Fluorescence imaging of *Dictyostelium discoideum* with a hard X-ray nanoprobe

K. Giewekemeyer¹, #, M. Hantke¹, C. Beta²,³, R. Tucoulou⁴ and T. Salditt¹

¹Institute for X-ray Physics, Georg-August-University, Friedrich-Hund-Pl. 1, 37077 Göttingen, Germany
²Max-Planck-Institute for Dynamics and Self-Organization, Am Faßberg 11, 37077 Göttingen, Germany
³Institute of Physics and Astronomy, University of Potsdam, Karl-Liebknecht-Str. 24/25, 14476 Potsdam/Golm, Germany
⁴European Synchrotron Radiation Facility (ESRF), BP 220, 38043 Grenoble, France

E-mail: #k.giewek@phys.uni-goettingen.de

Abstract. The preparation and a novel sample environment for X-ray based imaging of freeze-dried *Dictyostelium discoideum* cells are presented. As a first application a fluorescence imaging experiment with a nanofocused hard X-ray beam has been performed. The successful preparation was verified in elemental mappings with sub-200nm resolution, which allowed for the isolation of several ionic components specific to the cell body.

1. Introduction

The ability of cells to respond to an external chemical stimulus by directed motion (chemotaxis) is an essential requirement for cellular differentiation and complex cooperative behaviour. It has been studied very successfully on the molecular level in the eukaryotic amoeba *Dictyostelium discoideum* [1]. However, an equally-detailed view of the structure and dynamics on a (sub)cellular level, i.e. in the range of 10-500 nm, has not been achieved yet.

Here we report on the preparation and a novel sample environment for X-ray-based imaging of freeze-dried *Dictyostelium discoideum* cells. The successful preparation is demonstrated in an X-ray fluorescence imaging experiment at the ESRF (Grenoble) paving the way for a general application to X-ray imaging experiments of biological cells. X-ray fluorescence imaging has proved a powerful imaging technique in the recent past due to its specificity to any given metal ion and the possibility to measure ion concentrations of various elements for the same sample without staining simultaneously at high resolution [2].

2. Instruments and Methods

Cells of the *Dictyostelium discoideum* wild-type strain AX2-214 were used in our experiments. The cells were cultivated in shaking suspension of HL5 nutrient medium (7 g/L yeast extract, 14 g/L peptone, 0.5 g/L KH2PO4, 0.5 g/L Na2HPO4). Prior to preparation, cells were transferred to a Petri dish for transport. They were allowed to attach to the bottom surface of the Petri dish for at least 1h. Before transfer to the sample holder the supernatant nutrient solution was removed and cells were resuspended in 1-2 ml phosphate buffer (2 g/L KH2PO4, 0.36 g/L Na2HPO4 · 2H2O, pH 6.0). As sample holders microfabricated polyimide foils of 10-20 μm thickness and ca. 300 x 800 μm lateral dimensions (Mitegen, USA, cf. also [3]) were used. After cleaning in an ultrasonic bath of methanol and immediately prior to use the substrates were rendered hydrophilic by a 5-min-treatment in a plasma cleaner (Harrick Plasma, USA). A drop of cell suspension was placed onto the substrate, which was then injected...
Figure 1: Cell preparation by rapid freezing in liquid ethane (a). A small metal container filled with liquid ethane is kept in a bath of liquid nitrogen. The sample holder consisting of the polyimide foil on a metal pin is mounted onto a magnetic base, which is attached to a metal rod movable in the vertical direction. Before plunging the cell suspension is transferred onto the foil with a micropipet (b), and after a resting time of 1-3 min (c) the supernatant suspension is soaked up with a thin paper rod (d). Immediately afterwards, the metal rod is released, so that the polyimide tip is injected into the liquid ethane by gravitational force. During this process, the cells attached to the foil are covered with a thin water film of less than 1 \( \mu \text{m} \) thickness at the tip (magnified inset in (a)) allowing for rapid cooling of the solution into a glassy state.

into liquid ethane to prevent crystalization [4] (cf. Fig. 1). After transfer into liquid nitrogen the sample holders were placed in a freeze-drier (Christ, Germany) and removed after 40h under vacuum (0.001 mbar) and -76 \(^\circ\text{C}\) (temperature of the condenser).

The experiment was performed at the ID22NI undulator beamline of the ESRF (Grenoble, France) [5]. Quasi-monochromatic radiation of photon energy 17.5 keV was generated in the so-called pink mode using the intrinsic monochromaticity of the undulator source. The energy bandpass of the incoming radiation was further reduced to about \( \Delta \lambda/\lambda \sim 0.02 \) by two-dimensional focusing of the beam with a Kirckpatrick-Baez (KB) mirror system and a flat, horizontally deflecting Pd-coated Si-mirror for higher-harmonics rejection. The focus of the KB-mirror system was characterized by translation of an Au stripe of a nano-fabricated test pattern (Xradia, USA) recording both the transmitted intensity by a diode and the Au-L\( _{\alpha} \) fluorescence by a silicon drift detector (Vortex-EX, SII NanoTechnology Inc., Japan). The measured focal spot size was 160 nm (FHWM) in the vertical and 146 nm in the horizontal direction, respectively. The total intensity in the focal spot was on the order of \( 10^{11} \) cps. The sample was placed directly into the focal plane of the KB system and translated by two piezo stages in two directions perpendicular to the beam. During the scanning process the fluorescence signal was detected with the silicon drift detector (see above). Before and after the experiment optical micrographs of the sample could be obtained with an online microscope at the beamline. Great care was taken on limiting the radiation dose impinging on the sample by scanning only previously non-illuminated regions on the substrate. Optical fluorescent micrographs of the illuminated regions of the sample were obtained after the experiment with a fluorescent microscope (Z1 Observer, Zeiss, Germany) at an excitation wavelength of 590 nm.

3. Results and Discussion

An X-ray fluorescence mapping of a freeze-dried *Dictyostelium* cell and the corresponding cumulative energy spectrum obtained from the total mapping area are shown in Fig. 2a and c. Energy calibration and further processing were performed with the freely available software package PyMCA [6]: As a first step the total spectrum was analyzed by fitting all contributing lines to yield a fit to the measured curve. Using the same fit parameters the spectra of each scan point were fitted individually in the ”batch fitting” mode of the software and summarized as elemental mappings of the illuminated area. Several elements specific to the cellular interior with respect to the intercellular area could be identified. For the two most prominent elements, i.e. K-K\( _{\alpha} \) and Zn-K\( _{\alpha} \), a combined elemental mapping is shown in Fig. 2a. As visible in the graph, the K and Zn content of the cell is well localized to the cellular area as visible in the optical (fluorescence) micrographs (cf. Fig. 2b). This indicates a successful preparation process without early disruption of the cell membrane.

As visible in the cumulative spectrum shown in Fig. 2 many parasitic fluorescent lines are excited by air-scattered high-energy photons interacting with the metal rod of the sample holder. The unambiguous attribution of the K and Zn fluorescence contributions to cellular material is supported by a comparison of two spectra resulting from areas with and without cellular
Figure 2: (a) Combined fluorescent yield of the K-Kα (red) and the Zn-Kα (green) lines for a freeze-dried *Dictyostelium* cell (on the right), on a logarithmic scale. (b1) Optical fluorescence micrograph of the illuminated area shown in (a). As the autofluorescence of the substrate decreases during strong x-ray exposure the illuminated area can be localized easily. Contrast has been enhanced for better visibility. (b2 and b3) Optical micrographs of the imaged region on the sample before and after the experiment taken with the online-microscope at the beamline. A significant shrinking of the cell on the right is observable, however, the sample was slightly out of focus here, making a quantification of the shrinking effect very difficult. (c) Total fluorescence spectrum of the illuminated sample areas shown in (a) and (b) as well as corresponding fit (energy resolution (FWHM) ca. 150 eV). The strongest lines specific of the cell are indicated by vertical lines.

material on the substrate (cf. Fig. 3). As further elements specific to the cellular interior Ca and P are identified.

The problem of radiation damage is regarded as the major effect ultimately limiting the resolution in X-ray imaging experiments [7]. Here the effect of radiation exposure was visible in optical micrographs by an overall shrinking of the cellular material and a reduced autofluorescence signal of the entire substrate. The total flux incident on the sample was 10\(^{12}\) photons per \(\mu m^2\) (area of the cell ca. 48 \(\mu m^2\), step size 100 nm, photons per step ca. 10\(^{10}\)). Although the observed damage appears to be rather small given these numbers, and although very high resolutions might be possible with lower dose [7], optimized experimental conditions, i.e. cryogenic cooling of the sample, would be desirable here. This can be readily achieved with the presented sample environment.

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