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Method and Protocol

X-ray diffraction recording from a small amount of fibrous protein materials oriented by a micro shear-flow cell

Hiroyuki Iwamoto[1], Kazuhiro Oiwa[2], Kogiku Shiba[3], Kazuo Inaba[3]

[1] *Scattering and Imaging Division, Japan Synchrotron Radiation Research Institute, Sayo, Hyogo 679-5198, Japan*
[2] *Advanced ICT Research Institute, National Institute of Information and Communications Technology, Kobe, Hyogo 651-2492, Japan*
[3] *Shimoda Marine Research Center, University of Tsukuba, Shimoda, Shizuoka 415-0025, Japan*

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This paper describes a method for recording X-ray diffraction patterns from a small amount of fibrous protein materials while being oriented by using a micro shear-flow cell. This cell consists of two concentrically arranged glass tubes. The inner tube is stationary, while the outer one rotates at a high speed. The gap between the two tubes is about 100 µm, into which the suspension of fibrous protein materials is injected. By using synchrotron-radiation X-ray microbeams (diameter, 10 µm), clear diffraction images from oriented protein materials can be recorded. The required volume of the sample is only about 10 µl. This method can also be combined with the laser-flash photolysis of caged compounds. Examples of application of this method to the flagella of a green alga *Chlamydomonas*, and sperm of a tunicate *Ciona* are presented.

Key words: synchrotron radiation, X-ray microbeam, shear-flow alignment, eukaryotic flagella, X-ray fiber diffraction

Significance

X-ray fiber diffraction is an effective method for analyzing the molecular structure of fibrous proteins or protein assemblies. It is shown that by applying shear force, the samples can be oriented, and informative fiber diffraction patterns can be obtained. However, this method often requires a relatively large amount of samples. Here we present a method to record diffraction patterns from a microliter amount of fibrous samples while they are oriented, by using a micro shear-flow cell in combination with intense synchrotron-radiation X-ray microbeams. Dynamic experiments using caged compounds are also possible.

Introduction

Many biological molecules or their assemblies take the form of fibers, including DNA, myofilaments, microtubules, bacterial flagella, and eukaryotic flagellar/ciliary axonemes. For these materials, X-ray fiber diffraction is an effective method for determining their molecular structures, and the double-helical structure of DNA was also determined by this method [1]. X-ray fiber diffraction is most effective when the fibrous samples are orientationally aligned. Except for a few examples such as myofilaments in muscle fibers, most fibrous samples are only available in the form of randomly oriented aqueous suspensions.

In 2009, Sugiyama et al. [2] showed that when methyl cellulose is present in the suspension, fibrous materials can be oriented under shear-flow, and good diffraction patterns can be recorded. It had been theoretically shown and

Corresponding author: Hiroyuki Iwamoto, Scattering and Imaging Div., Japan Synchrotron Radiation Research Institute, Sayo, Hyogo 679-5198, Japan. ORCID iD: https://orcid.org/0000-0002-4980-8495, e-mail: iwamoto@spring8.or.jp

experimentally demonstrated that rod-like rigid bodies are aligned under shear flow, as quoted in Sugiyama et al. [2], but the effect is insufficient with shear-flow alone. Sugiyama et al. discovered that the effect is greatly enhanced by methylcellulose or other similar polymers, but its exact mechanism is unknown. Using this method, they recorded diffraction patterns from the oriented samples of axonemes from sea urchin sperm tails, tobacco mosaic virus, and microtubules. Their shear-flow apparatus consisted of two thin glass disks, one was stationary and the other rotating against it. The gap between the two disks was filled by the suspension of the sample containing methyl cellulose. The amount of the suspension required was 50-100 μl.

By using this shear-flow apparatus, the structures of axonemes from the flagella from wild type and mutant strains of a green alga, *Chlamydomonas*, were analyzed [3,4]. These mutants lacked specific component proteins of axonemes, enabling the identification of the origin of each reflection in the diffraction pattern. Using this apparatus and its improved version, Kamimura and colleagues also studied the effects of temperature, nucleotides and drugs on the structure of microtubules [5-7].

Whether it is this type of shear-flow experiments or ordinary protein solution scattering experiments, an order of mg/ml concentration of sample is needed to obtain sufficient X-ray signals. Protein samples are often difficult to prepare in large quantities, and it is desirable to keep the sample volume minimum. For this reason, we have developed a micro shear-flow cell with a sample volume of less than 10 μl. To obtain sufficient shear-flow rates, it is rotated by a high-speed coreless DC motor. By irradiating with intense synchrotron-radiation X-ray microbeams, sufficient scattering signal is obtained within a short exposure time. This shear-flow cell is also designed to enable laser-UV flash photolysis experiments so that one can perform time-resolved measurements using caged compounds.

Materials and Methods

Micro Shear-Flow Cell

Figure 1 shows the photograph and anatomical drawings of the micro shear-flow cell. The sample part consists of two concentrically arranged borosilicate glass tubes. The inner one is stationary and its outer diameter is 1.0 mm. The outer one is rotated by a motor, and its inner and outer diameters are 1.4 mm and 2.0 mm, respectively. Therefore, the gap between the two tubes (where the sample is loaded) is 200 μm. The overlap between the two tubes is 8 mm along the X-ray beam path, so that a maximum of 6 μl of sample can be loaded. Both ends of the glass tube is open, and the sample is retained in the gap by surface tension. The rotation of the motor (SCL12-2005, Namiki Precision Jewel, Tokyo, Japan, with an unloaded rotation speed of 17,200 rpm at the rated DC voltage of 5V) is transmitted to the outer glass tube via two pulleys of equal diameters and a rubber belt. When incorporated into the micro shear-flow cell, the motor rotated at around 10,000 rpm as measured by a high-speed video camera, giving a shear rate of 3,660 s^{-1}. This value is comparable to the one used to align specimens by Sugiyama et al.'s apparatus (1,000 -6,000 s^{-1}) [2]. Usually, an operation voltage of 4V gave good results (6,700 rpm).

X-ray Recording

In the example experiments as shown in Figure 2 in the Results section (eukaryotic flagellar axonemes), the recording of X-ray diffraction patterns was carried out at the BL40XU beamline of SPring-8. The X-ray energy was 12.4 keV, and two pinhole apertures, made of 50-μm thick tantalum foil (aperture diameters, 10 and 20 μm), were placed upstream of the sample. The X-ray flux after these pinholes is estimated to be about 2 x 10^{10} photons/s. The specimen-to-detector distance was 3.5 m. The specimen-to-detector distance is determined by considering the spacing of the structure of interest and the area of the detector used. In the example in Fig. 2, the detectors were of visible light type, and they were used in combination with a 4-inch X-ray image intensifier (V7739P-MOD, Hamamatsu Photonics, Hamamatsu, Japan), which intensifies the X-ray signal and converts it to visible light. As a detector, a high-speed CMOS video camera (AX-200, Photron, Tokyo, Japan, 1024 x 1024 pixels) or a slower CMOS camera (Model C11440-22CU, Hamamatsu Photonics, Hamamatsu, Japan, 2048 x 2048 pixels) was used, depending on the time resolution required. In the example in Fig. 2, the AX-200 camera was used at a rate of 1 ms/frame, and the specimen-to-detector distance was 3,590 mm so that the 1st layer-line reflection of axonemal structures at 96 nm^{-1} was observable. The measurements were done at room temperature (25°C).

Experimental Protocol

Methyl cellulose (MC, 4000 cPs) is dissolved in a buffer for the protein specimen at a 2% concentration and left in a refrigerator overnight. On the day of X-ray recording, equal volumes of sample-containing buffer and MC-containing buffer are thoroughly mixed, and the mixture is lightly centrifuged to remove bubbles. An aliquot (~10 μl) of the mixture is applied to the opening of the outer glass tube of the shear-flow cell while the motor is turned off (Fig. 1C). It is recommended to apply slightly more mixture than the volume of the cell (6 μl), because some of the mixture will remain

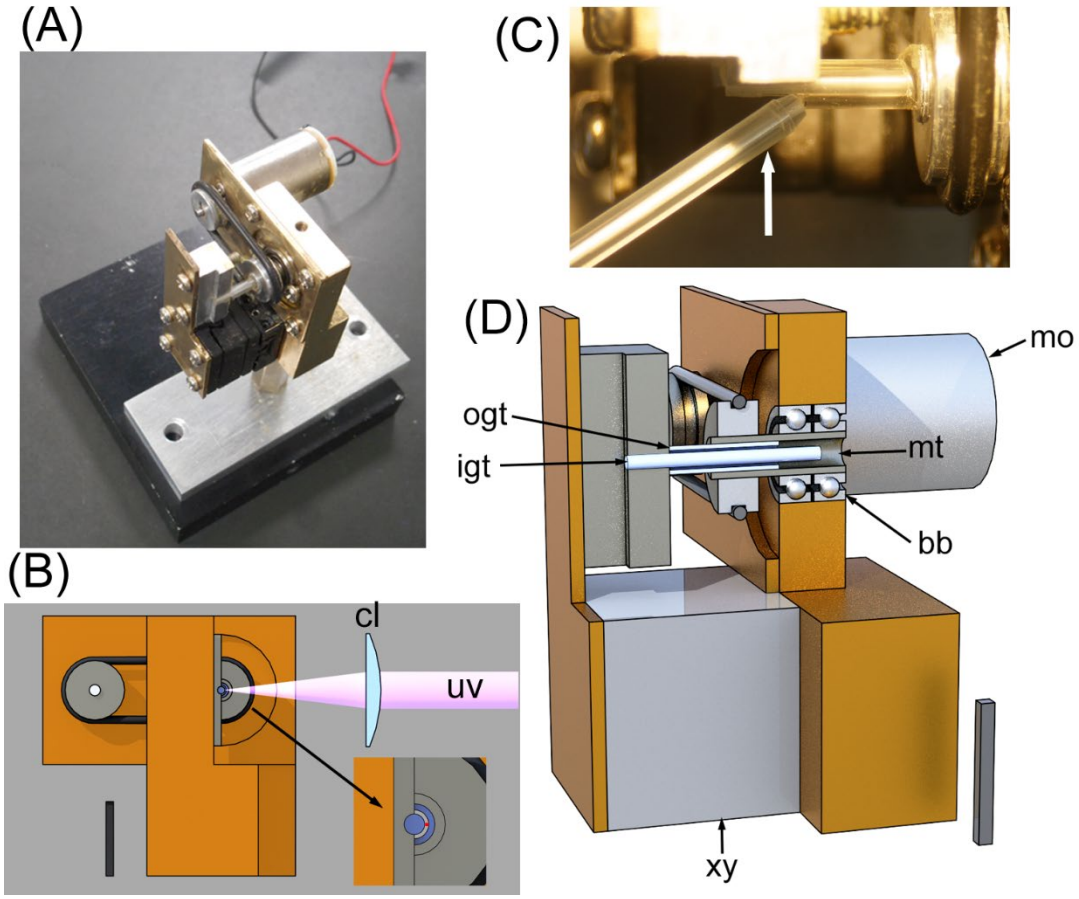

Figure 1 Photograph and anatomical drawings of the micro shear-flow cell. **(A)** Photograph. **(B)** Drawing of the cell viewed from the downstream side of the X-ray beams. This also shows the configuration for UV flash photolysis experiments. The UV beam (**uv**) is focused onto the glass tube by using a convex cylindrical lens (**cl**). In the magnified view of the central part (bottom right), the X-ray microbeam (red dot) passes through the gap between the two glass tubes. **(C)** Photograph showing how to load sample. The opening of the Microman pipetter tip (arrow) is made in contact with the end of the outer glass tube. Viewed from the top. **(D)** Anatomical drawing of the cell. The upper part of the cell is cut along the axis of rotation and removed to show the inside. **ogt**, outer glass tube; **igt**, inner glass tube; **mo**, motor; **mt**, metal tube; **bb**, ball bearing; **xy**, X-Y mechanical stage to fine-adjust the position of the inner glass tube relative to the outer glass tube. Screws are omitted from the drawings. In the drawings, the brown color represents parts made of brass, and the gray color represents made of other materials (aluminum, stainless steel, etc.). In (B) and (D), the height of the gray bar is 10 mm.

outside of the glass tube. The gap between the inner and outer glass tubes is filled with the sample by capillary action, so that no special skill is required. Because the mixture is highly viscous, the use of Microman (Gilson, Middletown, USA) with 10-µl tips is recommended. The glass tube is easily filled with the mixture if it is applied while the tube is manually rotated. After ensuring that the glass tube is fully filled with the sample and no bubble is trapped (bubbles cause parasitic scattering), the experimental hutch of the beamline is closed.

The motor is turned on immediately before X-ray exposure. The voltage should be adjusted so that best results are obtained by observing the recorded diffraction patterns. If the rotation is too slow, satisfactory alignment is not obtained. Excessive speed may cause the sample to splash off.

The exposure of the sample to X-ray should be kept as short as possible. Otherwise, it will cause radiation damage and temperature rise. In the experiments shown in Figs. 2 and 3, it was programmed so that the X-ray shutter, placed upstream of the sample, opened 30 ms ahead of trigger of the X-ray camera, and closed immediately after the recording was finished. If a long exposure time is required, it is desirable to split the exposure to several sessions to avoid temperature rise. The diffraction patterns in Fig. 2 were recorded in this way (200 ms x 4).

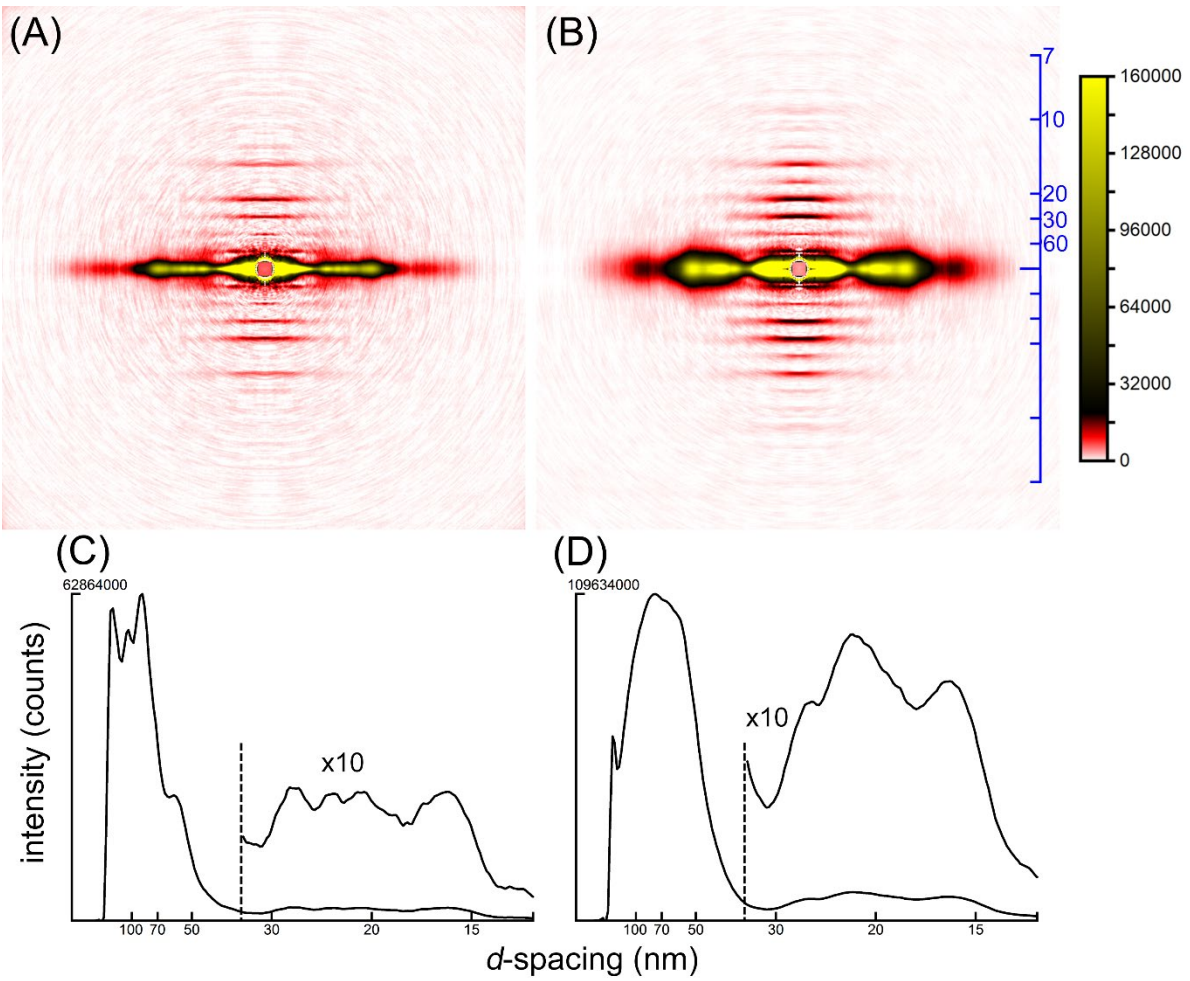

Figure 2 Diffraction patterns recorded by using the micro shear-flow cell. **(A)** *Chlamydomonas* (wild type), **(B)** *Ciona*. Recorded with an AX-200 high-speed camera at a speed of 1 ms/frame. For each, images of 800 frames were summed after individually correcting the orientations of images, and the background scattering was subtracted, and the 4 quadrants of the pattern were averaged. The blue scale on the right represents *d*-spacing (nm). The color bar indicates the intensity of X-ray scattering in a linear scale, and the number is counts/pixel. **(C, D)** Integrated intensity profiles of equatorial reflections. (C), *Chlamydomonas*, (D), *Ciona*. The vertical scale is linear.

By using a shear-flow cell, radiation damage is alleviated, because the irradiated volume of the sample is continually shifting [4]. The same benefit was observed for this micro shear-flow cell. Although the X-ray flux density in theBL40XU beamline is ~3 orders of magnitude greater than in the previous studies [2,4], the samples withstood ~1 s exposure without apparent radiation damage.

For laser flash photolysis experiments, mirrors should be arranged so that the laser beam comes to the side of the exposed part of the glass tube. As a UV light source, a Nd:YAG (Neodymium-doped Yttrium Aluminum Garnet) pulse laser is most commonly used, and the BL40XU beamline of SPring-8 is also equipped with this laser (Surelite, Continuum, USA; pulse duration, 6 ns; power, 120 mJ/pulse at λ = 355 nm). The linear absorption coefficient of borosilicate glass is -0.128 cm^{-1} at λ = 355 nm (based on Sigma Koki's data sheet), and the transmission of the UV light by the outer glass tube is calculated to be 99.6%. By using a convex cylindrical lens, the laser beam can be focused on the glass tube (Fig. 1B). Although photolysis on the side of the glass tube opposite to the UV laser may not be efficient, the photolyzed caged compound should start to reach this side in 3 ms if the motor rotates at 10,000 rpm, although it takes more time until the content is thoroughly mixed. Ideally, the timing of UV flash should be synchronized with the trigger of the high-speed X-ray detector.

Preparation of Test Samples

For test samples, the flagellar axonemes of a green alga, *Chlamydomonas reinhardtii* and the sperm tails of a tunicate, *Ciona intestinalis* (recently renamed as *Ciona robusta* or *Ciona intestinalis* type A), were used. The flagella of wild-type *Chlamydomonas* were collected and demembranated as described [4]. *Ciona* axonemes were isolated by homogenization of demembranated sperm (~1 ml undiluted sperm from 50 individuals) [8], basically according to the method as described previously [9]. The final concentration of the samples was 1.6 mg/ml for *Chlamydomonas*, and 5.4 mg/ml for *Ciona*. The diffraction patterns in Fig.2 were collected in the presence of 1 mM ATP and 100 μM vanadate (pCa = 8.0).

For flash photolysis experiments, demembranated flagellar axonemes of wildtype *Chlamydomonas* were used as described above. The experimental conditions were the same as for the samples in Fig. 2, except that the solution contained 1 mM caged ATP (Dojindo Laboratories, Kumamoto, Japan) instead of ATP.

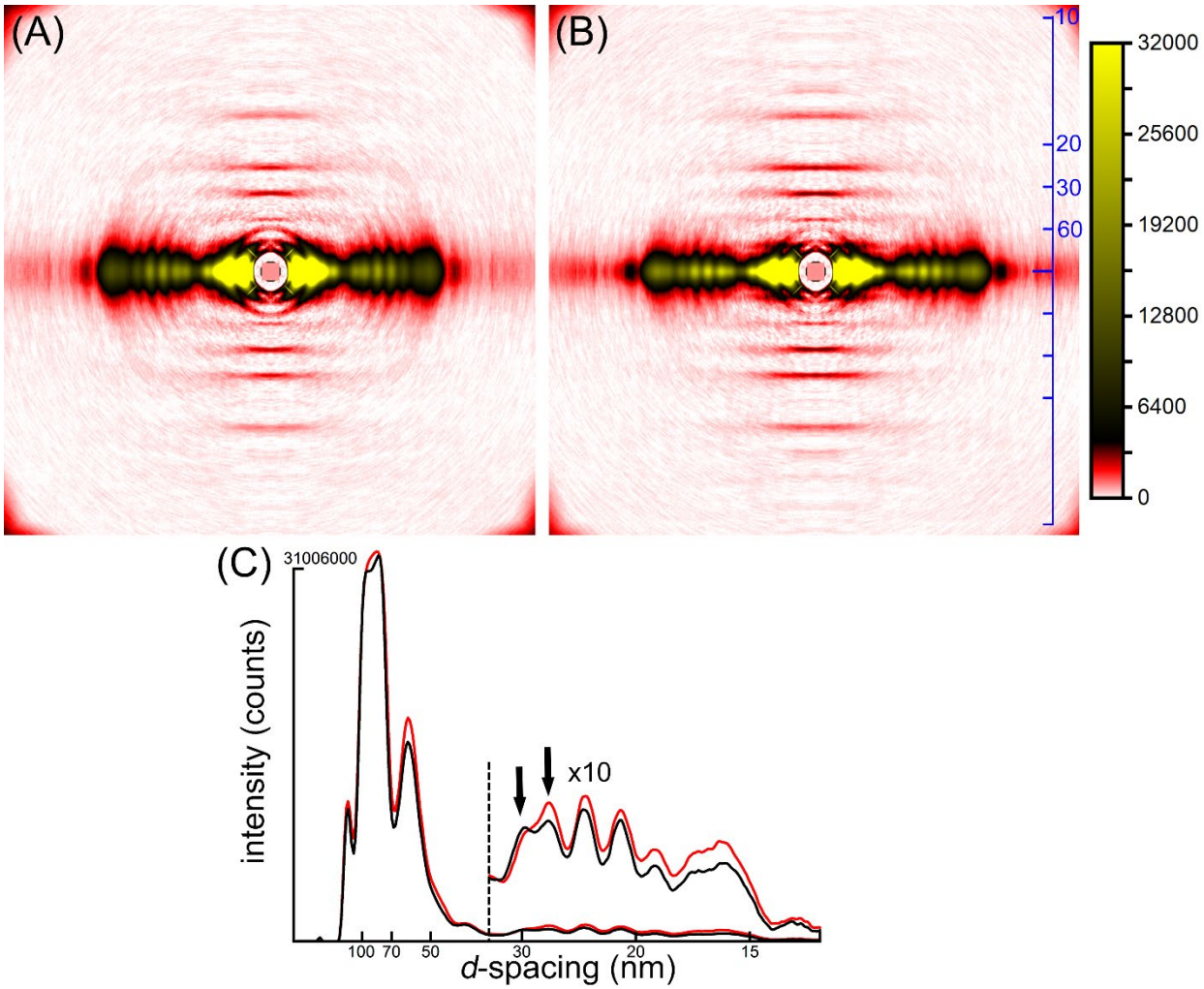

Figure 3 Effect of UV flash photolysis of caged ATP on *Chlamydomonas* axonemes. **(A)** Before photolysis, **(B)** after photolysis. To ensure thorough photolysis, UV flashes were repeated 20 times. Recorded with an AX-200 high-speed camera at a speed of 1 ms/frame. For each, images of 200 frames were summed after processing as described in the legend for Fig. 2. The scale on the right represents *d*-spacing (nm). Improvement of axonemal alignment is evident from the reduced extent of fanning out in the equatorial reflections, and reduced circular reflections originating from a population of disoriented axonemes. **(C)** Comparison of intensity profiles of equatorial reflections before and after photolysis. Black and red curves represent before and after photolysis, respectively. The most conspicuous change is observed at the peaks indicated by an arrow.

Results and Discussion

The diffraction patterns recorded from *Chlamydomonas* and *Ciona* axonemes are shown in Figure 2. Besides the strong equatorial reflections (yellow horizontal part at the center), a number of layer-line reflections are observed. They are indexed to the 96-nm repeat of the axonemal components, including radial spokes and inner dynein arms. The layer line closest to the center is the 1st layer-line reflection and it is clearly resolved. The 4th layer-line reflection at 24 nm^{-1} is strong in both *Chlamydomonas* and *Ciona*, because the dynein outer arms with a 24-nm repeat strongly contribute to this reflection [4].

Especially in *Chlamydomonas*, the layer-line reflections are straight, and the vertical width of the equatorial equations does not increase with the distance from the center (X-ray beam position). This means that the orientation of the axonemes is very well aligned. Poor alignment results in the arcing of the layer-line reflections and the spreading of the equatorial reflections with the distance from the center. Depending on the mutations the axonemal structure has (see ref. [4]), *Chlamydomonas* axonemes are very often naturally bent and are not easily straightened even under shear flow. In extreme cases, a population of axonemes seems to be randomly oriented even under shear flow, and a part of the equatorial reflections becomes ring-like and overlaps with the layer-line reflections. The axonemes of the wild-type strain as used here is usually by no means easily aligned. Considering these factors, the present results seem to demonstrate that the micro shear-flow cell is very effective in aligning eukaryotic flagellar axonemes.

In Fig. 2C and D (and also in Fig. 3C), the intensity profiles of the equatorial reflections are shown for *Chlamydomonas* and *Ciona* samples. Although they were recorded with a high-speed camera, the quality of the data is equivalent to that presented in ref. [4], and can be quantitatively analyzed as was done in ref. [4]. There are substantial differences in the outer part of equatorial reflections between *Chlamydomonas* and *Ciona* (x10 enlarged part), and this part is considered to reflect dynein, radial spoke and other axonemal components. These differences will be discussed in detail elsewhere.

Figure 3 shows the results of the flash photolysis of caged ATP. The diffraction pattern in Fig. 3A was recorded before photolysis, and the one in Fig. 3B was recorded after photolysis. The axonemes before photolysis were in rigor (strong bonds were formed between microtubules and dyneins), and were bent and stiff. For this reason, the extent of axonemal alignment is lesser than in the presence of ATP, as is evident from the fanning out of the equatorial reflections (Fig. 3A). After photolysis, the axonemes became more flexible, and the extent of alignment was improved (Fig. 3B). Apart from this change, the changes in the intensity profiles of reflections were expected to be subtle, and UV pulses were flashed 20 times to ensure thorough photolysis. The intensity profiles of equatorial reflections (Fig. 3C) shows that relative heights of the two peaks (indicated by arrows) changed after photolysis (red curve). After photolysis, the layer-line reflections were laterally extended, but this change could be the result of improved alignment of axonemes. Experiments using caged calcium were also conducted, and changes were observed in layer-line reflections after a single flash. Details of these experiments will be presented elsewhere.

To summarize, we have developed a micro shear-flow cell that requires 10 μl of sample or less at a time, and can effectively align suspended fibrous protein samples. The drawbacks are that it requires brilliant X-ray microbeams and their alignment is stringent. The glass tube must be strictly parallel to the beam path. In SPring-8, of all the public beamlines (beamlines for which every researcher can submit experimental proposals), only BL40XU fulfills the requirement. BL40XU can generate brighter X-rays than other beamlines, owing to the wider bandwidth of X-ray energies (2%). Because of this wide bandwidth, the quality of diffraction patterns is compromised to some extent. However, a renovation of BL40XU is scheduled in late 2024, and after this, BL40XU will be reborn as a beamline solely dedicated to small-angle scattering. After this renovation, better monochromatized (bandwidth = 0.01%) X-ray beams will be available. Microbeams will be generated by focusing the X-rays rather than using pinholes, thereby increasing the X-ray flux. By using the micro shear-flow cell as described here, it is expected that higher-quality diffraction patterns can be recorded.

Conclusion

We described the method and protocol for the use of the micro shear-flow cell for X-ray diffraction recording in the beamlines of synchrotron radiation facilities. Eukaryotic flagellar axonemes from *Chlamydomonas* and *Ciona* were used as test samples. They showed good orientational alignment when shear force was applied using the cell. The potential of the cell will be further improved after the scheduled renovation of the beamline.

Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

HI designed and built the micro shear-flow cell. HI and KO performed X-ray experiments. KI, KS and KO prepared samples. All authors contributed in writing paper.

Data Availability

The data are available upon request to the corresponding author.

Acknowledgements

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