Achievement of protein micro-crystallography at SPring-8 beamline BL32XU

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Abstract. A micro-focused X-ray beam with size ranging from 1 x 1 to 10 x 10 µm has been achieved at beamline BL32XU at SPring-8, Japan. Combining the available micro-beam with newly developed techniques has enabled efficient protein micro-crystallography.

1. Introduction
Recently, micro-focus X-ray beams have been proven to be effective for protein micro-crystallography [1]. The micro-focused beam enhances the signal-to-noise (S/N) ratio of diffraction peaks and reduces background noise from the non-crystal irradiated volume. Several beamlines worldwide have achieved a beamsize smaller than 10 µm for this purpose. The dedicated protein micro-crystallography beamline BL32XU at SPring-8 has been developed in the Japanese National Project, Targeted Protein Research Program. The concept of this beamline was described in our previous proceeding of SRI2009 [2]. By the end of 2009, we had completed the beamline commissioning and had achieved 1 µm focusing at the sample position. Since 2010, through two years of user operation, efficient methodologies for diffraction experiments using micro-beams have been developed.

In this manuscript, we describe achievements in protein micro-crystallography at BL32XU focusing on beamline capabilities, data collection strategy, enhancing S/N ratio and high speed screening for tiny protein crystals.

2. Fundamental abilities of the beamline BL32XU
The beamline consists of a primary slit, a monochromator, four-bladed slit for producing a virtual source, focusing K-B mirror and diffractometer [2]. A set of elliptically-shaped mirrors were fabricated with Elastic Emission Machining technique. Their demagnification factors for the horizontal and vertical directions correspond to 40 and 26, respectively. By producing a 40(H) and 26(V) µm aperture with the four-bladed slit, a 1 µm focused beam can be achieved. The minimum beam size is 0.9 x 0.9 µm with a corresponding flux of 6.2 x 10¹⁰ photons/sec at 12.4 keV [Fig.1]. A comparable photon flux density is available over the energy range 8.5 to 18.0 keV. The maximum beam size is 10 x 10 µm with 5.4 x 10¹² photons/sec at 12.4 keV, achieved by setting the four-bladed slit to the fully open position. Modifying the slit aperture enables selection of beam shape and size from 1 x 1 µm to 10 x 10 µm within 15 seconds.
3. User friendly GUI system for suggesting data collection strategy

We have been developing the ‘KUMA’ GUI software, with the capability to suggest data collection strategies for reduced radiation damage. A recommended data collection method at BL32XU is so-called ‘helical data collection’ [3] using 1(H) x 10(V) μm beam. We measured the propagation length of radiation damage on standard protein crystals at various X-ray energies and beam sizes, as reported by APS staff [4]. From the results, an empirical model function estimating the accumulated dose on the crystal was established. Using this model, KUMA suggests an optimal strategy taking into account X-ray energy, crystal size, beam size, desired data redundancy and aimed quantity of dose [to be published]. The KUMA system enables users to make the maximum use of crystal volume for data collection. Several successful structure determinations from tiny lipidic cubic phase (LCP) crystals proved the efficiency of this system. In the case described in reference [5], helical data collection using a 1(H) x 10(V) μm beam and 0.5 μm step was conducted. The total absorbed dose was kept below 5 MGy for each of three MAD datasets by using this concept.

4. Developments for enhancing S/N ratio in data collection

It is exceedingly important for enhancing S/N ratio to reduce background noise originating from both air and parasite scattering. A high-precision diffractometer and newly developed helium gas chamber have been installed into the end-station of BL32XU.

4.1. Diffractometer

At beamline BL32XU, a pinhole collimator located 25 mm upstream of the sample position cuts off both parasitic scattering from mirrors and air. The pinhole diameter of 30 μm can cover the utilized beam sizes. The scattering from the pinhole is reduced by a 10 mm-long brass pipe with a diameter of 300 μm.

A key component for data collection from tiny protein crystals is the crystal goniometer. We adopted an air-bearing goniometer having a sphere of confusion of less than 0.5 μm (KOHZU Precision Co., Ltd.). The minimum step resolution of each crystal translation axis corresponds to 0.1 μm. The absolute position of each translational axis can be monitored with the linear encoder (RENISHAW) with a resolution of 10 nm.

4.2. Helium chamber

We have developed a helium gas chamber, now available to users, for reducing the air scattering along the beam path (Figure 2(a)). The rigid part of the chamber consists of acrylic plates and encloses all devices around the sample position. A paper-made bellows part connects the acrylic box with the CCD detector surface. Helium gas is introduced into the chamber using a helium cryo-stream at a defined temperature between 30-100 K. With the chamber in place, the detector to sample distance may be set between 180-500 mm. The chamber was designed so that our sample changer robot, SPACE [6], can access sample pins on the goniometer with minimal loss of helium gas from the chamber. A small door, the so-called ‘cat flap’, was installed in the rigid part of the chamber and the arm of the SPACE robot opens this door when it exchanges sample pins (Figure 2(b)).

With this system, the user can conduct diffraction experiments in a similar manner as in routine data collection. The helium environment has been confirmed to reduce background noise from air scattering by a factor of 10 at 12.4 keV [Figure 2(c)]. One user experiment at this X-ray energy demonstrated that even an incident beam attenuated to 25% gave the same quality of diffraction dataset as the normal setting, which utilizes nitrogen gas flow.
Figure 2. (a) The overall view of the helium chamber. The X-ray beam travels from right to left. The goniometer and other devices around the sample are enclosed by the rigid part made of acrylic plates. The paper-made bellows part connects the rigid part and X-ray CCD detector. (b) The function of the ‘cat flap’. The sample changer robot, SPACE, can gain access to the goniometer head through a small hinged door, the ‘cat flap’. SPACE can exchange sample pins via this door with a very small change of helium gas concentration in the chamber. (c) Radially averaged background ADUs with and without helium chamber. The ‘normal’ trace shows background without the helium chamber. Both images were acquired using an exposure of $5 \times 10^{11}$ photons at 12.4 keV. The detector was a Rayonix MX225HE and the camera length was set to 300 mm from the sample position with an offset ADU of 10. The inner 200 pixel-length region was affected by the direct beam stopper.

5. Plate screening for micro crystals

In protein crystallography, obtaining a good quality crystal is an essential yet challenging step. Therefore, huge numbers of crystallization conditions are tried in the initial screening process and a number of small objects are often observed in these crystallization drops. However, it is difficult to establish whether these are protein crystals or not. In particular, crystals obtained with the LCP (lipidic cubic phase) method [7] often do not grow large enough to give high quality diffraction. Such crystals are easily deformed in the freezing process for cryo-crystallography because the phase condition of the lipid is very sensitive to variations of humidity and temperature. Furthermore, a major difficulty with LCP crystals is that LCP becomes opaque upon freezing and hence it is very difficult to visually align crystals. For these reasons, there is strong user demand for an in-situ crystal screening system at room temperature using X-rays [8].

We have developed an in-situ plate screening system at beamline BL32XU dedicated to the standard SBS-format crystallization plate, mounted manually (Figure 3(a)) (KOHZU Precision Co., Ltd.). Installation of this equipment is carried out exclusively using the goniometer stage and cryogenic equipment, with no other disturbance to the beamline. This system has three translation axes, with a positional repeatability of 1 μm, to cover the whole area of the SBS plate. The area covered with these translational axes is 25 x 120 x 100 mm with a maximum scan speed of 1.5 mm/sec.

An in-situ plate screening system for LCP crystals was also developed. For this system, the translation axes are equipped onto the goniometer (Figure 3(b)) (RIGAKU Aihara Precision Co., Ltd.). It can be more easily mounted than the SBS system described above. To utilize this system, the crystallization plate is required to be cut and mounted on a standard 25 x 75 mm glass slide. Oscillation images can be also collected using the spindle axis of the goniometer.

By combining these systems with an X-ray CMOS detector [9], it is possible to scan whole one well area within one minute. In our trial experiment, 3Å resolution diffraction spots were obtained from hen egg white lysozyme crystals smaller than 10 μm.
6. Challenges for automatic detection of protein micro-crystals

Generally, it is difficult to visualize tiny protein crystals because the refractive indices of the protein crystal and mother liquor are similar. We have developed an optical system to visualize such invisible protein crystals. The concept of this system is to visualize fluorescently labeled protein micro-crystals using an excitation light source and a modified co-axial microscope with emission filters currently installed on the beamline. Utilized fluorescent dyes include DyLight350 (Thermo Scientific Co., Ltd), FITC (fluorescein isothiocyanate), TAMRA (Carboxytetramethylrhodamine) and Cy5 [10]. The emission filter and excitation light source are manually changed according to the type of fluorescent dyes. Post-crystallization soaking with fluorescent labels was successful without detectable changes to crystal shape or diffraction data quality.

A newly developed program coded with C++ and OpenCV library has enabled automatic recognition of fluorescently labeled protein crystals. The positions of crystals are identified from digital images captured using a co-axial microscope. The program can interact with the beamline control system BSS [11] to quickly and easily position the identified crystal at the irradiation position for data collection.

7. Conclusion

We have achieved a focused X-ray beam of 1x1 μm with a flux of 6 x 10^{10} photons/sec at the beamline BL32XU at SPring-8. Combining this micro-beam with the integrated systems described in this manuscript has enabled users to conduct efficient data collection and crystal screening of protein micro-crystals.

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