Proceedings

Damage-free imaging of human chromosomes

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Received: July 16, 2015 / Accepted: August 20, 2015
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Abstract

Microscopy methods have provided most of our knowledge to date on the structural organisation of chromosomes. Even after decades of research, the high order structure of human chromosomes is still under investigation. The new generation of X-ray sources, X-ray Free Electron Lasers (XFELs) have opened an opportunity for imaging these complex structures with higher than optical resolution but without the radiation damage that usually accompanies X-ray imaging. Here we report our first experimental steps towards imaging micron-sized human chromosomes using the SACLA XFEL facility in Japan using the MAXIC chamber. The paper highlights the sample preparation of chromosomes, staining and drying conditions used, as well as the imaging optimisations and shows our first results.

Keywords: Chromosome, X-ray Free Electron Laser, Coherent Diffractive Imaging

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Introduction

The question of high order human chromosome organisation has been under scrutiny for decades (Fukui, 2009; Kornberg, 1974). The human genome contains 23 pairs of chromosomes that are made up of DNA (genes) and a number of complex proteins (e.g. histones), making chromatin. There is a gap in our knowledge between the level of the crystallographic structure of the nucleosome (Schalch *et al*., 2005) (11 nm diameter) and the optical resolution of the fully condensed mitotic chromosome (Fukui, 2009). This intermediate level of structure has not been resolved, between 11 and 250 nm, with the existence of a 30 nm fibre structure being still widely debated (Robinson *et al*., 2006; Nishino *et al*., 2012; Widom, 1989).

To resolve this long-standing question, X-ray imaging methods have been applied to the problem of chromosomes, notably Coherent Diffractive Imaging (CDI) (Nishino *et al*., 2009). X-ray methods have advantage over other microscopy methods as X-rays have high penetration and short wavelengths. Further advantages are that staining is not needed and intact samples can be imaged without sectioning. Even though X-rays can reach the resolution range of the hierarchical structures expected in a metaphase chromosome, studies to date (Nishino *et al*., 2009) have not provided any dramatic advance over optical imaging on the internal structural details. This is possibly because of radiation damage processes inherent to X-ray methods. Damage can be overcome by using a new kind of source, an X-ray Free Electron Laser (XFEL) which provides enough X-ray photons in a single short pulse (around 30 femtoseconds long) to obtain complete snapshot diffraction images of large molecules before their atoms can move (Neutze *et al*., 2000). This "diffract before destroy" concept should completely remove radiation damage from the picture (Chapman *et al*., 2011).

CDI relies on converting the diffraction pattern of a sample into a real-space image. This is possible because of "oversampling": if the sample is sufficiently small and the beam sufficiently coherent (*i.e.*, coherence length bigger than the sample), the X-ray diffraction pattern on a far-away detector will consist of fine fringes, called speckles. The spatial size of the speckles from a sample of size d is given by $\lambda D/d$, were λ is the X-ray wavelength and D is the detector distance. So long as the pixels of the detector are less than half the size of the speckles, the data are oversampled with respect to their spatial Shannon

frequency (Sayre, 1952). The number of measurements (detector pixel values) then exceeds the number of unknowns (pixel values in the image) and the data can be inverted or "reconstructed" into an image. This form of CDI was first demonstrated for X-rays by Miao *et al*. (1999).

XFELs have recently been used for imaging biological samples (Seibert *et al*., 2011; van der Schot *et al*., 2015). There is a report of an interphase nucleus (Song *et al*., 2014), but to date no report of mitotic chromosomes imaged by this method. This paper reports our first attempts at imaging human chromosome using the SCALA XFEL facility in Japan with an experiment which took place in June 2015.

Material and methods

Cell culture was used to prepare mitotic chromosomes from a b-lymphobalstoid male Yoruba cell line (passage 4) by growing at 37 °C in a 5 % CO2 atmosphere in RPMI medium (Sigma, UK) supplemented with penicillin/streptomycin (Sigma, UK) and 20 % FBS (Sigma, UK) was grown. 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 6 hours. Polyamine mitotic chromosomes were prepared following the previously described procedures (Yusuf *et al*., 2014). Briefly, the cells were treated with a prewarmed hypotonic, 0.075 M KCl (VWR BDH Prolabo, UK) for 15 minutes and then resuspended into polyamine buffer. Other preparations were placed into methanol acetic acid (3 washes in total) following a procedure described earlier (Shemilt *et al*., 2014). The polyamine prepared chromosomes were recovered after placing the suspension onto ice for 10 minutes and then vortexing for 2 minutes to allow the chromosomes to burst out of the cells. Such chromosome preparations can be stored for a month at 4 °C.

Samples were also prepared using HeLa S3 human cervical cancer cell line. HeLa S3 cell was cultured at 37 °C in a 5 % CO2 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 (Hayashihara *et al*., 2008) and applied onto 0.01 % poly-L-lysine coated silicon nitride window, 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 to the samples for 1 min incubation (Dwiranti *et al*., 2012). Afterwards, the excess ionic liquid was removed by filter paper and the chromosome samples were air dried.

Chromosomes were prepared for X-ray imaging according to a previously published protocol by Nishino *et al*. (2009). Chromosome samples were fixed in glutaraldehyde and placed onto silicon nitride windows containing 150 μM of SYBR gold stain. The samples were washed in water to remove residues of dye and then left to air dry. Chromosome preparations were verified by imaging using a Zeiss AxioZ2 fluorescence microscope with ISIS software. Chromosomes on the same membrane were stained with Platinum blue, a dye synthesized in-house following the protocol in Shemilt *et al*. (2014), at a concentration 5 mM for 30 min and washed for 5, 10, and 15 min in water. Chromosome samples were left to air dry or dried using HMDS.

Samples were prepared on 36x36 silicon nitride windows membrane arrays as needed for insertion into the MAXIC chamber (Song *et al*., 2014), which was installed in BL3 of the SACLA XFEL facility (Tono *et al*., 2013). Our collaboration had a 48-hour run at SACLA in June 2015, which resulted in 400,000 exposures (12TB) of diffraction data. For CDI data collection, we used 10 Hz 4 keV (λ = 0.31nm) X-ray pulses to have a strong signal from the biological samples just one micron thick. The 4-quadrant Multi-port Charge-Coupled Device (MPCCD) detector (Kameshima *et al*., 2014), installed at D=1.5 m, with its 50 μm pixels permitted us to measure diffraction patterns from objects as large as d=4.2 μm to maintain the oversampling condition. A 60x60 pixel hole through the centre of the 4 quadrants avoided damage to the detector but meant there was missing data which our phasing algorithms will eventually have to overcome.

Results

Beam damage effects are clearly seen in Fig. 1 which shows optical microscope images of one of the 150 nm Silicon Nitride (SiN) membranes coated with a dispersed, concentrated solution of chromosome sample K4. Ionic liquid has been added in an attempt to preserve the 3D structure by preventing drying out of the sample. As seen, the ionic liquid, visible from its Newton's interference rings under the microscope, even survives insertion of the membrane sample into the MAXIC chamber and out again. It does not, however, survive the X-ray beam, which is seen to puncture the membrane. Of the eight SACLA shots shown, seven are "misses", which do not hit any of the chromosomes. One of them is a direct hit, presumably leading to a diffraction pattern recorded on the X-ray detector, and also creates a much larger hole in the membrane.

The XFEL-drilled hole size is consistently about 3 μm for the empty regions of the membrane, which is a little larger than the measured beam size of 2.1 μm. The hole around the chromosome "event" is about 6 μm in diameter. It is understood that the heat pulse of injected X-ray energy will cause a thermal bump spreading across the membrane, which will melt out to a certain diameter. When one of the chromosomes is hit, more X-rays are absorbed and a much bigger impulse of heat results, causing a larger melted circle.

It is also notable that there is a 5 μm coloured ring around each of the holes where damage has appeared. The ionic liquid has been cleared away from a similar sized area, suggesting that the temperature pulse had reached that far, even though it was apparently insufficient to melt the SiN membrane. The elongated shape of the coloured regions suggests it might be related to tails on the beam, which are stronger in the vertical direction.

Turning now to the diffraction patterns recorded by the MPCCD detector, we consider two examples in Fig. 2, from sample K4, frames 340821 and 341746. These are examples of "hits" where the diffraction became considerably

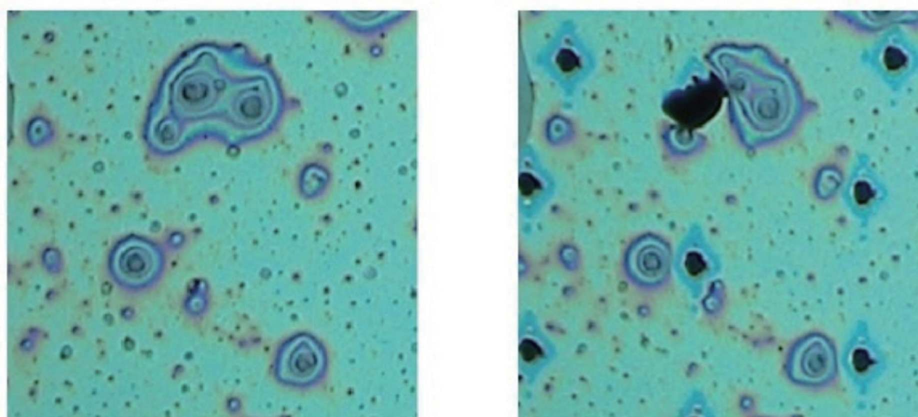

Figure 1. Enlarged optical microscope views of the 150 nm Silicon Nitride membrane coated with ionic liquid on sample K4. Image size is 60x60 µm. Left: before exposure. Right: same region after exposure to the SACLA beam. The spacing between shots is 25 µm.

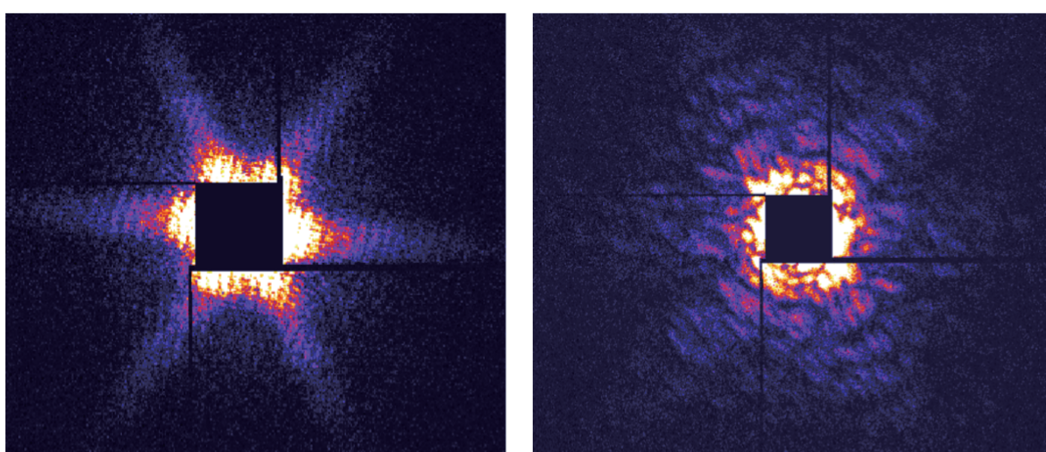

Figure 2. XFEL CDI diffraction patterns recorded on the MPCCD detector from sample K4, frames 340821 (left) and 341746 (right). The central hole in the detector is 60x60 pixels wide.

stronger than the background. The hit rate was found to be about 6 %, suggesting we had correctly optimized the density of sample coating on the membranes. This fraction varied strongly from one preparation to the next and with region of the membrane array. During the beamtime, we learned to make maps of the heavily coated regions and program them into the LabView scanner software interface. With experience, the operators found they could detect when the coverage was too low and move on to the next region of the membrane.

The intensity distribution recorded in these CDI diffraction patterns is the modulus squared of the Fourier transform of the chromosome that was hit in the experiment. This image density will be revealed eventually once the patterns have been reconstructed. The 2-fold symmetry of the data is clear; this is expected by Friedel's law and indicates that the density is a mathematically real quantity. The shapes and sizes of the two objects measured in Fig. 2 can be estimated from their speckle sizes, 4x4 pixels for 340821 and 10x25 pixels for 341746. The elongated speckles in the latter case arise from an object which is extended rectangularly in the opposite direction, which is the expected shape of a metaphase chromosome. Converting to real space, we get sizes of 2.3x2.3 µm for 340821 and 0.9x0.37 µm for 341746.

Conclusions

We have obtained a large number of promising CDI diffraction patterns from a variety of preparations of metaphase chromosomes with a hit rate of over 5 %. The object sizes deduced from the CDI speckle sizes are within the expected range for isolated chromosomes. Occasionally bigger objects, possibly whole cell nuclei, were hit, but these could be identified by their larger puncture hole in the SiN membrane.

Phasing the data and reconstructing the images still has to be completed, but it is expected these will have good resolution, around 45nm, based on seeing significant diffraction typically 190 pixels from the centre of each pattern. Once the images are obtained, the results of various trends of sample preparation, foe example Mg^{++} concentration, will be investigated.

Acknowledgements

This work was partially funded by the UK Biotechnology and Biological Sciences Research Council (BBSRC) and Engineering and Physical Sciences Research Council (EPSRC). JC was supported by a fellowship from the Volkswagen Stiftung. The experiments were performed at BL3 of SACLA with the approval of the Japan Synchrotron Radiation Research Institute (JASRI) under Proposal

No. 2015A8029. We thank the SACLA staff, particularly Yasumasa Joti and Kensuke Tono for their excellent help with operation of the beamline during our visit.

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