Cryogenic-stage experiments with *Chlamydomonas*

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Introduction

Soft x-ray microscopy has been shown to provide a novel insight into the structure of biological specimens since the images obtained are, unlike transmission electron microscopy where material is chemically fixed, dehydrated, embedded, sectioned and stained, of living hydrated cells (Kirz et al., 1995; Stead et al., 1995). For example, in the unicellular green alga *Chlamydomonas*, there appear to be numerous spherical inclusions which are not seen when transmission electron microscopy is used to study cell ultrastructure (Ford et al., 1994).

To date the main soft x-ray imaging modes have been either contact microscopy using a laser plasma source, or scanning or transmission x-ray microscopy using either a laser plasma or synchrotron source. However, living specimens suffer from radiation damage if the imaging times are too long. For example, measurements of metaphase chromosomes from beans (*Vicia faba*) have shown them to be very radiation sensitive when wet but relatively insensitive when dry (Williams et al, 1993). Viability of yeast cells and functionality of myofibrils were both destroyed by doses of soft x-rays well below the doses used for microscopy (Fujisaki, et al., 1996). Images obtained previously at the ALS have shown not only mass loss but also gross alteration in cellular structures, for example, bacterial spores are damaged by a single short exposure to soft x-rays (2.4nm) but dry spores show little or no sign radiation-induced damage (Stead et al., 1997, 1998). The novel spherical cellular inclusions seen in *Chlamydomonas* are themselves particularly radiation sensitive (Stead et al.,1997). Although images of living *Chlamydomonas* cells have previously been obtained with exposure times of 1-4s some of the initial images still appear to be damaged, that is to say the images appear similar to those which had been irradiated previously. This suggests that the observable damage occurs during the exposure and that any reduction in the exposure time should reduce the likelihood of recording radiation damaged images (Ford et al., 1998). Moreover since damage occurs within this time it is not possible to alter either the field of view or the focus and take a second image. The situation is exacerbated when images are to be recorded at wavelengths other than 2.4nm, as is the case when imaging either side of an absorption edge to determine the elemental composition, because the efficiency of the x-ray optics is diminished therefore the exposure times are greatly increased. In early experiments to determine the oxygen distribution in cells of *Chlamydomonas*, for example, exposure times were often about 30min. and therefore the specimen had to be chemically fixed and/or dried. Such treatment of the specimen negates the very considerable advantage that x-ray microscopy has over conventional electron microscopy as it prevents the imaging of living material. Any increase in the x-ray flux would clearly reduce imaging times and allow hydrated images to be studied without the fear of introducing radiation-induced artefacts. However, the improvements needed to facilitate the recording of two images, each sufficiently short to avoid radiation damage, to give information on elemental distribution in a living specimen are technically too difficult at the present time. Furthermore, during the time it takes to alter the wavelength, any damage caused to the specimen may continue to proliferate even though the specimen is not, at that time, exposed to x-rays.

Figure 1: The liquid nitrogen-filled dewar through which helium is passed. The specimen holder, onto which the silicon nitride windows are mounted, is placed at the bottom of this assembly. Thermocouples monitor the temperature of the mount in several positions as well as the incoming jet of helium.

There is considerable interest, therefore, in treatments which preserve the structural integrity of the tissue and reduce radiation sensitivity. In transmission electron microscopy cryo-fixation, in which the specimen is frozen rapidly and then sectioned and examined whilst remaining frozen, has offered the possibility that chemical fixation could be avoided. Images of cryo-prepared material usually show the cellular structures to be more regular in outline and better preserved. Micrographs, therefore, differ from those of conventionally prepared tissues but cryo-fixation is technically demanding and still only examines a very thin section of the material in question furthermore the material still requires staining to enhance contrast as electrons are scattered, not absorbed as is the case in x-ray imaging.

Several methods are currently employed in electron microscopy which use frozen biological samples (e.g. freeze-fracture; freeze-etching; freeze-substitution) and the cryo-preservation of micro-organisms is routinely used in culture collections. The experience already gained by these

techniques needs to be borne in mind when freezing samples for ultrastructural studies using soft x-ray microscopy.

Water can account for 40-90% of a biological sample though micro-organisms such as bacteria, yeast and unicellular algae usually have a much lower water content. When such samples are cooled below zero, ice crystals form which, as they expand, can damage the ultrastructure of cells. Solid water can exist in three main states, hexagonal crystals, cubic crystals and a vitreous or amorphous state. Ultrastructure is preserved best in the vitreous state though this requires rapid freezing at rates of $>2x10^{5}$°C.s^{-1} over the range 20 to -100°C i.e. within a fraction of a ms. In practice, the accepted criterion for freezing speed is that it should reduce ice crystal size to a point where there is no visible structural distortion (which will depend on the resolution of the microscopy). Established freezing methods such as plunge freezing into liquid Freon or propane cooled by liquid nitrogen can produce freezing rates of 10^{4}-10^{5}°C.s^{-1} though the fastest rates are only achieved with cell monolayers. Liquid nitrogen alone is not satisfactory since it can achieve rates of only 10^{3}°C.s^{-1} due to an insulating layer of evaporated nitrogen gas. Ultrastructural damage can be reduced by including a cryoprotectant such as glycerol or prior fixation of the sample with glutaraldehyde. However, both of these have the potential for introducing artefacts into the image. Spray or jet freezing using propane cooled with liquid nitrogen is particularly suitable for suspensions of single cells which, due to their low thermal mass, can usually be satisfactorily frozen without using a cryoprotectant.

Cell organelles vary in their sensitivity to ice crystal damage with mitochondria, endoplasmic reticulum and the Golgi apparatus showing best preservation due to their low water content or because of their intra-organelle solute concentration. The nucleus has a relatively high water content and is the most sensitive to ice crystal damage.

The freezing system used for a particular technique will depend of the type of specimen and the constraints of the instrumentation but should aim to freeze within the limits quoted above in order to produce a vitreous state (the ideal) or ice crystals which will not cause damage up to the resolution limit if the microscope. This report relates to the progress made in developing a cryo-stage for soft x-ray microscopy at the ALS and the initial results that have been obtained using this equipment.

Material & methods

Beam line improvements. The use of zone plates to focus the x-rays results in a considerable loss of fluence, since the high resolution zone plates used at XM-1 can only be fabricated with relatively low diffraction efficiency. A new condenser zone plate with an efficiency that was improved by a factor of 15 has now been introduced and thus imaging times are reduced by the same factor.

Cryo-preparation. A monolayer of *Chlamydomonas* cells was sandwiched between two silicon nitride windows, each 50nm thick. This was then frozen by jet-freezing with two jets of helium cooled by passing through liquid nitrogen, one onto each window. The flow rate of the helium could be adjusted to provide different freezing rates. In general, however, rates of cooling of 10^{3}°C per sec were routinely obtainable with a final temperature of c.-130°C. Throughout the freezing process the frame holding the silicon nitride windows was maintained at 21°C by means of several 'heaters'. Once frozen the complete assembly (Fig. 1) was transferred to the beam line. The flow of liquid nitrogen cooled helium enabled the specimen to be maintained at the desired temperatures whilst the metal frame was kept at 21°C to prevent any thermally induced expansion or contraction of the beamline which would adversely affect the specimen focus.

Results & Discussion

Recently zone plates with a much greater efficiency have been installed on XM-1 and basic imaging times for hydrated *Chlamydomonas* cells have been reduced to approx. 0.2s. This reduction in the exposure time, by more than an order of magnitude, over previous imaging times (Stead et al., 1997) is short enough to prevent radiation-induced structural damage from being evident. However, using a series of very short exposures (ie reduced x-ray dose) it is apparent that radiation-induced specimen damage continues during the intervals between successive images (Fig. 2). Thus there is little damage apparent in either of the initial images (Fig. 2a,c) or in an image taken immediately after the initial image

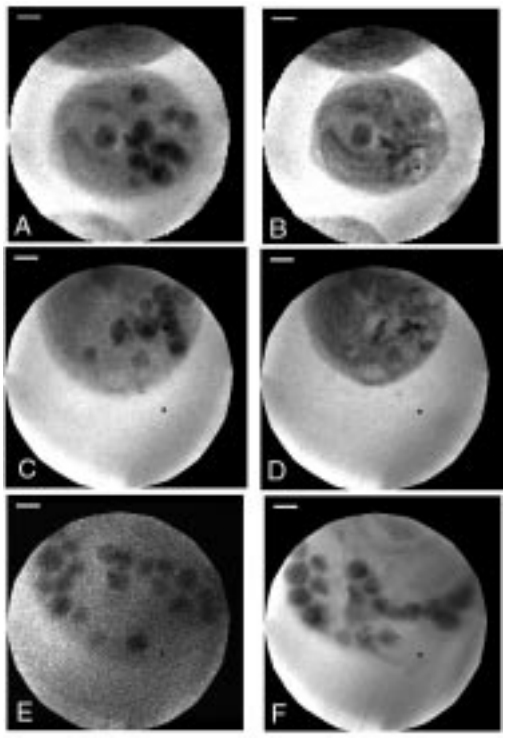

Figure 2: Images of *Chlamydomonas* showing that radiation damage accumulates within the cells between exposures. Scale bar 1μm. a) Initial image of a cell, exposure 0.2As. b) Second image taken immediately after the above image, exposure 0.2As. c) Initial image of a cell, exposure 0.1As.
d) Second image taken 1min after the above image, exposure 0.1As. e) Initial image of a cell, exposure 0.05As. f) Second image taken 1min after the above image, exposure 0.2As.

(Fig. 2b). However, the damage, especially to the x-ray dense spheres, is very obvious (Fig. 2d) if there is a short interval (eg a few seconds) between recording sequential images. Therefore, whilst the decreased exposure time avoids observable radiation damage when only one image is recorded, sequential imaging of the same cell is still impossible radiation damage artefacts. Consequently if the area of interest of a particular cell is not in the field of view, or if it is out of focus, it is not possible to move a few mm and take a second image. If the initial image is very short (Fig. 2e) then it seems that a second image can be obtained (Fig, 2f) without apparent radiation damage even if there is a lag considerable between the images. However, the signal:noise ratio is so poor that it is impossible to ascertain if the image is in focus and unless the object of interest is very distinct it is impossible to be certain that the field of view is appropriate.

The development of the cryo-stage has considerably increased the exposure time that can be used without observable radiation damage (Fig. 3). Indeed even after many exposures totalling in excess of 1.0As subsequent images appear similar, if not identical, to those taken at the very beginning of the sequence (Fig. 3a,b). Because it is possible to increase the exposure time the image quality (signal:noise ratio) can be greatly improved, thus details of the cell structure which were unclear in images of samples held in an aqueous environment become very much more obvious in images of frozen material. For example the flagella are visible and indeed the internal structure (ie microtubules) of these is evident in some images, especially if the exposure time is increased to further improve the signal:noise ratio (Fig. 3c).

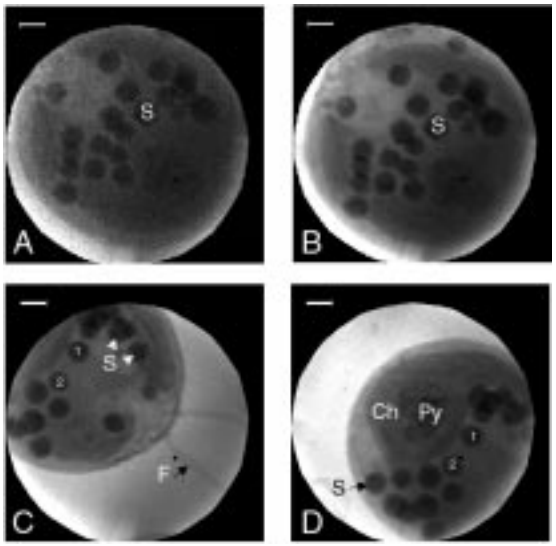

Figure 3: Soft x-ray images of frozen hydrated *Chlamydomonas* cell. a) Exposure 0.02As but had previously been exposed for a total of 0.08As. b) Image of same cell; exposure 0.5As having previously been exposed for 1.5As. c) Anterior part of cell showing flagella and x-ray dense spheres. Exposure 0.5As. d) Posterior of the same cell showing the chloroplast with pyrenoid and the x-ray dense spheres. Exposure 0.2As but by this time the cell had been irradiated for 2.1As. The spheres labelled 1 and 2 and the same on each image. The images show the x-ray absorbing spheres (S), flagella (F), chloroplast (Ch) with pyrenoid (Py). Bar = 1µm.

The chloroplast and pyrenoid within it are also identifiable (Fig. 3d), but the most striking features are the numerous x-ray absorbing spheres each up to 1µm in diameter. Unlike material held in an aqueous environment (Stead et al., 1997; Ford et al., 1998) the images of the spheres show no sign of radiation damage even after multiple exposures. Such images are very similar to those previously reported by Schneider et al (1995) in which internal cellular details were also visible but in which exposure times of at least 10s were needed because of the lower x-ray fluence that was available.

The greater stability of the specimens has allowed series of images to be taken which cover adjacent areas of the specimen, these can then be tiled together to give an image of the entire cell (Fig. 4). However, in this case the specimen was frozen slowly (a freezing rate of between 10 and 10^2 °C per sec as opposed to 10^3 °C per sec for the specimen in Fig. 3) and held at between -50 and -80°C (as opposed to -150°C) to avoid the problems of ice crystal contamination on the window. Under these conditions the cell contents appear somewhat shrunken leaving a clear area around each structure which did not absorb x-rays. More importantly however, is the ability to perform elemental mapping on hydrated, as opposed to dried cells, without any concerns about the introduction of radiation damage. Images taken either side of the oxygen absorption edge (Fig. 5) show that the spherical inclusions are rich in oxygen. The exact function of these structures, however, remains unresolved, in the literature there are only two reports of any structures which initially might be thought to resemble the spheres seen by x-ray microscopy. Wolfe et al. (1997) reported the over production and accumulation of some of the photosynthetic light harvesting proteins in a mutant strain of *Chlamydomonas.* However, all the present images, in which these x-ray absorbing spheres have been observed have been obtained using the wild type of *Chlamydomonas reinhardtti* in

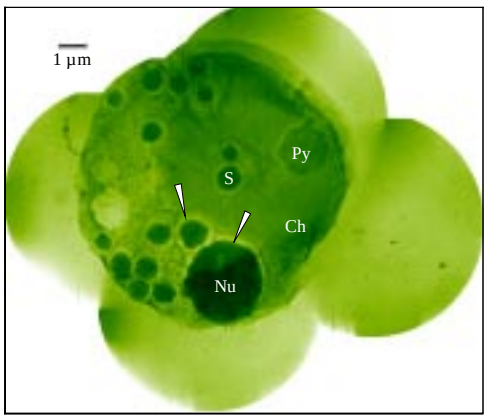

Figure 4: Composite image comprising 5 individual images tiled together. This was produced directly by the software programme and has not received any further manual adjustments. Several organelles other than the x-ray absorbing spheres (S) are visible, including the nucleus (Nu), chloroplast (Ch) and pyrenoid (Py). This specimen was cooled slowly (10^2°C per sec) and whilst radiation damage of the spheres is not obvious there is some shrinkage around each sphere and around the nucleus (arrowed) suggesting that there was shrinkage during the freezing process. Bar = 1µm.

which there is no over production of these proteins. The only other report relates to an accumulation of calcium phosphate at one particular stage of the cell cycle in a related species of *Chlamydomonas* (Siderius et al., 1996), but in the present soft x-ray microscopy study the spheres are not only somewhat larger but they persist throughout the cell cycle. Therefore, neither of these reports seem to be looking at the same structures that we have observed with soft x-ray microscopy. Further work to characterise the composition of these spheres is clearly necessary and this, coupled with biochemical studies, should give a better insight of their possible function.

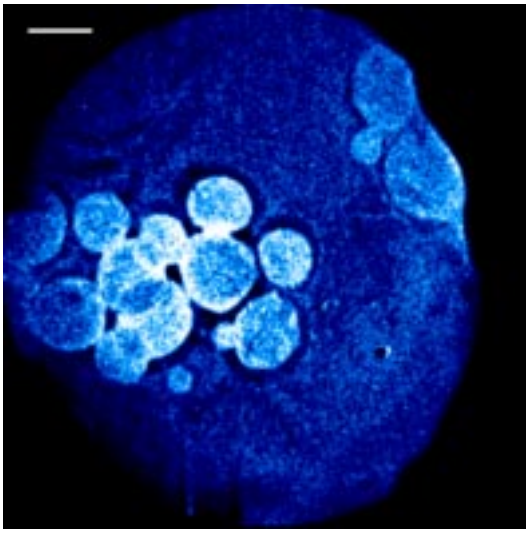

Figure 5: Oxygen distribution in a slowly frozen cell. Two images were taken below and at the oxygen K- absorption edge and processed to show the oxygen distribution. Bar = 1μm.

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