2 Structural Biology Research Center

2-1 Introduction

The Structural Biology Research Group was formed in May 2000. The aims of the research group are user support in synchrotron-radiation X-ray crystallography of macromolecules, the development of highly advanced techniques, and in-house research in structural biology. The group has grown steadily during the last five years, with the number of members increasing from four (one professor and three research assistants) to about thirty (Fig. 1). The core staff members of the group are currently the group leader Professor Soichi Wakatsuki, Associate Professor Ryuichi Kato, and five Research Associates (Noriyuki Igarashi, Naohiro Matsugaki, Masato Kawasaki, Masahiko Hiraki and Yusuke Yamada). The structural biology building was extended from 429 m^2 to 643 m^2 in area, and will be re-extended to about 850 m^2 in FY2006 to accommodate the increasing number of group members. While about half of the group are engaged in beamline operation/development and the remaining half in biological research, the synergy between the two activities is a unique aspect of this group. In accordance with the recent change of the status of KEK from a governmental institute to an agency in April 2004, the Structural Biology Research Group became the Structural Biology Research Center in May 2003. Three graduate students have successfully obtained a Ph.D. based on studies in the Structural Biology Research Center. Two graduate students of the Graduate University for Advanced Studies (SOKENDAI) are currently carrying out their own research relevant to the group's research fields under the guidance of the group staff.

During FY2001-FY2003, a "Special Coordination Funds for Promoting Science and Technology" fund from MEXT (Ministry of Education, Culture, Sports, Science and Technology) supported the activities of our group together with other universities (Hokkaido, Tokyo, Kyoto and Osaka) and the research institute of NHK (Japanese national broadcasting corporation). We built and commissioned a new high-throughput beamline BL-5A, developed an assortment of technologies for the automated handling of protein crystals, constructed a prototype of a next-generation two-dimensional X-ray HARP (high-gain avalanche rushing amorphous photoconductor) detector, and developed software to facilitate rapid and accurate structure determination. We also made improvements to the experimental environment associated with the beamlines and sample-preparation laboratories using the same research fund. Subsequently, a five-year national project "Protein 3000" was started by MEXT in FY2002. The project consists of two programs; a "Comprehensive Program" carried out by RIKEN and "Individual Analysis Programs" carried out by eight consortia of universities and institutes including the Structural Biology Research Center. In addition, in FY2004 a new research and development program "Development of Systems and Technology for Advanced Measurement and Analysis" was launched by JST (Japan Science and Technology Agency). We proposed a project to develop a next-generation detector combined with a micro-focus beamline. This project was selected and commenced in the same year for initial 3 years. In this project we will develop and construct a new beamline BL-17A optimized for data collection from small crystals, and an advanced prototype of an X-ray HARP detector in collaboration with NHK Engineering Service (NHK-ES) and associated companies.

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

Highlights of the research and development projects and biological research in FY2005 are presented below. The highlights demonstrate the synergistic approach of the group in pursuing active structural-biology in-house research, improving and developing fundamental research techniques for synchrotron-based protein crystallography, and operating the user facilities.

2-2 Protein 3000 Project — Individual Analysis Programs —

FY2005 was the fourth year of the five-year Protein 3000 Project. The Structural Biology Research Center serves as one of the eight consortia of the national project, pursuing structural and functional analyses of proteins in the field of post-translational modification and transport. Three new members were selected to join our consortia to promote the field of medical applications at the end of FY2005. At present, our consortium consists of eleven universities and four research institutes (Table 1).

Cell signaling and intracellular trafficking are the means by which eukaryotic cells deliver cargo proteins

Functional Analyses	Intracellular trafficking	Akihiko Nakano (RIKEN, Univ. of Tokyo) Kazuhisa Nakayama (Kyoto Univ. Pharmaceutical) Hiroshi Ohno (RIKEN Laboratory of Epithelial Immunobiology), Hiroaki Kato (Kyoto Univ. Pharmaceutical) Masayuki Murata (Univ. of Tokyo, Arts and Sciences) Syuya Fukai (Tokyo Inst. of Technology) Soichi Wakatsuki (KEK-PF)
	Post-translational modification	Shogo Oka (Kyoto Univ. Pharmaceutical) Naoyuki Taniguchi (Osaka Univ. Medicine) Yoshifumi Jigami (AIST) Koichi Kato (Nagoya City Univ. Pharmaceutical) Sumihiro Hase (Osaka Univ. Science) Soichi Wakatsuki (KEK-PF) Tamao Endo (Tokyo Metropolitan Institute of Gerontology) Kenji Yamamoto (Kyoto Univ. Graduate School of Biostudies) Akira Kurosaka (Kyoto Sangyo Univ. Faculty of Engineering)
	Medical applications	Tatsuo Shioda (Osaka Univ. Institute for Microbial Diseases) Hirohito Tsubouchi (Kagoshima Univ. Medical and Dental Sciences) Akio Ido (Kyoto Univ. Hospital. Translational Research Center)
Structural Analyses	X-ray crystallography	Takamasa Nonaka (Nagaoka Univ. of Technology) Nobutada Tanaka (Showa Univ. Pharmaceutical) Hiroaki Kato (Kyoto Univ. Pharmaceutical) Shuya Fukai (Tokyo Inst. of Technology) Soichi Wakatsuki (KEK-PF)
	NMR, Small angle X-ray scattering, Bioinformatics	Koichi Kato (Nagoya City Univ. Pharmaceutical) Mikio Kataoka (Nara Inst. of Science and Technology) Kei Yura (JAERI CCSE)

Table 1 Members of the "Post-Translational Modification and Transport" network in Protein 3000 Project.

to various organelles, cell membranes, and extracellular destinations (Fig. 2). During the trafficking process, more than half of 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. A more profound understanding of the biological and biomedical functions of transport and modification proteins is indispensable for making progress in the medical treatment of human diseases.

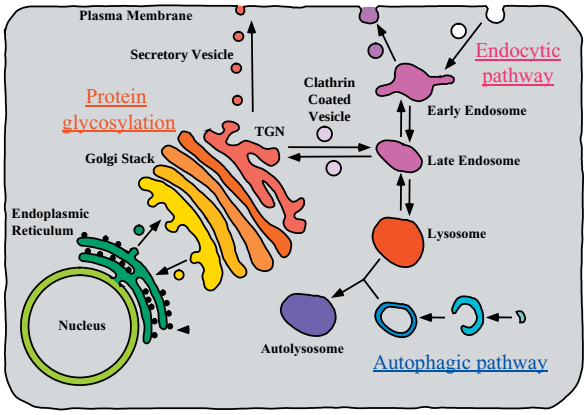

Figure 2
Schematic drawing of protein glycosylation and transport in cells.

To facilitate the current research program, the Structural Biology Research Center has taken the lead by coordinating the research network. As part of the project, we have sought to maximize the efficiency of the large-scale expression, purification, and crystallization of proteins by trying to eliminate bottlenecks in each process. Crystallographic studies using synchrotron X-rays are conducted at KEK-PF, NMR experiments at Nagoya City University, and small-angle X-ray scattering experiments at Nara Institute of Science and Technology using beamlines at KEK-PF and SPring-8. Each structural-analysis project maintains close contact with the groups responsible for functional analyses. Our initial research plan was to accomplish the structural and functional analyses of more than 70 proteins in five years. To date, the number of structures determined has reached over 100, with the number of obtained data being 50 (Table 2). In the near future the initial target will be exceeded by a factor of two. In addition, we are currently studying about 150 additional targets. Eleven domestic and two international patent applications have been submitted based on our R&D and structural and functional studies

Table 2 Progress of solved protein structures of the "Post-Translational Modification and Transport" network in Protein 3000 Project.

status	in progress			Number of structures solved	Total
	Number of expression system construction	Number of establishment of purfication procedure	Number of data collected		
X-ray	140	104	49	98	147
NMR	9	8	1	4	5
Total	149	112	50	102	152

Table 3 Beam time used at KEK-PF in Protein 3000 Project.

Network Committee	FY 2003	FY 2004	FY 2005	Total
Metabolism	12	25	15.5	52.5
Development and Cell differentiation	3	8.5	10.5	22
Transcription and Translation (Hokkaido University)	1	7.5	11.5	20
Transcription and Translation (Yokohama City University)	8	14	8.75	30.75
Higher Order Biological Functions	0	1	7.5	8.5
Signal Transduction	1	3.5	5.5	10
Brain and Neurology	3	8	11	22
Protein Transport and Modification	13	18	16.5	47.5
Total (days)	41	85.5	86.75	213.25

of the target proteins.

KEK-PF is one of two synchrotron-radiation facilities with high-throughput protein-crystallography beamlines in Japan. To cooperate with other groups in promoting the Protein 3000 Project, the Structural Biology Research Center has established an operation scheme to reserve about 30% of the beam time at our beamlines for users of the eight university consortia under an S2 proposal (see page 145). As summarized in Table 3, a total of 213 days of beam time has been allocated to users since the beginning of the project. A web-based beam-time reservation system has also been developed to facilitate the project.

Post-translational modification of proteins is closely related to intracellular transport systems, and new protein-protein and protein-ligand interactions are being identified and characterized, thus providing new targets for our project. A logical extension of the current proteomics studies is the incorporation of post-translational modification and transport machineries, particularly membrane complexes, which play a key role in membrane trafficking. Our future research plans thus include the structural analyses of these complexes. With this in mind, we plan to further develop our integrated and synergistic approaches within our consortia and establish new research collaborations with groups in other research networks.

2-3 Development of Systems and Technology for Advanced Measurement and Analysis

The progress of structural-biology research, including structural genome projects such as the Protein 3000 Project, has recently led to a request for structure determinations of proteins which are difficult to solve, such as membrane proteins and protein-protein complexes. Such difficult targets are not easy to crystallize in sufficient sizes. Even with the latest third-generation synchrotron facilities such as SPring-8 (Japan), APS (US), and ESRF (EU), structural analyses using protein crystals of a few micrometers or smaller in size are currently out of reach. While every effort has been made and more advanced techniques are being developed to push the limits of ring-based synchrotron facilities, the next (fourth)-generation synchrotron light sources, such as the X-ray free electron laser (XFEL) and energy recovery linac (ERL), are expected to provide X-ray beams with unprecedented quality. X-rays of much higher brilliance and shorter pulses emitted from such light sources will be undoubtedly powerful tools for the structural analyses of single or clustered molecules. To develop next-generation two-dimensional X-ray detectors with high-speed readout and high sensi-

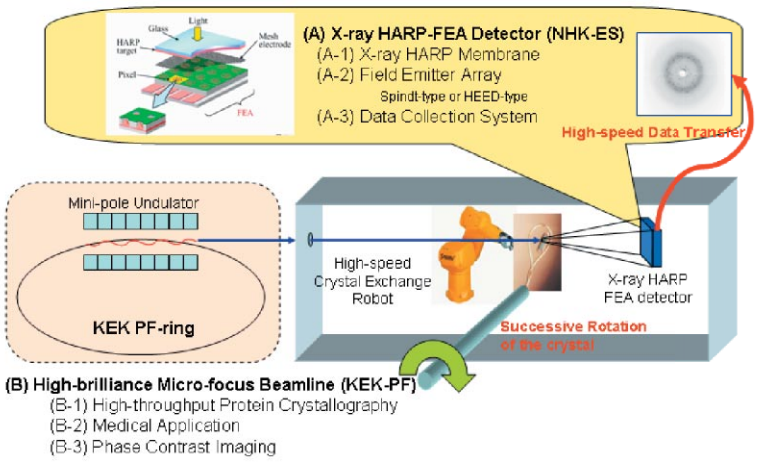

Figure 3
Outline of the "Development of Systems and Technology for Advanced Measurement and Analysis" program in the KEK-PF. In this project, we are mainly developing (a) an X-ray FEA-HARP detector in collaboration with NHK-ES and (b) a high-brightness micro-focus beamline.

tivity is crucial for making full use of the characteristics of next-generation synchrotron light sources. Such detectors will be also useful for collecting weak diffractions from small crystals using micro-focus beamlines.

A new measurement system is under development for biological macromolecules based on the next-generation X-ray detector. It is combined with a micro-focus beamline optimized for micron-size crystals, funded by the "Development of Systems and Technology for Advanced Measurement and Analysis" program (FY2004-2009) of JST (Fig. 3). Based on previous developments during FY2001-2003, we are now improving the X-ray HARP detector in collaboration with NHK-ES. The core of the detector system consists of a matrix field emitter array (FEA) and a HARP membrane, which is made mainly of amorphous selenium and utilizes an avalanche multiplication effect under a high-voltage field to amplify the signals. These characteristics have the following advantages over currently available CCDs and other area detectors: (1) higher sensitivity owing to the amplification effect by the HARP membrane; (2) higher spatial resolution achieved by the single-driven FEA; (3) a higher framing rate of 10-120 images/s. The X-ray FEA-HARP detector will be used for experiments with next-generation light sources because of this higher sensitivity and framing rate. In fact, the maximum framing rate of 120 images/s matches the proposed frequency of the Linac Coherent Light Source (LCLS), an XFEL being developed at the Stanford Linear Accelerator Center (SLAC), California, USA. The detector is also intended for other applications such as low-dose medical imaging and real-time phase-contrast imaging methods, which will also benefit from the high sensitivity and high framing rate of the detector.

Figure 4 shows a view of a test experiment using the X-ray HARP detector at the new micro-focus beamline BL-17A. Diffraction images covering a 180° range from a protein crystal could be recorded within only 12 s. The prototype showed a higher spatial resolution and sensitivity than those of a commercial CCD detector (Fig. 5).

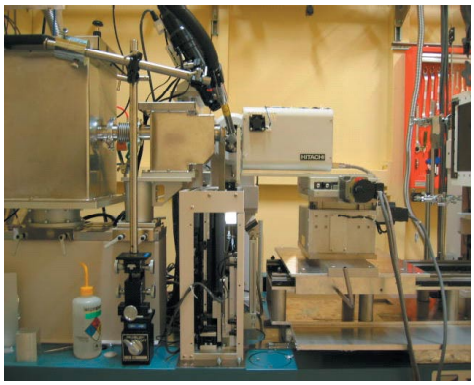

Figure 4
View of a test experiment at BL-17A using a prototype X-ray HARP detector.

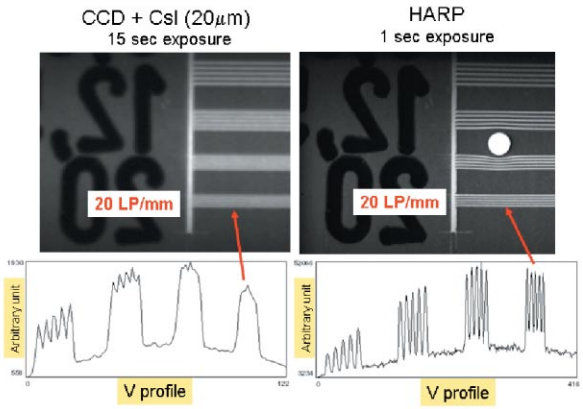

Figure 5
Comparison of HARP and CCD X-ray detectors in spatial resolution and sensitivity. Experiments were carried out at AR-NE5A using a 25-keV X-ray beam. Images of chart slit taken with CCD (left) and HARP (right) are shown. Each line at an interval of 20 line-pairs / mm, which corresponds to a 25-μm spatial resolution, can be clearly seen in the HARP but not in the CCD (indicated by the red arrows and vertical profiles). Although a voltage of 1300 V applied on the HARP membrane is lower than an avalanche voltage of 1500-1600 V, the sensitivity is better than that of the CCD. A white dot in the right image shows a defect of the HARP membrane.

2-4 Highlights of In-House Structural Biology Research

Double-sided ubiquitin interacting motif of Hrs in protein sorting

Ubiquitin is a highly conserved 76 amino acid protein expressed ubiquitously in eukaryotic cells. Ubiquitin can be covalently bonded to target proteins and functions as a signal for various cellular processes such as protein degradation. For example, ubiquitination of growth factor receptor proteins on the cell membrane surface triggers endocytosis of the receptors. Internalized receptors are recognized by a cascade of protein complexes which possess ubiquitin binding domains, and finally transferred to lysosome for degradation. Hrs (Hepatocyte growth factor-regulated trypsine kinase substrate) is one of the essential proteins responsible for this degradation cascade. Hrs has ubiquitin interact-

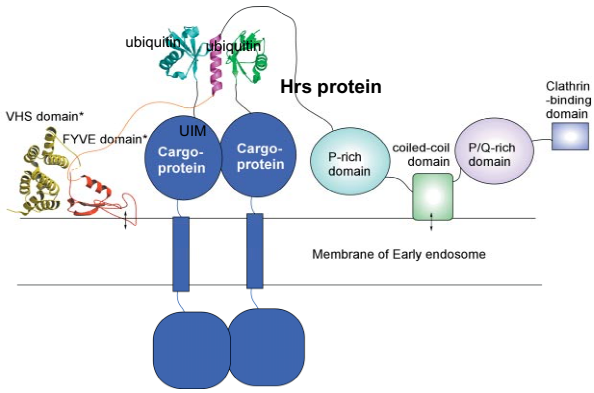

Figure 6
A model for the overall structure of Hrs interacting with ubiquitinated cargo proteins. Two ubiquitin molecules bind to Hrs-UIM at the same time. (*)Crystal structure of VHS-FYVE domains of Hrs (Protein Data Bank ID: 1DVP)

ing motif (UIM) which interacts weakly but specifically with ubiquitins. The result of crystal-structural analysis of Hrs-UIM in complex with ubiquitins revealed that Hrs-UIM is a short helix with two binding faces almost identical on either side, both interacting with ubiquitins (Fig. 6). This is the first observation of UIM interacting with two ubiquitins at the same time. See Highlights in this issue for details [1].

Novel metal ion independent glycoprotein cargo receptors, Emp46p and Emp47p

Newly synthesized secretory proteins are glycosylated with high mannose-type sugar chains in endoplasmic reticulum and transported to Golgi apparatus via secretory vesicles. A lectin-type domain is recurrently used as a sugar-binding module in both extracellular and intracellular proteins. Emp46p and Emp47p are intracellular lectin-type receptor proteins in yeast. These two proteins are 45% identical in sequence and are required for the transport of some glycoprotein cargos from the endoplasmic reticulum to Golgi apparatus. We have solved the crystal structures of lectin-type domains (carbohydrate recognition domains; CRDs) of Emp46p and Emp47p (Fig. 7). The CRD of Emp46p unexpectedly binds a potassium ion, unlike the related mammalian proteins which bind calcium ions. In contrast, Emp47p binds no metal ions. See Highlights in this issue for details [2].

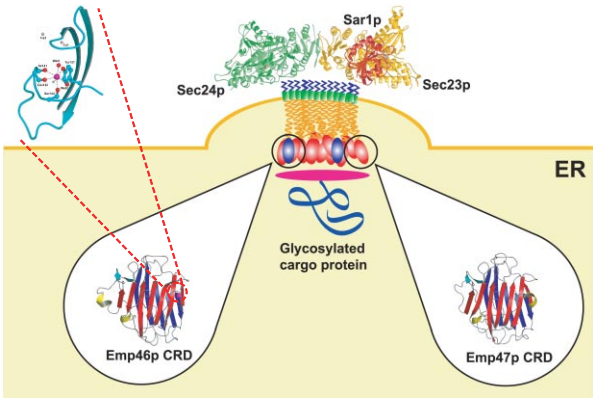

Figure 7
A model for glycoprotein transport by yeast cargo receptors Emp46p and Emp47p. The overall structure of an oligomeric complex of Emp46p and Emp47p is drawn in the middle. The crystal structures of Emp46p CRD (lower left) and Emp47p CRD (lower right) and close-up view of potassium binding site of Emp46p (upper left) are shown. The Sar1/Sec23/Sec24 complex of the COPII vesicle coat is shown on the top (Protein Data Bank ID: 1M2O, 1M2V).

References

[1] S. Hirano, M. Kawasaki, H. Ura, R. Kato, C. Raiborg, H. Stenmark and S. Wakatsuki, *Nature Struct. Mol. Biol.* **13** (2006) 272.

[2] T. Satoh, K. Sato, A. Kanoh, K. Yamashita, Y. Yamada, N. Igarashi, R. Kato, A. Nakano and S. Wakatsuki, *J. Biol. Chem.*, **281** (2006)10410.

2-5 High-Throughput Modern Protein Crystallography Beamlines

Two insertion-device (ID) beamlines and one bending-magnet (BM) beamline are operated for protein crystallography (Table 4). The ID beamlines AR-NW12A and BL-5A designed for high-throughput MAD (multiple-wavelength anomalous diffraction) experiments have been optimized to be more stable and more efficient.

Table 4 Specifications of the PF protein crystallography beamlines.

	BL-6A	BL-5A	BL-17A	AR-NW12A
Starting year	1987	2004	2006	2003
Status	operational	operational	operational	operational
Synchrotron ring	PF	PF	PF	PF-AR
X-ray source	BM	MPW	SGU	Undulator
Wavelength range (Å)	0.9-1.3	0.7-1.9	0.97-1.11 1.37-2.05	0.7-1.9
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}	1×10^{11}	6.6×10^{9}(@1.0 Å) 1.3×10^{10}(@2.0 Å)	2×10^{11}
Slit size (μm)	100	200	20	200
Detector	Quantum 4R	Quantum 315	Quantum 4R	Quantum 210r
Type	CCD	CCD	CCD	CCD
Active area (mm^2)	188 × 188	315 × 315	188 × 188	210 × 210
Pixel size (μm^2)	81.6 × 81.6	102 × 102 , (51 × 51)	81.6 × 81.6	51 × 51
Pixel number	2304 × 2304	3072 × 3072 (6144 × 6144)	2304 × 2304	4096 × 4096
Frame data size (MB)	11	19 (75)	11	34
Readout time (sec)	8	1	8	0.9
Typical exposure time (1.0° oscillation)	30 s	5 s	10 s	5 s
Typical data collection time (180 frames)	120 min	20 min	60 min	15 min
Camera distance (mm)	50 ~ 400	60 ~ 1000	40 ~ 700	60 ~ 1000
Detector vertical offset	0 ~ 100 mm	0 ~ 180 mm	0 ~ 100 mm	0 ~ 100 mm

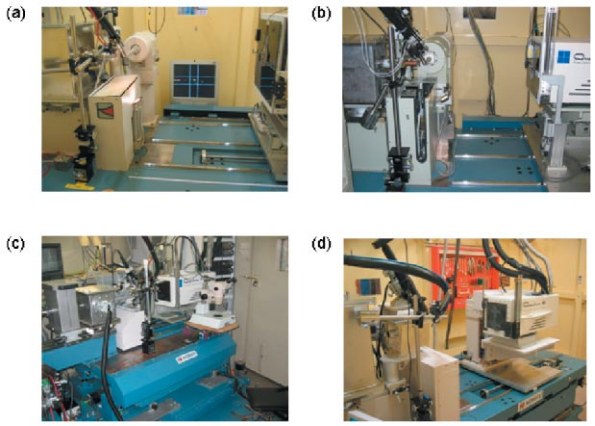

Figure 8
Diffractometers used at the protein crystallography beamlines.
(a) BL-5A (b) NW12A (c) BL-6A (d) BL-17A.

In addition, the diffractometer at the BM beamline, BL-6A, has been improved with the installation of a high-speed shutter, a high-precision sample-rotation axis and a "Fancybox" interface, allowing users to carry out their experiments in the same manner as at the ID beamlines (Fig. 8). All of the beamlines have been quite stable and productive throughout FY2005.

At the end of FY2004, we started constructing a new micro-focus beamline at BL-17A. BL-18B, previously a BM beamline for protein-crystallography, was shut down. The light source for the new BL-17A is a newly developed mini-pole (short-gap) undulator which can deliver a highly brilliant beam to the experimental station. The beamline is designed for measurements of very small protein crystals and for low-energy experiments (Table 4). We observed the first beam in October 2005, and succeeded in the first diffraction experiment using a protein crystal in March 2006. Public use of BL-17A will commence in FY2006.

The beamline control system has been improved in functionality and ease-of-use. We have developed a unified graphical user interface (GUI) for beamline operation, which provides several functions to assist users, for example, automatic sample alignment. The GUI is common to all the protein crystallography beamlines so that all beamlines can be operated in the same manner. To use the ID beamlines more efficiently, SSRL (Stanford Synchrotron Radiation Laboratory)-type sample-exchange robots were installed. These robots have now been commissioned and will be available in FY2006.

We are also developing an integrated control system based on a unified database, the PCCS (Protein Crystallography experiment Control System), which can manage all experimental information including diffraction measurement and sample preperation. The system allows fully automated measurements and multiple access from inside/outside the PF. Diffraction experiments using PCCS are currently under commissioning. The system will run on a central server connected to the beamlines and the experimental facilities through a high-speed network.

2-6 Robotics for High-Throughput and Automated Protein Structural Research

Large scale protein crystallization and monitoring system

Crystallization of samples still remains one of the bottlenecks in crystallographic analyses of macromolecules. We have developed a large-scale protein crystallization and monitoring system that is unrivalled in speed compared with other systems in the world [1]. The system has achieved more than a 100-fold shortening of the time required for crystallization setups; the time required to setup 480 crystallization conditions is only 10 min. compared to the 20 hours necessary to perform the same work manually. Users can view images of the crystallization drops remotely using web-based browsing software and search quickly for appropriate crystallization conditions. About 1,800 crystallization plates (each plate contains 96 crystallization drops) have been set up for initial screening of crystallization conditions from the beginning of the operation in the autumn of 2003. We succeeded in making a large number of crystals using the crystallization system. In some cases, we could directly determine the protein structures from the crystals grown by the system without further optimization. In other cases, crystals with size and quality sufficient for X-ray diffraction experiments were obtained by further optimization (manual screening) based on the conditions found by the system.

To improve the crystallization system, we developed a desktop monitoring system and installed it in the cold room of the Structural Biology Research Center (Fig. 9). This monitoring system takes images of the crystallization drops in the plates set by users, and sends them to the file server of the large-scale crystallization system. Users can also access these images via web-based browsing software. In addition, we are developing another high-speed monitoring system with 96 cameras in parallel which captures images of all the drops in the crystallization plates in tenths of a second.

We also plan to develop a nanoliter dispensing sys-

Figure 9
Desktop monitoring system installed in the cold room.

Figure 10
Novel sample-exchange robot for R&D. Inset shows an inside view of the Dewar holding three SSRL cassettes in place.

tem to reduce sample volume and dispensing time, and an automated crystal scoring system based on various image processing techniques. As a longer term development project, we also plan to develop a fully-automated system which encompasses the whole range of crystal preparation: protein crystallization, crystal observation, crystal scoring, picking up crystals, soaking crystals to a cryoprotectant, and freezing and storing crystals into a cassette. To this end, we have already developed a seal-cutting system to cut out the seal of the specified wells containing prospective crystals. A cryoprotectant exchange system and a subsidiary system for supplying consumables are under development.

Automated sample exchange robots

The high-flux ID beamlines BL-5A and AR-NW12A need significantly shorter exposure time for X-ray protein crystallographic experiments than BM beamlines. Combined with fast-readout CCD detectors and shutters, each user (group) can collect 20-30 high-quality X-ray diffraction datasets during a 24-hour beam time. To further improve the overall throughput, the time for other steps must be reduced as much as possible, notably the time required for manual crystal exchange in the experimental hutches. To this end, we have adopted the automated system developed by the Stanford Synchrotron Radiation Laboratory (SSRL) for picking up samples from a liquid nitrogen Dewar, and mounting them on the diffractometer. We chose this system because of its reliability, with about four years of track record of user operation, large capacity (continuous operation of 288 crystals using three cassettes with 96 crystals each) and compatibility with commercially available sample holders. In addition, we are developing a cryo-tong with two slots which can reduce the number of trips between the Dewar and the diffractometer by a factor of two. We have already developed two exchange robots and installed them on BL-5A and AR-NW12A during the summer shutdown of 2004. We are modifying the calibration procedure of the sample cassettes in the Dewar in order to improve the stability of the system. In addition, we have prepared an R&D exchange robot (Fig. 10) to develop the hardware and the software during beam time. These robots will increase the throughput of the beamlines from 20 to 30 datasets per day to over 100.

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