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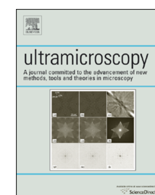

Rapid, low dose X-ray diffractive imaging of the malaria parasite *Plasmodium falciparum*

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ABSTRACT

Phase-diverse X-ray coherent diffractive imaging (CDI) provides a route to high sensitivity and spatial resolution with moderate radiation dose. It also provides a robust solution to the well-known phase-problem, making on-line image reconstruction feasible. Here we apply phase-diverse CDI to a cellular sample, obtaining images of an erythrocyte infected by the sexual stage of the malaria parasite, *Plasmodium falciparum*, with a radiation dose significantly lower than the lowest dose previously reported for cellular imaging using CDI. The high sensitivity and resolution allow key biological features to be identified within intact cells, providing complementary information to optical and electron microscopy. This high throughput method could be used for fast tomographic imaging, or to generate multiple replicates in two-dimensions of hydrated biological systems without freezing or fixing. This work demonstrates that phase-diverse CDI is a valuable complementary imaging method for the biological sciences and ready for immediate application.

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1. Introduction

X-ray coherent diffractive imaging (CDI) is under intensive development for biological imaging where the short wavelength and specific interaction of X-rays with matter is exploited to image intracellular features [1–7]. Unlike most other X-ray microscopy techniques the spatial resolution of CDI exceeds the limits imposed by image-forming optics [8]. Compared to transmission X-ray microscopy (TXM), CDI allows a lower radiation dose on the specimen by removing post-specimen optics and maximising the sensitivity to variations in the sample density and composition [9,10]. In application to biological samples, the practical resolution in CDI will generally be limited by radiation damage rather than the available coherent flux of the source [11,12], and, as such it is crucial to optimise the sensitivity and dose efficiency of the technique.

Phase-diverse CDI extends CDI ptychography [13] by overlapping a curved illumination both transversely and longitudinally to enable increased contrast with reduced radiation dose [10]. As with ptychography, extended areas can be imaged virtually free from artefacts, however, typically far fewer measurements with less overlap are required in the phase-diverse case due to the phase-curved incident illumination [10,13,14]. Phase-diverse CDI has recently been shown to provide high-sensitivity, high-resolution images (i.e. sub 100 nm) while significantly reducing the radiation dose compared to other CDI methods [10,15]. Utilising these advantages, the application of this technique to cellular imaging will therefore allow the collection of data much more rapidly than currently possible using CDI and with much less sample damage than is incurred with full-field X-ray imaging.

In this manuscript, we apply phase-diverse CDI to high resolution imaging of a parasite cellular sample demonstrating that both the dose and time for data acquisition are dramatically reduced compared to previously published cellular data using CDI. Surprisingly, we find that the necessary dose required for nanoscale resolution phase-diverse CDI is sufficiently low so that imaging of

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hydrated cells without fixing, freezing, staining, or fluorescent labelling will be possible using this approach. This method will permit high throughput whole cell, quantitative, and phase contrast tomography with similar acquisition times to transmission X-ray microscopy, albeit with a significantly lower radiation dose [16]. To illustrate this, we imaged two whole unstained red blood cells (RBCs) infected with the malaria parasite *Plasmodium falciparum* at the sexual (gametocyte) life cycle stage at a level of resolution sufficient to identify key biological features. This proof of principle experiment paves the way to X-ray microscopy finding greater application within the life sciences.

2. Methods

2.1. Parasite culture

P. falciparum-infected RBCs were cultured in a medium containing 4% human serum, 0.5% AlbuMAX in RPMI 1640 supplemented with hypoxanthine and glutamate as described previously [17], using blood donated by the Red Cross Blood Service, Melbourne. A high gametocyte producing 3D7 parasite strain was used for this study [18]. Asexual replication was inhibited with N-acetyl glucosamine (62.5 mM) and gametocytes were cultured to the desired stage and purified by magnetic separation [19].

2.2. Phase-diverse CDI data collection

X-ray data were collected at Beamline 2-ID-B at the Advanced Photon Source [20] using an in vacuo imaging endstation [21]. The sample was placed in a diverging 2.5 keV X-ray beam formed by coherently illuminating a 160 μm diameter Fresnel zone plate (FZP) with an outer zone width of 30 nm, yielding a FZP focal length of 9.8 mm. The combination of a 40 μm central beamstop at the FZP and a 10 μm order sorting aperture (OSA) at the focal plane was used to isolate the first order focus. At this X-ray energy the variation in the phase component of the complex refractive index dominates that of the absorption component by more than an order of magnitude for most biological materials. Hence our analysis here focuses on the reconstructed phase component only.

The sample was prepared as above, before being fixed in 0.01% paraformaldehyde and mounted on a gold electron microscopy grid coated with Formvar and freeze-dried for 30 min. The X-ray diffraction dataset consisted of a 4×4 and 4×3 1.5 μm^2 spaced overlapping grid of measurements collected at distances from the FZP focus of 1.055 mm and 1.045 mm respectively [10]. At the sample position, the diameter of the illumination was approximately 16 μm with Fresnel numbers in excess of 30 at the sample. The lateral overlap fraction [10] was approximately 90%, and the total reconstructed field of view exceeded 15×15 μm^2. A single image with an exposure time of 0.25 s was collected at each scan position on a cooled charge-coupled device (CCD) detector (Princeton MT-MTE) with 2048×2048 square pixels each with a 13.5 μm side length placed 83 cm downstream of the sample, giving a total exposure time of 7 s with an absorbed radiation dose on the sample of less than 1.2 kGy. The experimental arrangement is shown in Fig. 1. The data were treated according to Williams et al. [22], and combined using the phase-diverse CDI algorithm [15]. Images were reconstructed in just 200 iterations of error reduction [23] using the NADIA software package [24].

2.3. Optical and electron microscopy data collection

Live stage III–IV gametocytes were prepared for optical imaging by overnight incubation with the lipid stain, BODIPY-TR-ceramide (1.5 μM) [25]. The gametocytes were harvested by centrifugation,

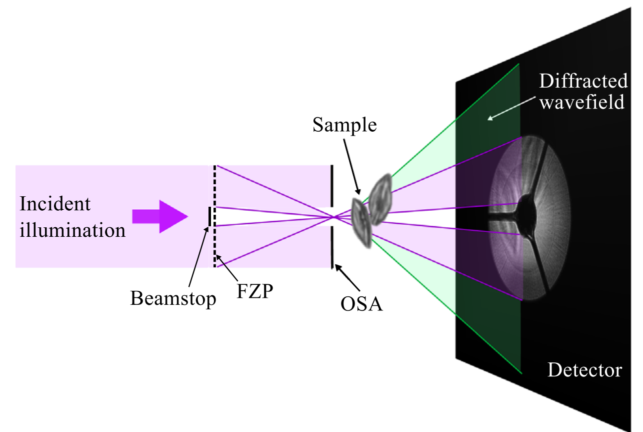

Fig. 1. Experimental arrangement. An order sorting aperture (OSA) is placed at the focal plane of 160 μm diameter Fresnel zone plate (FZP) with a 40 μm central beamstop. The sample is placed approximately 1 mm downstream of the focus and the diffracted illumination is recorded on the CCD detector placed 83 cm downstream of the focal plane. A single intensity measurement is shown on a linear scale. Figure not to scale.

washed in RPMI-HEPES and mounted on glass slides. The samples were viewed on the Leica SP2 confocal microscope with 488 nm excitation. Gametocyte samples were prepared for electron microscopy using an adaption of previously described methods [26]. Cells were fixed in 2.6% glutaraldehyde in PBS, pH 7.3, overnight and embedded in 3% agarose. The sample was post-fixed in osmium tetroxide (1% in PBS) for 1 h followed by progressive dehydration in ethanol. Samples were further dehydrated using progressive ethanol:acetone ratios as described [19] and finally immersed in acetone for 10 min before gradual replacement with epoxy resin. 70 nm sections were cut and stained with 2% w/v uranyl acetate in water for 5 min and Reynolds lead citrate for 10 min before image acquisition using a JOEL2010 Transmission Electron Microscope at 120 keV.

3. Results

P. falciparum is named for the elongated ‘falciform’ shape it adopts when preparing to undergo transfer into a mosquito vector. It has been suggested that cell elongation may facilitate the circulation of late stage gametocytes in the blood stream without being removed by the mechanical filtering mechanisms in the host's spleen [19].

The X-ray image (Fig. 2A) shows the phase of the reconstructed complex transmission function of the sample, represented as a two dimensional projection. As the sample of interest was isolated on the membrane, the original 15×15 μm^2 field of view was cropped to include only the area of interest. Analysis of the power spectrum indicates a spatial resolution of approximately 25 nm. The shapes of each of the host RBCs (yellow) are distorted by the intracellular parasite. The parasites are evident as regions of increased phase retardation with an elongated crescent shape inside the host cell (blue). Features with medium level of phase retardation are observed that likely represent membranous extensions of the parasite and independent membranous features in the host RBC (Fig. 1A, black arrows). Regions of very small phase retardation (red) are observed that likely represent regions of flattening of the host cell in what is known as the Laveran's bib [27].

The phase-diverse CDI image data can be compared with transmission light micrographs and matched fluorescence micrographs of gametocytes labelled with the lipid probe BODIPY-ceramide (Fig. 2B). The membrane probe reveals the outline of the parasite, which is also evident in the brightfield images due to

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