X-ray fiber diffraction: a tool for understanding the structural dynamics of tubulin dimers in native microtubules

When we are going to know structural details of biological molecules, there would be two possibilities; electron microscopy and X-ray crystallography. Owing to remarkable improvements in cryo-electron microscopy techniques using direct electron detectors, impressive progresses showing high-resolution (~0.3 nm) images of various biological molecules in action are now appearing regularly in leading journals. Since details of protein architectures at the atomic scale have been obtained by X-ray crystallography, we can now combine them with electron micrographs to gain more direct insights into the functions of biomolecules than we expected by conventional tools used over a decade ago. This would also be the case for X-ray fiber diffraction/X-ray solution scattering analysis, which we expect will become the third major method used for structural biology.

One of the main advantageous points to use X-ray diffraction is that it enables us to acquire information on native biological molecules functioning in aqueous solutions at moderate temperatures. Here, two main drawbacks should be briefly mentioned, that is, we cannot completely prevent damage induced by focused high energy X-ray beams, and we can only collect indirect information on molecular structures such as structural repeats, mean size and shape. However, owing to the following five improvements achieved for our fiber diffraction technique, X-ray fiber diffraction is now one of the most powerful tools for investigating tubulin molecule dynamics in microtubules [1-3].

First, in our technique of X-ray fiber diffraction, we have successfully revived a flow-aligning technique to collect data from live axonemes of sea-urchin spermatozoa [1,2]. The technique of shear-flow alignment, first theoretically described by Jeffery [4,5], seems to be old-fashioned as it was applied to TMV [6,7] and actin fibers [8] in the 1960s. However, we found that including methylcellulose or other harmless polymers that induce high viscosity of the buffer medium enabled us to accomplish the homogeneous orientation of biological filaments [1]. This polymer technique could be applied to other types of biological filaments, e.g., axonemes [1,2], microtubules [1,3], collagen and TMV [1]. Second, damage to biological materials can be neglected or minimized even under irradiation with high-flux beam of 1×10^{15} photons/s (BL40XU) with 100×0.25 s exposures, because a specimen under the shear flow is continuously renewed at the millisecond time scale (see below). Thus, every time we are looking at diffractions from a fresh specimen with little beam damage. Third, as the use of shearing flows to align fibers are also stirring up the specimen continuously in solution, we can change the medium conditions at a fast time scale (<100 ms). This is quite convenient for investigating the effects of chemicals on biological filaments, e.g., stabilizer or destabilizer of microtubules. Fourth, a rheometer-type shear-flow apparatus composed of two flat disks (round microscope cover slips) was placed separately in parallel (Fig. 1), where small air bubbles contaminating the viscous solution are immediately excluded from the X-ray beam path by circumferential flows occurring in the apparatus chamber. Finally, the most dramatic improvement to be stressed is the time course of fiber alignment. In the case of microtubules, a homogeneous orientation can be accomplished in a few seconds and structural information can be acquired after about a second (exposure time of 0.2–0.3 s). This is highly advantageous when we are going to analyze the structure of labile filaments such as microtubules.

We estimated the total amount of microtubule specimen required for our experiments performed at two SPring-8 beamlines, **BL45XU** (2×10^{11} photons/s) for SAXS and high-flux **BL40XU** (1×10^{15} photons/s). The volume of the sample placed in the X-ray beam path (0.1×0.2 mm^2) was approximately 7 nL. As the total volume of the specimen placed between the two round cover slips was 80 μL (between a/b and c/d in Fig. 1), only 0.009% of total specimen in the shear-

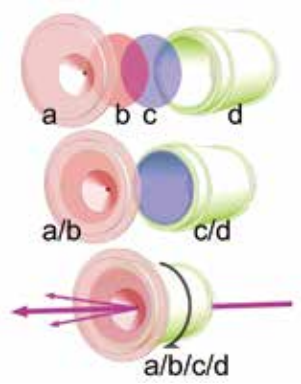

Fig. 1. Schematic drawing of the apparatus for the shear-flow alignment of microtubules showing a copper plate **(a)** holding a round cover slip **(b)**. Another round slip **(c)** was glued on a long tube made of machinable ceramic **(d)**. A medium containing porcine brain microtubules was placed in the narrow gap (0.35 mm) between the two cover slips (between a/b and c/d). After starting to spin (black arrow) the long tube (c/d), the X-ray beam (magenta) was incident to the specimen at a site 7.5 mm away from the center of rotation. Diffraction signals were acquired on the left side of the apparatus.

flow chamber was irradiated. This small portion of the sample solution containing around 1×10^7 microtubules with an average length of 10 μm provide us enough intensity of diffraction signals with 0.25 and 30 s exposure by BL40XU and BL45XU, respectively. In particular, from the strong fourth-order reflections, approximately 1 nm, we could precisely determine the changes in the axial tubulin repeat (Fig. 2). Under the present experimental conditions, where the round cover slip is spinning at 10 – 20 rps to induce a shear flow (shear rate, 1300 – 2600 s^{-1}), each microtubule within the specimen medium remains the beam path of the X-ray for only 0.2 – 0.4 ms on average. Thus, damage of the microtubules caused by the X-ray beam would be negligible, if any.

Figure 3 shows an example of the dynamic changes in an axial tubulin repeat. In this case, a downward shift (lower angle) of the fourth-order reflections was clearly observed after applying paclitaxel, which indicates that the mean axial tubulin repeat became longer within 30 s after the addition of a microtubule stabilizer. Microtubules are composed of self-assembled tubulin heterodimers stacked in a cylindrical wall having a two-dimensional unit arrangement. Our observation revealed that the microtubule structure has some flexibility and that each tubulin dimer inside microtubules can change its molecular configuration immediately after binding to paclitaxel. In the present study, the structural change was finished within 30 s. Our recent preliminary observations with higher time resolution (<0.25 s) indicated that the actual structural shift was completed faster (<1 s).

The examples shown here are the dynamic changes in an axial tubulin repeat, but we also observed structural dynamics in the diffraction of equatorial signals [3] corresponding to a change in the microtubule diameter or spacing between protofilaments, the longitudinal arrays of tubulin units. Along with other detailed information of the static structure of tubulin dimer molecules obtained by crystallography [9] and cryo-electron microscopy [10,11], we expect to obtain more insight into the dynamics of tubulin structure, i.e., how the molecular configuration of tubulin dimers is flexible and adaptive depending on their chemical and physical states, and how it is correlated to the microtubule functions and the control of eukaryotic cell shapes and motility.

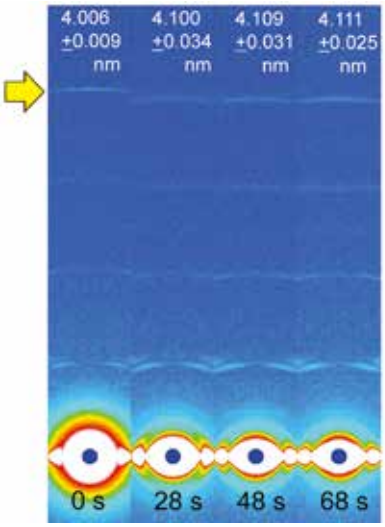

Fig. 3. Structural changes of microtubules after mixing with paclitaxel. Patterns of meridional diffraction signals obtained by 20 s exposure are shown. From the fourth-layer-line (yellow arrow), tubulin axial repeat was determined.

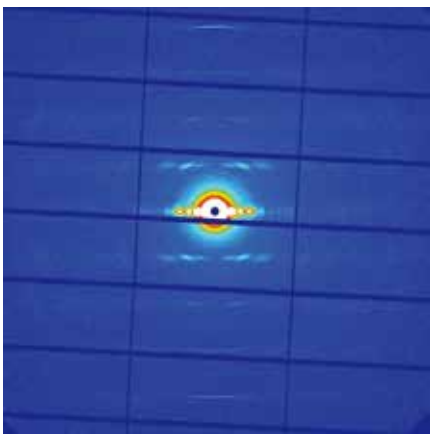

Fig. 2. Example of X-ray fiber diffraction of microtubules. The image was acquired by PILATUS 3X 2M at BL45XU using microtubules assembled with GTP at 37.0°C. Yellow arrow indicates the fourth-order reflection (~1 nm) of axial tubulin repeat (~4 nm).

Shinji Kamimura

Department of Biological Sciences, Chuo University

Email: skam@bio.chuo-u.ac.jp

References

[1] T. Sugiyama *et al.*: Biophys. J. **97** (2009) 3132.
[2] S. Toba *et al.*: Biophys. J. **108** (2015) 2843.
[3] *S. Kamimura, Y. Fujita, Y. Wada, T. Yagi and H. Iwamoto: Cytoskeleton **73** (2016) 131.*
[4] G.B. Jeffery: Proc. R. Soc. Lond. A **102** (1922) 161.
[5] R.G. Larson: The Structure and Rheology of Complex Fluids (1999) Oxford Univ. Press.
[6] E.W. Taylor & W. Cramer: Biophys. J. **3** (1963) 143.
[7] J.D. Bernal & I. Fankuchen: J. Gen. Physiol. **25** (1941) 111.
[8] K. Maruyama: J. Biochem. **55** (1964) 277.
[9] B. Gigant *et al.*: Cell **102** (2000) 809.
[10] H. Li *et al.*: Structure **10** (2002) 1317.
[11] G.M. Alushin *et al.*: Cell **157** (2014) 1117.