoattraction, and apoptosis of murine thymocytes and T cells and human melanoma cells.

We determined the crystal structures of the mouse galectin-9 N-terminal CRD (NCRD) in the absence and presence of carbohydrates [11]. The results demonstrated that the mouse galectin-9 NCRD forms a unique dimer that differs significantly from the canonical two-fold symmetric dimer seen for galectins-1 and -2 (Fig. 5A). Next, we determined the crystal structures of the human galectin-9 NCRD in complex with lactose or Forssman pentasaccharide [12]. Human galectin-9 NCRD exists as a monomer in crystals, despite a high sequence identity to the mouse homologue (Fig. 5B). Comparative frontal affinity chromatography analysis of the mouse and human galectin-9 NCRDs revealed different carbohydrate binding specificities, with disparate affinities for complex glycoconjugates. Human galectin-9 NCRD exhibited a high affinity for Forssman pentasaccharide; the association constant for mouse galectin-9 NCRD was 100-fold less than that observed for the human protein. The combination of structural data with mutational studies demonstrated that non-conserved amino acid residues on the concave surface were important for the determination of target specificities. We also observed that the human galectin-9 NCRD exhibited greater inhibition of cell proliferation than the mouse NCRD. These findings suggest that the biological difference between close species such as human and mouse is caused by glycoconjugate specificity invoked from the minor but important structural deference of galectin-9.

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2-4 Beamlines for Protein Crystallography

Current Status of Beamlines

The Structural Biology Research Center currently operates 4 structural biology beamlines. AR-NW12A, BL-5A and BL-17A are insertion device (ID) beamlines, while BL-6A is a conventional bending magnet beamline (Table 2). Among the ID beamlines, AR-NW12A and BL-5A are high-throughput structural biology beamlines. A micro-beam beamline BL-17A was newly constructed and opened to general users in 2006. It was designed for micro-crystal structure analysis and also for SAD phasing with light atoms using a low energy X-ray beam at around 6-8 keV [6].

In the next two years, two more beamline constructions are scheduled. A new high-throughput beamline, AR-NE3A, will be built in summer 2008 and opened to users in April, 2009. The beamline is expected to show a higher performance than current high-throughput beamlines. The beamline will be mainly dedicated to drug design. Another new beamline, BL-1A, will be built in FY2009. The goal is to deliver a brilliant lower energy

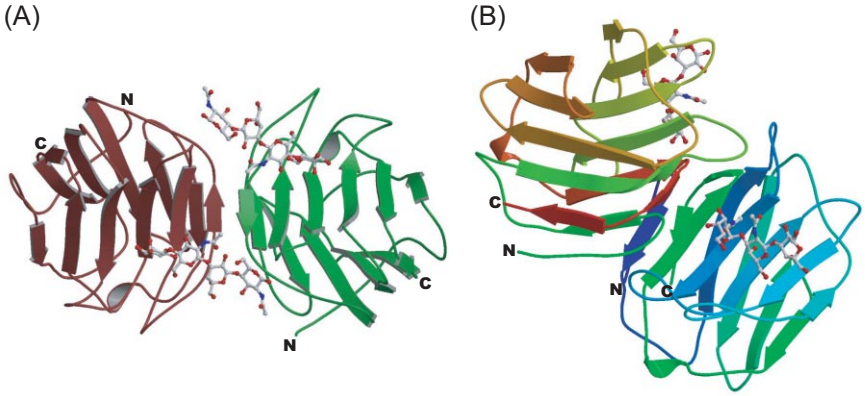

Figure 5
N-terminal carbohydrate recognition domain (NCRD) of animal lectin galectin-9.
(A) Mouse NCRD (ribbon diagram) in complex with tetra-saccharide (stick model). The mouse NCRD forms a dimer in the crystal and also in solution.
(B) Human NCRD (ribbon diagram) in complex with Forssman pentasaccharide (stick model). There is no prominent interaction between the two molecules. Human NCRD forms a monomer in the crystal and also in solution.

Table 2 Summary of protein crystallography beamlines at KEK-PF

	BL-6A	AR-NW12A	BL-5A	BL-17A
Starting year	1987	2003	2004	2006
Synchrotron ring	PF	PF-AR	PF	PF-AR
Injection	twice a day	twice a day (10:00, 22:00)	twice a day (9:00, 21:00)	
X-ray source	Bending Magnet	Undulator	Multi Pole Wiggler	Short Gap Undulator
Wavelength range (Å)	0.91-1.33	0.7-1.9	0.7-1.9	0.95-1.3, 1.6-2.2
Energy resolution (ΔE/E)	1×10^{-3}	2.5×10^{-4}	2.5×10^{-4}	2.5×10^{-4}
Photon flux (photons/sec @ 1.0 Å)	1.0×10^{10}	2.0×10^{11}	1.0×10^{11}	6.6×10^{9}(@1.0 Å) 1.3×10^{10}(@2.0 Å)
Slit size (mm)	0.1	0.2	0.2	0.02
Detector	Quantum 4R	Quantum 210r	Quantum 315	Quantum 270
Type	CCD	CCD	CCD	CCD
Active area (mm^2)	188 × 188	210 × 210	315 × 315	270 × 270
Pixel size (μm^2)	81.6 × 81.6	51 × 51	51 × 51	64.8 × 64.8
Pixel number	2304 × 2304	4096 × 4096	6144 × 6144	4096 × 4096
Frame data size (MB)	11	34	75	34
Readout time (sec)	8	0.9	1	1.1
Typical exposure time (1.0° oscillation)	30 sec	5 sec	5 sec	5 sec
Typical data collection time (180 frames)	120 min	14 min	20 min	24 min
Camera distance (mm)	50 ~ 400	60 ~ 1000	60 ~ 1000	40 ~ 700
Detector vertical offset	0 ~ 100 mm	0 ~ 100 mm	0 ~ 180 mm	0 ~ 100 mm
Image processing software	HKL2000, DPS/mosflm			

X-ray beam at around 4-5 keV (dedicated to sulfur SAD experiments) and more photon flux at around 12 keV than BL-17A. After completion of these two new beamlines, we will operate three high-throughput beamlines, two micro-crystallography beamlines, and one conventional beamline.

The X-ray area detectors used at the beamlines have been upgraded step by step. The ADSC Quantum 210 CCD detector at AR-NW12A was upgraded to the Quantum 210r in summer 2006, giving a reduction in readout noise. An ADSC Quantum 270, a brand-new, high-gain CCD detector was installed at BL-17A in summer 2007. In summer 2008, the Quantum 315 at BL-5A will be upgraded to a Quantum 315r, the same as that used at AR-NW12A. We have developed the beam feedback system to stabilize the X-ray beam, and the high-speed and high-precision sample rotation axis using the air bearing in order to realize and improve the micro-beam beamlines. With the combination of these developments, we will accomplish the very good signal-to-noise data collection system.

For further high-throughput protein crystallography, we have made improvements in the automation of beamline operation, developing sample changer robots, an automatic sample centering system, and unified beamline control software. These developments, based on stable beamlines and a reliable network, will contribute to reaching the goal of a fully-integrated structure determination pipeline. We are also developing a new WEB-based remote monitoring system (PReMo). Users will be able to check the experimental progress of their group on the WEB or a cellular phone.

AR-NE3A: New Beamline for Pharmaceutical Researches

In recent years, advancements in high-throughput techniques for macromolecular crystallography have heightened the importance of structure-based drug design, and demand for synchrotron use by pharmaceutical researchers has increased. In order to meet this demand, we are constructing a new high-throughput macromolecular crystallography beamline AR-NE3A (Fig. 6). This is funded in partnership with Astellas Pharma Inc. The construction of the beamline will be completed in the summer shutdown of the PF-AR in 2008. Beamline commissioning will be finished by the end of March 2009, and the first user operations are expected in April 2009.

The light source is an in-vacuum undulator in the PF-AR 6.5 GeV ring, providing a high flux X-ray beam. Ray-tracing simulations suggest that new AR-NE3A affords higher X-ray beam flux at the sample position than the high-throughput beamlines AR-NW12A and BL-5A. In the experimental hutch, there will be a high precision diffractometer, a fast-readout and high-gain CCD detector and a sample exchange robot which can handle more than two hundred cryo-cooled samples in a liquid nitrogen Dewar. In order to realize the high-throughput data collection required for pharmaceutical research, we are developing a fully-automated data collection system, which is described below.

Automation of Data Collection

Fully-automated X-ray diffraction data collection and data analysis have been strongly demanded by structural biology researchers. The key technologies are

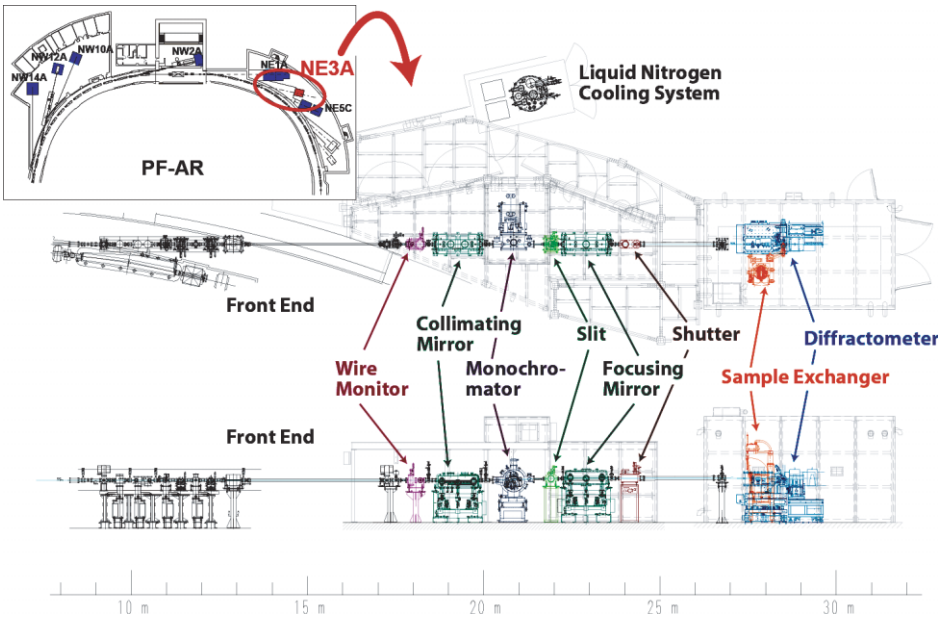

Figure 6
Schematic view of the pharmaceutical beamline AR-NE3A.
The AR-NE3A beamline is being constructed at the PR-AR 6.5 GeV ring (inset). The optics consist of three main components, a collimating mirror, a double crystal monochromator with a liquid nitrogen cooling system, and a toroidal double-focusing mirror.

automated sample exchange and automated sample centering. In addition, we have advanced the development of automated data evaluation software, aiming at an automated data analysis system.

We have developed sample exchange robots, named “PAM” (PF Automated Mounting system). These were first installed at BL-5A and AR-NW12A in collaboration with the SSRL (Stanford Synchrotron Radiation Lightsource, USA) macromolecular crystallography group, and have been available to users since October 2006. In order to reduce the time required for sample exchange, we developed a double-tongs system “Gemini”, which can hold two sample pins at the same time [13]. A double-tonged PAM was installed at BL-17A at the end of FY2006, and user operations began in May 2007 (Fig. 7). The new system can exchange samples in less than 10 seconds. To make the most efficient use of the double tongs, it is necessary to achieve parallel operation between the data collection and “pre- and post-sample exchange”, i.e. the preparation of the next sample and the storage of used sample. The three PAMs were used by 11.2% of users in FY2007, and 2,282 sample pins were mounted using them. The PAMs can currently only handle SSRL cassettes, but the “SPACE” sample exchange robot developed at SPring-8 uses different types of sample pins and cassettes. A project to develop compatible cassettes and pins started with SPring-8 and Kyoto Univ. as part of the “Targeted Proteins Research Program”.

Figure 7
Sample exchange robot installed at BL-17A.
The inset shows a magnified image of the double tong system developed at KEK-PF, which improves sample exchange throughput compared to using a single tong.

Data collection experiments require users to align a huge number of sample crystals with the X-ray beam. We have improved an automated loop centering function based on image processing to assist this alignment. The automated loop centering can be automatically executed after the PAM mounts a sample. We are also developing an automated crystal centering function, based on low-dose diffraction patterns, for fully-automated X-ray diffraction measurements.

For estimation of the best diffraction conditions, we are developing a diffraction image evaluation client, PROCESSOR, which will be incorporated into the beamline controlling system. The client also enables the system to process the collected data sets automatically. In order to monitor the X-ray diffraction experiments, we have developed the PReMo (PF Remote Monitoring) system [14]. In this system, another client of our beamline controlling system, REPORTER, collects the states and the results of present experiments, and stores them into a database. The information can be browsed from a

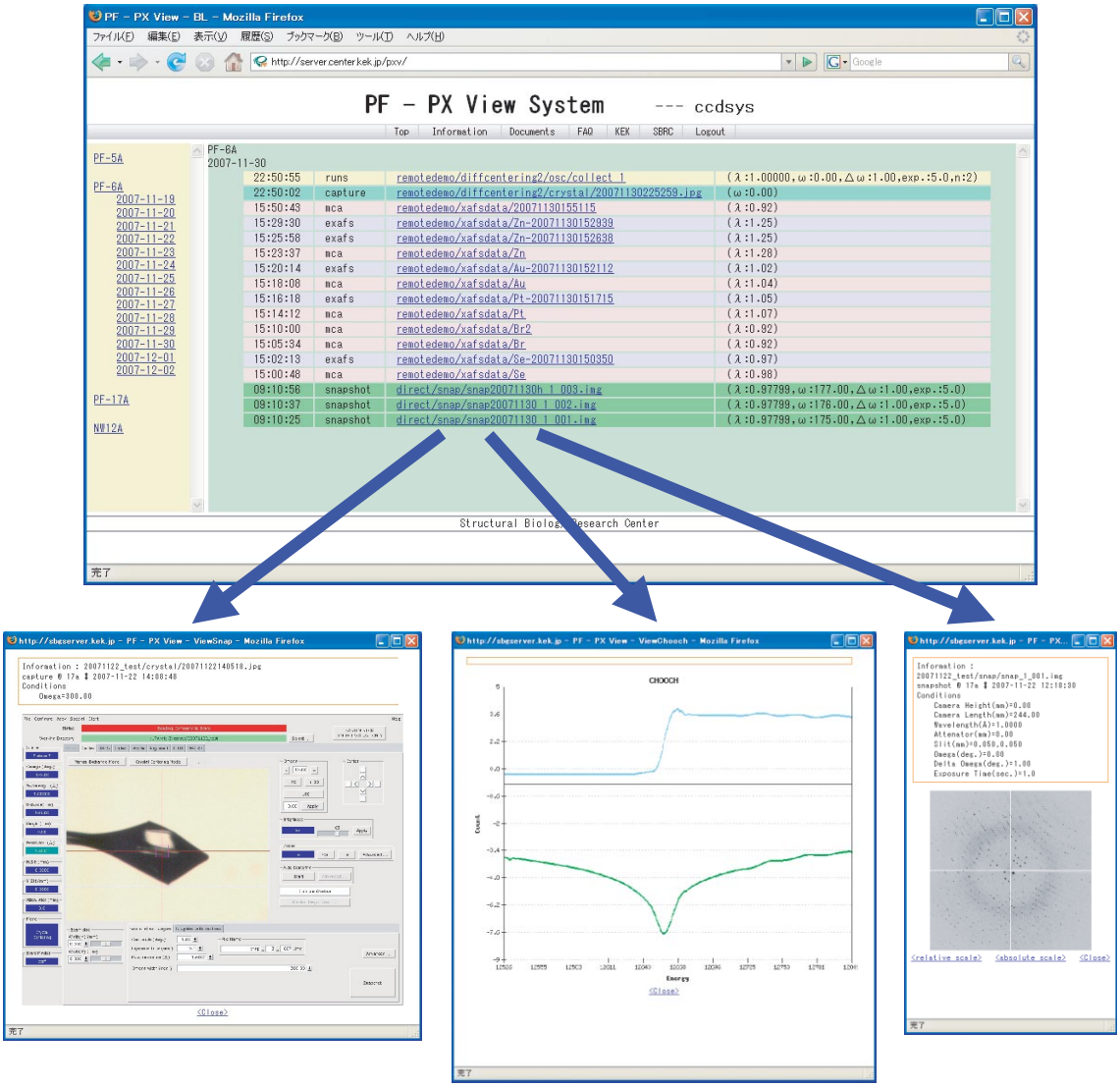

Figure 8
Remote monitoring system for diffraction experiments.
After login to the PReMo (PF Remote Monitoring) system using a WEB browser, all user experiments which are currently executing or have been completed are listed (top panel). Each line is a link to the experimental data or results viewer, and the user can view for example crystal images, energy scan plots, and diffraction images and so on (lower panels).

WEB browser (Fig. 8), and selected information can be checked by cellular phone. The PReMo system permits users to access the information even from the outside of the beamlines, using the HTTPS protocol.

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