The Stockholm laboratory cryo x-ray microscope: towards cell-cell interaction studies

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Abstract. We describe recent improvements in the Stockholm laboratory x-ray microscope and the first experiments aiming towards studies of cell-cell interaction. The shorter exposure time due to a higher brightness laser-plasma source will become of large importance for tomography while the reproducible cryo preparation of few-cell samples is essential for the interaction studies.

1. Introduction
X-ray microscopy (XRM) in the water window ($\lambda=2.3-4.4$ nm; $E=284-540$ eV) has the capability to image whole unstained cells with unprecedented resolution and contrast [1,2]. Results with high biological relevance are presently emerging [3,4] from microscopes located at high-average spectral brightness synchrotron sources. Typically exposure times are in the seconds range. Also at the laboratory microscopes, synchrotron-quality images are now produced but with longer exposure times due to the lower source brightness [5,6]. With the latest upgrade of our compact soft x-ray source we have demonstrated stable operation with a brightness of $>1.5 \times 10^{12}$ ph/(s×mm$^2$×mrad$^2$×line) and 10-second exposure-time imaging [7], i.e. approaching the performance of microscopes at early synchrotrons.

The primary goal of our laboratory XRM is to study cryo-fixated cells in their near-native environment. In such studies the high-brightness source will be of particular importance for tomography, significantly reducing the exposure time compared to in the early proof-of-principle experiments [8], and, thus, making studies of biological relevance possible. In the present paper we describe the current state of the microscope and some early attempts towards studies of cell-cell interactions.

2. Microscope arrangement
Figure 1 shows the experimental microscope arrangement, with the source, the condenser and imaging optics, the cryo sample holder and the detector. Below we briefly describe these sub-systems, with emphasis on the source due to its recent improvement.

The laser-plasma source is based on a regenerative liquid-nitrogen jet target allowing high-repetition-rate operation. The 20 µm diameter jet is formed by driving liquid nitrogen through a fused-silica capillary nozzle with a backing pressure of 10-20 atm, resulting in a jet velocity of approximately 60 m/s. The beam from a 2 kHz, 100 mJ, 600 ps, Nd:YAG master-oscillator power-amplifier (MOPA) laser [9] is focused onto the jet to form the plasma. The nitrogen plasma emits strong peaks in the water-window corresponding to electron transitions in the highly ionized nitrogen.
The emission line utilized in our microscope is the $\lambda/\Delta\lambda>500$ hydrogen-like (NVII, 1s-2p) line at 2.478 nm [10]. The source size is typically $14\times20\ \mu m^2$ FWHM, and it radiates the flux $1.1\times10^{15}\ ph/(s\times sr\times line)$ of the 2.48 nm line uniformly into $4\pi$ sr, resulting in an average spectral brightness of $>1.5\times10^{12}\ ph/(s\times mm^2\times mrad^2\times line)$ [7].

Figure 1. The Stockholm soft x-ray microscope with the high-power liquid-jet source, a Cr/V multilayer condenser mirror, cell sample in sample holder, nickel zone plate and CCD detector.

The source is imaged by a Cr/V multilayer condenser mirror onto the sample plane with a magnification of $1.6\times$. The narrow mirror bandpass ($\lambda/\Delta\lambda\approx300$) matches the 2.48 nm emission line so that it also serves as monochromator. At the sample plane cryo-prepared samples on TEM-grids are inserted using a modified TEM-sample-holder stage with both cryo and tilt capabilities. Thus, it facilitates cryo tomography operation. The illuminated object is imaged onto the detector by a 25-50 nm outermost zone-width, 50-100 $\mu m$ diameter nickel zone-plate. The image is recorded by a cooled back-illuminated CCD detector.

3. Towards cell-biological studies
The goal of our XRM activity is to allow high-resolution studies of cells and cell-cell interactions. For such studies cryo sample preparation is necessary to avoid radiation damage on the samples, see, e.g. [5]. Thus, a robust protocol of cryo preparation for the laboratory microscope has been developed. Sample quality and reproducibility have been the key goals. The method is demonstrated on parasites and for immune system natural killer (NK) cell-target cell interaction studies.

3.1. Cryo preparation for laboratory XRM
The method used for cryo fixation is plunge-freezing, i.e., fast immersion of the sample into liquid ethane in order to obtain a cooling rate higher than $10^4\ K/s$. At this cooling rate vitreous ice is formed, thereby avoiding damage to the sample substructures. We use 3 mm gold TEM grids as sample carriers in the laboratory x-ray microscope. To avoid ice crystal formation it is essential that the water layer on the grid is thin. The removal of excess liquid is done by blotting the sample carefully from the sides. With non-adherent cells or cells that are not given proper time to adhere to the carriers this method often results in removal of a majority of the cells. However, with the use of adherent cells the number of cells remaining on the grid after blotting and cryo fixation is reasonably reproducible and reasonably high.

In the next two sections we show two examples of imaging based on the protocol. First single-cell images of two types of parasites are demonstrated. They were grown according to standard procedure and seeded onto the TEM grid before plunge freezing [5] but not given the proper time to adhere to the carrier. Still, they remained in sufficient numbers to allow imaging.
The second example is aimed at cell-cell interaction studies. Here the cancer target cell was the adherent cell line HEK 293T. The target cells were cultivated on 3 mm gold TEM grids with a thin carbon support layer. After 24 hours incubation at 37 °C in an atmosphere of 5% CO₂ the NK cell solution was added to the grid. Subsequently, after another hour of incubation, the NK-target cell sample was imaged in the x-ray microscope or in a confocal laser scanning microscope. Figure 2 shows two light microscope images taken just before the NK cells were added in order to investigate the plunge freezing. Here the HEK cells can be identified on the grid before and after cryo fixation.

![Figure 2](image)

**Figure 2.** Light microscopy image before (a) and after (b) cryofixation.

### 3.2. Cryo imaging of parasites, 2D and 3D

Figure 3 shows recent images of parasites [5]. Figure 3a shows the protozoan parasite *Spironucleus Salmonicida*. The flagella as well as subcellular structures in the carbon-rich cell body are clearly visible. Vesicular structures, not seen in light microscopy, are clearly visible, as are the flagellar canals. Figure 3b shows another protozoan parasite, *Giardia intestinalis*. Several flagella and an intact adhesive disc and nuclei are observed.

![Figure 3](image)

**Figure 3.** Parasites *Spironucleus Salmonicida* (a) and *Giardia intestinalis* (b) (from [5]).

### 3.3. Towards cell-cell interaction studies

Natural killer cells are cells of the immune system that have the ability to release toxins and kill virus-infected cells and cancer cells [11]. NK cells play an important role in host defence and immune regulation. When a NK cell encounters another cell, it forms an immune synapse in order to investigate whether the cell is a threat. If there is a downregulation of major histocompatibility
complex (MHC) class I molecules on the target cell surface this indicates that the cell is malignant and the NK cell will kill the target cell. It delivers granules carrying cytolytic effector molecules to the target cell, inducing apoptosis. This process is of great interest in order to improve our understanding of the immune system. However, the resolution of light microscopy is not high enough to resolve small structures such as the lytic granules since they typically have a diameter smaller than 100 nm. Therefore x-ray microscopy potentially could shed light on this transport process and perhaps even show the reorganization of the microtubule organization centre (MTOC) that is responsible for the intracellular transport.

Figure 4 shows the first XRM image of a NK and a HEK 293T target cell in close proximity to each other. The image shows promise for studies of the interaction area, especially in tomographic imaging.

![Figure 4. XRM image of NK cell with adjacent target cell. FOV = 25 µm.](image)

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