Single Particle Imaging of human chromosomes at SACLA

Authors and addresses

Ian Robinson[1,2,3], Joerg Schwenke[1,2], Mohammed Yusuf[1,2], Ana Estandarte[1,2], Fucai Zhang[1,2], Jesse Clark[4,5], Changyong Song[6], Daewoong Nam[6], Yasumasa Joti[7,8], Kensuke Tono[7,8], Makina Yabashi[7,8], Gina Ratnasari[9], Kohei Kaneyoshi[9], Hideaki Takata[9] and Kiichi Fukui[9]

[1] London Centre for Nanotechnology, University College, Gower St, London, WC1E 6BT, UK

[2] Research Complex at Harwell, Rutherford Appleton Laboratory, Didcot, OX11 0FA, UK

[3] Materials Science and Engineering, TongJi University, Shanghai, China

[4] Stanford PULSE Institute, SLAC National Accelerator Laboratory, Menlo Park, CA 94025, USA

[5] Center for Free-Electron Laser Science (CFEL), Deutsches Elektronen Synchrotron (DESY), Notkestrasse 85, 22607 Hamburg, Germany

[6] Pohang University of Science and Technology, 77 Cheongam-ro, Nam-gu, Pohang-si, Gyeongsangbuk-do, South Korea

[7] Japan Synchrotron Radiation Research Institute, 1-1-1 Kouto, Sayo, Hyogo Prefecture, 679-5198, Japan

[8] RIKEN SPring-8 Center, 1-1-1 Kouto, Sayo, Hyogo Prefecture, 679-5148, Japan

[9] Osaka University, 2-1 Yamadaoka, Suita 565-0871, Osaka, Japan

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Abstract

Single Particle Imaging (SPI) is one of the front-page opportunities which were used to motivate the construction of the first X-ray Free Electron Lasers (XFELs). SPI's big advantage is that it avoids radiation damage to biological samples because the diffraction takes place in femtosecond single shots before any atomic motion can take place in the sample, hence before the onset of radiation damage. This is the "diffract before destruction" theme, destruction being assured from the high X-ray doses used. This article reports our collaboration's first attempt at SPI using the SACLA XFEL facility in June 2015. The report is limited to experience with the instrumentation and examples of data because we have not yet had time to invert them to images.

Introduction

There are two proposed methods of presenting samples to the XFEL beam for SPI: particle injection and membrane-scanned samples. Particle-injection SPI was the main design goal of the SXI instrument at Stanford's LCLS project, but this beamline has evolved into a "serial femtosecond crystallography" facility and its SPI capabilities have not been so strongly developed. SPI was also an early application of the AMO beamline of LCLS using the user provided CAMP chamber. Virus structure was an early promise of SPI because the high symmetry of some viruses could be used to

obtain multiple views of a sample and achieve single-shot 3D imaging. This is the same idea as "non-crystallographic symmetry". For signal reasons, it was found that very large viruses were needed for the experiment to work well. Impressive 3D images of a mimi-virus, obtained by the Hajdu group, show significant internal structure [1,2]. Another important direction is "Live cell imaging" with single XFEL shots applied to previously living cells in a thin liquid environment. Live cyanobacteria [3] and other cells [4] were sandwiched between two SiN membranes and imaged with single shots in an impressive demonstration of this application.

Carboxysomes are polyhedral particles involved with the fixation of CO_2 in cyanobacteria. They appear icosahedral in the electron microscope with sizes from 90-500 nm. They are difficult to crystallize because of this size variation. The particles contain a large number of ribulose bisphosphate carboxylase/oxygenase (Rubisco) clusters (11 nm diam), sometimes forming internal arrays. Rubisco is the most common protein on our planet and is vital to life. Hantke et al imaged Carboxysome particles by injecting them into the CXI station at LCLS [5]. The individual images avoided the size dispersity problem, but clearly showed their icosahedral symmetry. This work demonstrated the utility of non-damaging X-ray imaging filling the gap between optical and electron microscopy resolutions.

The particle injection method is very wasteful of material, with gram quantities of protein sometimes needed for a run of a few hours, collecting diffraction patterns at 120Hz at LCLS. Less than 1 in 10^9 particles typically gets hit directly. Technical improvements such as using a lipidic-phase propellant have improved this situation [6], but are not always compatible with the samples.

An alternative method was developed by the group of Changyong Song at the SPring-8 Angstrom Compact Free Electron Laser (SACLA) XFEL facility [7], adjacent to the Spring8 synchrotron facility in Japan. The MAXIC chamber [8] uses a fast-scanning mechanical raster system to move membrane samples through the beam at up to 30Hz repetition rate. The scanner is fast enough to separate each shot beyond the circle of debris (Fig 1), but some seconds are wasted moving from one membrane window to the next. Imaging of the mammalian nucleus has been achieved in this way [9]. In the work reported here, because of the difficulty of handling whole chromosome samples, we used the membrane-scanning, rather than particle-injection, method to measure human chromosomes with single shots of X-rays from SACLA in order to obtain damage-free images.

Methods

A b-lymphobalstoid male Yoruba cell line (passage 4) was grown at 37 °C in a 5% CO_2 atmosphere in RPMI medium (Sigma, UK) supplemented with penicillin/streptomycin (Sigma, UK) and 20% FBS (Sigma, UK). Mitotic cells were enriched using a thymidine block with the addition of 2 mM thymidine for 16 hours and then arresting the mitotic cells after treating the cells with colcemid 0.2 µg/mL (Gibco Life Technologies, UK) for 16 hours. Polyamine mitotic chromosomes were prepared by treatment with a prewarmed hypotonic, 0.075 M KCl (VWR BDH Prolabo, UK) for 15 minutes and then resuspended into polyamine buffer [10,11] or in Methanol acetic acid [12].

Chromosomes were prepared for X-ray imaging according to a previously published protocol [13,14]. The chromosome sample was fixed in glutaraldehyde and placed onto a silicon nitride window containing 150 µM of SYBR gold stain. The sample was washed in water to remove residues of dye. Chromosome preparations were verified by imaging using a Zeiss AxioZ2 fluorescence microscope with ISIS software or an Olympus LEXT-OLS4000. For some of the membranes, chromosomes were stained with Platinum blue [12], at a concentration 5 mM for 30 min and washed for 5, 10, and 15

min in water. Chromosome samples were either dehydrated using an ethanol series and left to air dry or were dried using hexamethyldisilazane (HMDS).

A parallel set of samples was prepared using HeLa S3 human cervical cancer cell line. HeLa S3 cell was cultured at 37°C in a 5% CO_2 atmosphere in RPMI 1640 medium (Nacalai Tesque, Japan) with 5% of fetal bovine serum (FBS). To obtain mitotic cells, colcemid was added (final concentration 0.1μg/mL) 16 h before harvesting. Mitotic chromosomes were isolated by polyamine method [15] and applied onto 0.01% poly-L-lysine coated silicon nitride windows, kept on ice for 10 min. After incubation in XBE0 buffer (10 mM HEPES, pH 7.7, 100 mM KCl and 5 mM EGTA) for 30 min, the chromosomes were fixed with 2.5% glutaraldehyde/XBE0 for 30 min. After washing with XBE0 three times for 5 min each, a solution of 0.5% ionic liquid, 1-butyl-3-methylimidazolium tetrafluoroborate (BMI-BF4, Merck), was applied onto the samples for 1 min incubation [16]. Afterwards, extra ionic liquid was removed by filter paper, and the chromosome samples were air dried.

The 36x36 window membrane arrays used by MAXIC were screened under an optical microscope to draft maps of the densely covered regions, which were then entered into the LabView interface of the MAXIC chamber. We scanned a total of 20 membrane arrays in five batches of 4. Measurements were made at BL3 of the SACLA facility [17]. With 1-hour downtime between batches for breaking vacuum, our collaboration's 48-hour run in June 2015 resulted in 400,000 exposures (12TB) of diffraction data. Some membranes were found not to diffract and were skipped by observant operators. SACLA ran very well during the entire beamtime with very few interruptions. We used 4keV X-rays at 10Hz to have a strong signal from biological samples one micron thick. The Multi-port Charge-Coupled Device (MPCCD) detector [18] worked well and gave data with low background.

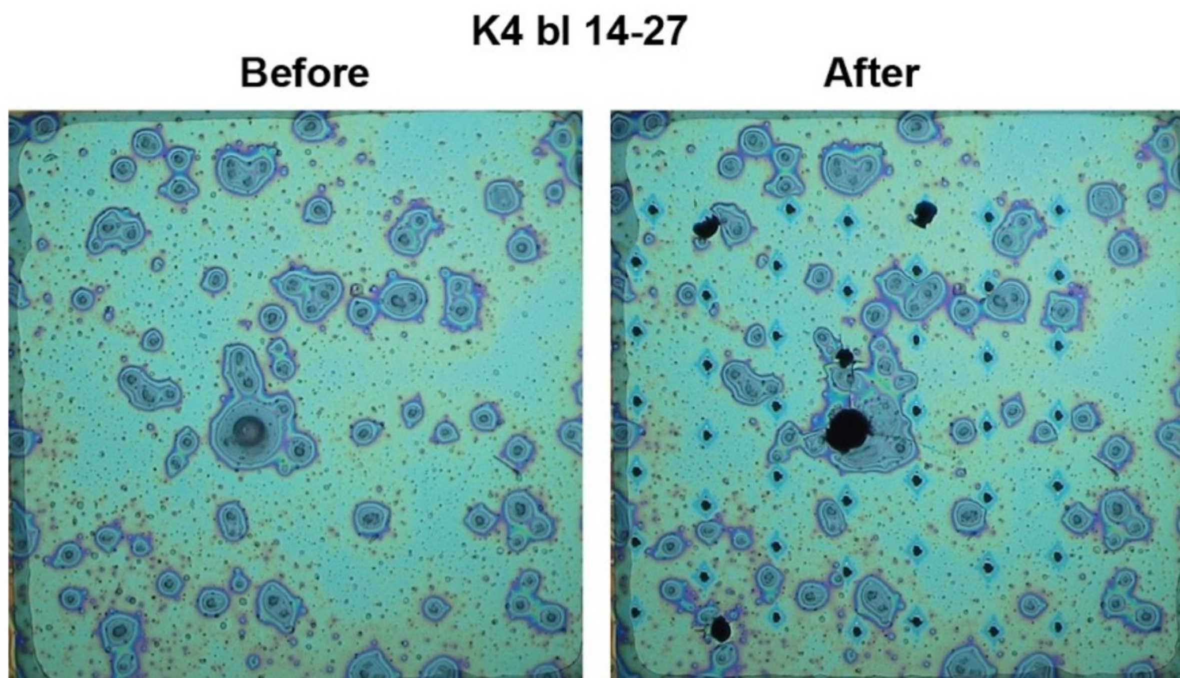

Figure 1. Optical microscope image of a 200x200 μm Silicon Nitride (SiN) membrane, 150nm thick, coated with a small density of human HeLa S3 chromosomes. The sample was prepared with a non-aqueous ionic liquid which survives insertion into vacuum. Left: before irradiation. Right: after irradiation with a 7x7 array of XFEL shots, spaced 25 μm apart.

The first 3 hours were used for beamline alignment and testing the effect of closing the Kirkpatrick-Baez (KB) mirror entrance slits located 4m in front of the sample chamber in order to enlarge the focus size. The KB focussing system produced a focus of 1.5µm, which is smaller than some of the chromosomes we wanted to study. Diffraction-limit effects at the slit allow the focus to be enlarged to match the size of the samples, several microns in some cases, and still stay within the oversampling range of the detector (50µm pixels at 1.5m). When the slit size was 1x1mm, the focus was measured to be 1.7µmx1.4µm with a wire scan. When the slits were closed to 0.25x0.25mm we found it gave 2.1µm, 0.1x0.1 gave 3.6µm, 0.06x0.06 gave 4.3µm and 0.04x0.04 gave 5.9µm, all in the vertical direction. In order not to lose too much flux, we decided on 0.25x0.25mm slits about 6 hours into the run.

Results

As can be seen in Fig.1, the sample we prepared contains only chromosome suspension, with almost no nucleus or chromosome cluster material. Almost all the chromosomes were individually suspended in the buffer solution and have been scattered on the substrate surface following sample preparation. The sizes of the isolated chromosomes ranged from 1 to 3µm.

Initially we were concerned about breaking membranes with the beam. Most exposed 100nm thick windows were found to have burst after seeing the full beam. This could have been partly due to the vacuum shock, since there were still diffraction patterns seen in some cases. But after closing the slits, the problem of burst windows was reduced to a rare event. 150nm and 200nm thick windows survived without breakage. In Fig 1 we observed that the extent of the damage caused by the beam depends on the size of the object that was hit - big objects lead to bigger holes, presumably because more energy is absorbed. Cracks can be observed around the biggest hole.

The damage surrounding each XFEL shot can be seen in the images of Fig 1. Empty regions of the membrane show a circle of visible alteration about 5 µm in diameter. Places where the ionic liquid pools are visualised by their Newton Rings remain unchanged outside this diameter. The drilled holes appear to be about 3 µm in diameter, not far from the size measure with wire scans. Three locations originally containing small objects, about the size of single chromosomes, give rise to holes (dark circles) about 5 µm in diameter. The one direct hit on a large object, 10 µm in diameter, probably an unburst cell nucleus, produced a hole about 13 µm in diameter and the appearance of cracks in the membrane.

A very rough scaling relation can be inferred from these observations. A metaphase cell nucleus contains a mass 46 times bigger than a single chromosome and shows a damage circle diameter, d=2.6 times bigger. We can understand this behaviour in terms of the two-dimensional heat diffusion equation applied to heat flow within the membrane,

$$\frac{\partial T}{\partial t} = D(\frac{\partial^2 T}{\partial x^2} + \frac{\partial^2 T}{\partial y^2})$$

where the symbol D represents the ratio of the thermal conductivity to the density per unit area of the membrane material. Following an impulse of heat at the origin, Q_0, the temperature follows a time-dependent Gaussian distribution,

$$T(x,y,t) = \frac{Q_0}{4\pi Dt} e^{-(x^2+y^2)/4Dt}$$

This will reach the melting point of the membrane everywhere within a circle of diameter d, where it can be shown that d scales with the square root of the heat input Q_0. We consider that the holes punctured in the membrane of Fig 1 are melted by the heat of X-ray absorption by the mass of sample in the beam. We can therefore deduce that in our experiment, the nucleus in Fig 1 received $(2.6)^2$ = 7 times the amount of heat input from the X-ray beam as the single chromosomes. This seems reasonable considering that the beam size of 2.1 μm falls well within the 10 μm nuclear diameter, so only about 7 of its 46 chromosomes would have been actually hit by the beam.

A large number of diffraction patterns was collected during the experiment running at 10Hz for most of the 48-hour run. The hit rate varied considerably from sample to sample. We used the "RunDataViewer" ImageJ plug in to view the 49 diffraction patterns from each membrane in turn [19]. Typically there were about 3 hits per membrane. We manually selected diffraction patterns for inversion into images. The size of the objects recorded was also found to vary. Sometimes only the central maximum of the diffraction pattern was lost in the in the 60x60 pixel central hole of the MPCCD detector. This corresponds to diffraction from an object 280nm in size. However on other examples as shown in Fig 2, the speckles were about 15 pixels across, from objects around 560nm across. But these examples have several fringes of missing data due to the central hole, which we expect will challenge the reconstruction algorithms [20].

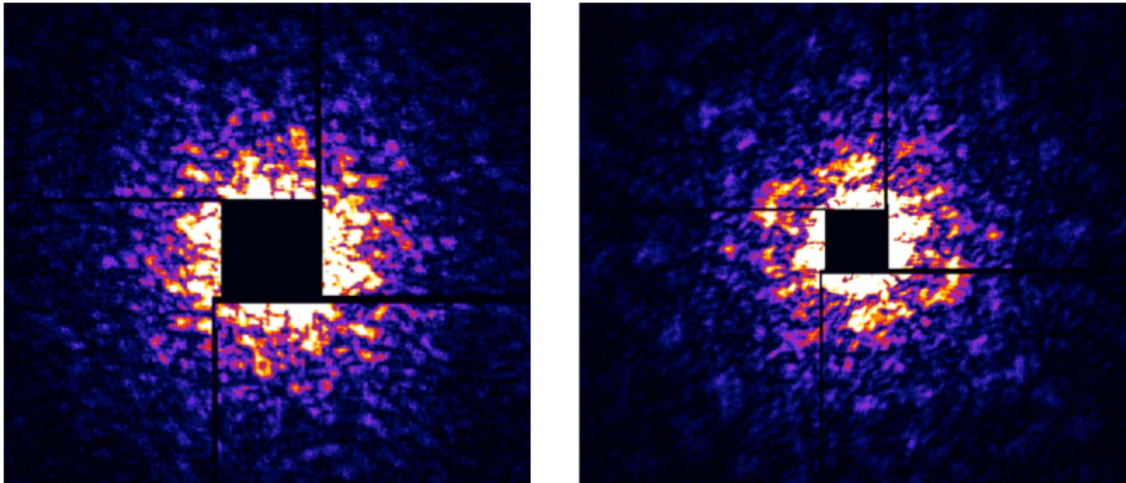

Figure 2. Examples of XFEL SPI diffraction data collected from human chromosome samples mounted on SiN membranes. The central square is the gap between the four panels of the MPCCD detector, which is 60x60 pixels wide. Good 2-fold symmetry of the patterns can be seen.

Discussion

The central hole in the MPCCD detector [18] is an unavoidable consequence of the forward scattering geometry. The four detector panels were adjusted carefully at the start of the experiment to get as close as possible to the direct beam without saturating the closest pixels. Nevertheless, this leads to missing 60x60 pixels in the centre of the diffraction patterns. A second MPCCD detector

was positioned at 3.0m from the sample behind the missing hole, but this was also protected by a beam stop which blocked most of the data recorded there.

While we expect to obtain good images from the diffraction patterns despite the missing data [18], there are potential improvements to the measuring system that could be implemented. Recording the direct beam behind an attenuator of the second detector could work. This would allow filling in of the missing region after scaling and adjustment. In our experience, in previous CDI and ptychography experiments performed elsewhere, this has not worked as well as we would have liked.

Instead, it might be interesting to consider putting an in-line holography setup inside the central hole instead of the second diffraction detector (which was mostly covered by a beam stop). We are developing a modulator-based single-shot imaging system (called Coherent Modulation Imaging - CMI), which could be employed here [21]. Getting a real space image would allow the synthesis of the missing diffraction data on the first detector.

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