SOFT X-RAY MICROSCOPY BEAMLINE FOR THE CESLAB

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Keywords:
microscopy, tomography, phase contrast, imaging

Abstract

Many questions of biology are to be answered with the help of new techniques that allow to reveal the structure of samples at micrometer range and bellow in a non-destructive way. The X-ray microscopy system that we propose should lead to continuation of experiments design in the supporting laboratory, and in addition, should increase the level of co-operation between the societies that is interested in Cell Biology and many other mostly biological applications in the Czech Republic and surrounding countries. Three-dimensional imaging plays a growing role in investigations of biological structure, where it is often important to understand the relationship between components in a cell that may be distributed in depth. Ideally this is done on a specimen which is minimally modified from its living state. We propose here a beamline for soft X-ray microscopy to record fluorescence maps, but as also tomographic data sets. The instrument will be operational in different imaging mode, but particularly optimized for Full-field transmission X-ray microscopy/tomography (TXM) and Scanning X-ray microscopy/fluorescence (SXM). Specimens of this thickness are likely to have many overlapping structures, so some form of 3D imaging becomes highly desirable to resolve the complexity. Based on recent development of Fresnel zone plate optics it is feasible to achieve resolution down to 20 nm or better in all three dimensions, and it is also possible to highlight specific structures based on their intrinsic chemistry using X-ray near-absorption-edge resonances. Efforts will be made to combine tomography with the elemental and chemical state mapping capabilities of the X-ray microscope to identify features of sample in three dimensions.

Introduction

Soft X-ray microscopes can be used to study hydrated cells and produce images of 30 nm resolutions. If the cells are imaged in the X-ray transmissive “water window”, where organic material absorbs approximately an order of magnitude more strongly than water, chemical contrast enhancement agents are not required to view the distribution of cellular structures. In such experiments, cells must be rapidly frozen to be studied on a cryostage, showing information which is closely similar to 4D-living cell observation by LSCM. Conversely to confocal microscope, X-ray microscope uses electromagnetic radiation in the soft X-ray band, which mostly origins from synchrotron radiation source. In both confocal and X-ray microscopy, images of high-resolution can be obtained. The resolution of X-ray microscopy is between that of the optical microscope and the trans-electron microscope. Conversely to conventional electron microscopy, X-ray microscopy enables to view biological samples in their natural state. TEM is used to obtain images with nanometer resolution; however, 3D-reconstruction of ultra-thin sections is a time-consuming and sophisticated process unlike in the case of X-ray microscopy where the specimen thickness greatly exceeds that which can be studied in electron tomography, so that whole cells can be studied.

Beamline design

As a model, the ALBA soft X-ray microscopy beamline proposal was used in agreement with the ALBA research team. We propose to use a bending magnet beam. The energy is selected by a double-crystal monochromator in the optics hutch or eventually if higher flux and lower energy resolution is required, a second, multilayer monochromator could be installed and moved into the beam instead of the double-crystal monochromator. The monochromator is mainly for the imaging with parallel beam in the CITB, while in divergent beam geometry the bending mirrors coated with multilayer structure would serve themselves as monochromators.

The end-station is planned to house two kind of setups: one is dedicated to scanning microscopy techniques (SXM) and the section to full-field transmission microscopy (TXM).

The focusing optics consists of a two Fresnel zone plates The field of view will be in the range of 10 ěm allowing higher than thousand fold magnification. A pinhole or additional pre-focusing element will define the beam that impinges the CZP which focuses the source to a spot of typical dimensions around 2–5 m. As these are smaller in some cases than the sample size, it is necessary to have the possibility of scanning the CZP perpendicularly to the incoming beam. After the CZP a central stop has to be installed to prevent direct illumination to reach the sample in order to reduce the background. The CZP have to be mounted on a precise z stage allowing translations parallel to the direction of the incoming beam, in order to change the wavelength. The CZP and associated mechanical translations will be installed in a high vacuum system which will have windows along the optical path. The whole vacuum chamber has to have the possibility of being removed from the beam path and accurately repositioned back on the optical axis in order to be able to exchange the zone plates when the energy range required is out of the limit of the one mounted in the beam path. This could however be realized in a different way by having two zone plates installed on translation stages and only moved in the beam the one required for the given experiment.

The microscope will cover a spectral range from a photon energy of 250 eV to 1.8 keV, so that primary K and L

atomic resonances of elements such as C, N, O, Al, Ti, Fe, Co and Ni can be probed. Beside this, the higher energies up to 4 keV should also be available for phase contrast imaging.

X-ray microscopy beamline must take into account the construction of a laboratory space fulfilling the requirements for cell culture, standard biochemical techniques, cold room, light microscopy and confocal microscopy.

Experimental approaches

Transmission X-ray microscopy (TXM)

The TXM or full-field microscopy itself works comparable to the different known scanning microscopes as shown in figure: The source – in this case the storage ring radiation emitted by the bending magnet – is demagnified by the condenser zone plate (CZP) into a focal spot with a diameter below 1 m. In the focal spot plane, the object is aligned and is scanned across the focal spot. The light diffracted by the sample is afterwards focused by the imaging zone plate (IZP). The up to 1000–2000× magnified image is detected by a back side illuminated CCD coupled with an optical phosphor or scintillator system. The specimen can be fully hydrated and positioned between two thin (100 nm) low-absorption silicon nitride membranes.

If the wavelength of the monochromatic beam matches one of the K and L resonances of a selected element, the contrast is based on absorption. However exploiting the region of higher energies (2–4) keV would give valuable complementary information in the form of phase contrast imaging. Moreover as the absorption decreases with increasing energy, thicker samples may be imaged. Strong absorption of ionizing radiation implies high radiation dose; the radiation dose delivered to a hydrated biological specimen in the course of acquiring a single 50 nm resolution image with a signal-to-noise ratio of 5:1 is about 100 mrad or 106 Gray. One can lower the dose by as much as an order of magnitude by using phase contrast. In this energy range the transmission X-ray microscope (TXM) or full field imaging microscope is basically conceived to work in Zernike phase contrast but the design of the microscope is intended to offer maximum flexibility for the use of various contrast modes.

Cryomicroscopy

Cells will be cultured on 100 nm-thick silicon nitride support films, rinsed in phosphate-buffered saline (PBS), covered with another 100 nm-thick silicon nitride support film and placed on the microscope. Then the cells will be rapidly frozen by exposure to a jet of liquid nitrogen-cooled dry helium gas. This approach produces freezing rates on the order of 3000 °C s^{-1}, generating images virtually free of ice crystal artifacts at the level of resolution of the X-ray.

Immunocytochemistry

For localization of cellular proteins and chromatin associated domains, cells will be cultured in appropriate medium on 100 nm thin silicon nitride support films, rinsed in PBS (37 °C), then fixed. After rinsing in PBS, cells they will be examined with the confocal microscope. For X-ray microscopy, cells will be then post fixed in 2% glutaraldehyde in PBS, rinsed in double distilled H2O, silver enhanced (or Li, Ag-enhanced, Nanoprobes Inc.), rinsed in double distilled H_2O, then imaged in the hydrated state with the X-ray microscope. Using this technique, the structural analyses of nuclear pores, splicing factor, nucleoli and other chromatin associated domains such as e.g. PML bodies will be studied.

3D imaging

Recovery of three-dimensional information is a major step forward for the full exploitation of microscopy techniques. The first demonstration of soft X-ray tomography of hydrated specimens have been performed at Stony Brook [3] Tomography experiments with a cryo-scanning transmission X-ray microscope of a mouse 3T3 fibroblast with 100 nm transverse resolution and 250 nm longitudinal resolution was obtained. The use of cryo methods allows the full data set to be recorded without observable specimen changes from radiation damage. Since then several groups have improved this technique and still further improvement is to be expected.

X-ray fluorescence spectroscopy

The setup described in can be readily modified for 2D mapping of samples and recording of fluorescence spectra at each point with an energy dispersive detector. The modification consists in placing the sample in the focal plane and not using the objective zone plate, but placing a energy dispersive detector at an appropriate angle to collect fluorescence photons. The different signals from interaction of X-rays with matter like fluorescence, absorption or XAS can also be detected with high spatial resolution using using the CCD camera behind the sample.

The wide spectral operating range of the microscope is attractive for spectro-microscopy. Whilst in its simplest form this might consist of taking multiple images of a single sample region at different incident energies, an interesting extension is to perform highly spatially resolved XAS scans on small regions of the sample. The spatial resolution of this mode is potentially limited by the probe size, convergence and the sample thickness, but requires careful mechanical design due to the energy dependence of the zone plate focal length. Spectromicroscopic applications are difficult to be used in fluorescence mode, this microscope should offer also the full capability to use XAS in transmission.

Fluo-tomography

Removing the objective zone plate from the beam path and rotating the sample during the acquisition of fluorescence spectra would allow to combine tomography with fluorescence spectroscopic studies. Conventional x-ray transmission tomography provides the spatial distribution of the absorption coefficient inside a sample. Other tomographic techniques, based on the detection of photons coming from fluorescent emission, Compton and Rayleigh scattering, are used for obtaining information on the internal elemental composition of the sample. However, the reconstruction

problem for these techniques is generally much more difficult than that of transmission tomography, mainly due to self-absorption effects in the sample. [4] presented an approach to the reconstruction problem, which integrates the information from the three types of signals. This method provides the quantitative spatial distribution of all elements that emit detectable fluorescent lines, even when the absorption effects are strong.

Cone-beam projection imaging – zoom-tomography

Again if the objective zone plate is removed and sample placed out of the focal plane, zoom-tomography by projection can be used. The advantage being that the flux will be higher since there is one optical element involved. Shorter exposure time will bring the possibilities to image dynamical processes and reduce the radiation damage of the sample. This will increase the potential of the beamline for a wider range of studies.

Applications

Biological research

Laboratory Molecular Cytology and Cytometry from the Institute of Biophysics, Academy of Science of the Czech Republic (as a laboratory supporting the X-ray microscopy beam line) have over ten years experience in image analysis and confocal microscopy, see relevant publications [3]. By the use of X-ray microscopy we would like to study cytoskeleton components such as lamin A/C, nuclear organization of nuclei as well as histone code modifications such methylation and acetylation [4]. Cell signaling, organelle movement, cell division, and interaction of cells with the environment should be studied during the supporting group co-operation with the Faculty of Medicine, Masaryk University in Brno.

Studies on relationship between cellular structure and cellular processes are a main objective of many cell biology. Confocal microscopy enables to analyzed the cells in their physiological conditions, and moreover, reconstruction of biological objects in 3D and/or 4D-mode. The limit of confocal microscopy is the spatial resolution on the boundary up to 200 nm for fluorescence imaging (and much worse for transmitted light imaging). An alternative used represents transmission electron microscopy (TEM) that is extremely successful to study the sub-cellular nuclear organization of specific structures at high-resolution level. Nevertheless, TEM has several important limitations: The sample has to be introduced in the vacuum required by the electron beam, the thickness of the samples is limited to very thin layers, and as mentioned above, the observation of whole cells is limited to 2D-mode, in majority of studies. Using described methodological approaches, it is possible to analyzed cytoskeleton components, proteins involved in the nuclear membrane and nuclear interior, as well. The resolution limit for this type of microscopic analysis is around 3–4 nm [5]. Due to limitation of TEM in 3D-reconstruction a new tools, how to precisely study the biological objets, are developed. A very attractive possibility to avoid the limitations of either confocal or electron microscopy is X-ray microscopy, that uses the native contrast mechanism (water window) of biological material in an aqueous environment. High flux of tunable X-rays produced in synchrotron facilities in the soft energy range (< 700 eV) can be used under different setups: Full field transmission X-ray microscopy (TXM), scanning transmission X-ray microscopy (STXM) and scanning fluorescence X-ray microscopy [6] and even the simultaneous combination of transmission and fluorescence mode. Soft X-ray microscopy has unique capabilities that make it a very useful tool to study hydrated cells. Meyer-Ilse et al. [7] obtained a high contrast image of whole hydrated cells at the level of 50 nm resolution. In addition, chemical fixation is not required for X-ray microscopy of cryo-fixed specimens, which are stained by the use of immunocytochemistry. In such specimens, whole cell tomography can be performed In this case, X-ray microscopy has ability to provide unique, 3D-information about the e.g. cellular or nuclear distribution of proteins, nuclear organelles and/or nuclear organization of chromatin and chromatin associated domains.

Another attractive use of a X-ray microscopy beamline is to study nuclear reorganization of chromatin and chromatin associated domains such as nuclear speckles involving splicing factors. Genomic instability is also a hallmark of cancer. It may affect chromosome number and integrity and is suspected to provide a source of variability that may be used by cancer cells to evolve towards more malignant states. Getting cell division back under control, or simply arresting division in cancer cells is a prime objective of current basic and applied research, and one that can benefit from new high-resolution, less invasive microscopy techniques. Therefore, X-ray microscopy seems to be useful tool for applied biology in cancer diagnosis and therapy.

Related to the co-operation, we will contact the plant biology groups in our region, in order to study e.g. a slug (Pb) concentration in plant as an indicator of toxicity or poisoning. Such experimental studies seem to be also a very interesting for eco-toxicologists, plant cell physiologist and for further biotechnological applications.

When preparing for experiments with X-rays on biological samples one must consider the radiation damage on these samples. There are several methods (e.g. phase contrast imaging) to lower the dose deposited in the sample, but radiation damage would still lead to changes in the specimen during the collection of a 3D data set unless one were to consider schemes involving multiple beams from a single, fast-pulse X-ray source. This is true both of living specimens, and also of chemically fixed specimens at room temperature. One solution is to use the cryo methods developed in the electron microscopy community, where the sample is rapidly frozen to liquid nitrogen temperatures so as to obtain ice in an amorphous state and thereby avoid ice crystal artifacts.

Environmental studies

The study and understanding of man-made pollution and investigation of decontamination procedures is of increasing concern, with significant funding now being made by governments to develop effective clean-up strategies. A

major aspect in understanding pollution is the study of the transport mechanisms through soils, since a great variety of chemical reactions within soil systems occurs in an aqueous phase. Due to its ability to image specimen directly in their aqueous environment, X-ray microscopes that combine sub-micron spatial resolution and high spectral resolution in the one instrument are very suitable instruments for these studies, since they not only allow access to the physical microstructure, but also give information about the chemical processes involved.

Chemistry and material science

A future area of research in which x-ray microscopy could well be of benefit is that of carbon-based electronics, and the electronic properties of polymers, since fundamental questions about the processes which take place at the surfaces and interfaces involved in forming electronic junctions in mixtures of polymers remain to be addressed. Polymer electronics, including both sensors and displays, promise to be of long-term technological importance, with the capability to incorporate structures on a finer scale than is possible with silicon. In this context, the ability to carry out imaging at absorption edges from the carbon K edge up to the sulphur K edge will help to provide high spatial resolution and quantitative compositional mapping of multi-component polymeric materials and polymer nanocomposites.

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