Cryo X-ray microscope with flat sample geometry for correlative fluorescence and nanoscale tomographic imaging

Gerd Schneider, Peter Guttmann, Stefan Rehbein, Stephan Werner, Rolf Follath


Helmholtz-Zentrum Berlin für Materialien und Energie GmbH, Institute for Nanometre Optics and Technology, Albert-Einstein-Str. 15, 12489 Berlin, Germany

Abstract

X-ray imaging offers a new 3-D view into cells. With its ability to penetrate whole hydrated cells it is ideally suited for pairing fluorescence light microscopy and nanoscale X-ray tomography. In this paper, we describe the X-ray optical set-up and the design of the cryo full-field transmission X-ray microscope (TXM) at the electron storage ring BESSY II. Compared to previous TXM set-ups with zone plate condenser monochromator, the new X-ray optical layout employs an undulator source, a spherical grating monochromator and an elliptically shaped glass capillary mirror as condenser. This set-up improves the spectral resolution by an order of magnitude. Furthermore, the partially coherent object illumination improves the contrast transfer of the microscope compared to incoherent conditions.

With the new TXM, cells grown on flat support grids can be tilted perpendicular to the optical axis without any geometrical restrictions by the previously required pinhole for the zone plate monochromator close to the sample plane. We also developed an incorporated fluorescence light microscope which permits to record fluorescence, bright field and DIC images of cryogenic cells inside the TXM. For TXM tomography, imaging with multi-keV X-rays is a straightforward approach to increase the depth of focus. Under these conditions phase contrast imaging is necessary. For soft X-rays with shrinking depth of focus towards 10 nm spatial resolution, thin optical sections through a thick specimen might be obtained by deconvolution X-ray microscopy. As alternative 3-D X-ray imaging techniques, the confocal cryo-STXM and the dual beam cryo-FIB/STXM with photoelectron detection are proposed.

1. Introduction

Modern microscopic techniques are important tools for structural biology. Structural information about biological cells can be obtained with light microscopes operating in phase-contrast mode, fluorescence detection mode, confocal scanning mode for three-dimensional (3-D) imaging, and spatially resolving Raman spectroscopy mode. Independent of the imaging mode, the spatial resolution obtainable with these far-field microscopes is limited by the wavelength of the visible light or UV radiation used for imaging. Advanced fluorescence microscopes overcome the Abbe resolution limit, as for example STED or PALM (Betzig et al., 2006; Hell and Wichmann, 1994). However, these techniques rely on the location of fluorescent dyes to visualize indirectly the cell structures. The approach to selectively visualize labeled structures has many advantages but does not give a complete picture of the whole cell.

Recent instrumental developments are the scanning near-field optical microscope (SNOM) and the atomic force microscope (AFM). These techniques overcome the diffraction limitation of the far-field light microscopes, but deliver only information of structures near the surface of an object. Much higher resolution can be obtained by using electrons with short de Broglie wavelengths. Transmission electron microscopes (TEM’s) can resolve single atoms in radiation stable materials like crystals. Despite its excellent resolution, electron microscopy applied to biological cells has some limitations. Living biological cells are in an aqueous environment. However, cells are commonly dried for standard TEM studies. Caused by the surface tension during the drying process, the cells can structurally be altered. The cryo TEM overcomes this problem; however, the strong inelastic scattering of the electrons in the samples restricts the maximum sample thickness for TEM investigations to the sub-μm range, i.e. only thin sections of cells can be imaged with high spatial resolution in the sub-10 nm range (Baumeister, 2002; Dubochet et al., 1988).

To extend the microscopic techniques for the examination of matter and to overcome the diffraction limit of visible light microscopes, it is obvious to image objects with electromagnetic radiation of shorter wavelengths, e.g., with X-rays. For biological applications already Wolter (1952) found that organic structures possess a natural elemental contrast against water in the so-called cryo-TEM.
water window wavelength range (4.37–2.29 nm) between the K-absorption edges of carbon (284 eV) and oxygen (543 eV) (see Fig. 1). Water is an order of magnitude more transparent than organic compounds at these wavelengths; therefore, whole hydrated cells with about 10 μm thickness can be studied by soft X-ray microscopy (Kirz et al., 1995; Schmahl et al., 1993).

As cells represent complex three-dimensional nanostructures, imaging of whole cells requires a 3-D technique. Tomography is a key method for obtaining 3-D information. It is well established in electron tomography of thin sections or small particles and in X-ray tomography for medical applications at larger scale. For X-rays this method provides excellent 3-D information also from thick objects, because the refractive index is close to unity and, therefore, no refractive index-mismatch of cellular structures and water exists.

High spatial resolution in X-ray tomography can be achieved by combining nanoscale X-ray microscopy and tomography. With this approach the resolution is in a first approximation determined by the spatial resolution of the X-ray microscope. X-ray tomography on artificial samples was first demonstrated by Haddad et al. (1994) using a scanning transmission X-ray microscope (STXM) and by Lehr (1997) using a TXM. At the same time, it was experimentally demonstrated that biological cells under cryogenic conditions are structurally unaltered up to an X-ray dose of nearly \(10^{10} \) Gy (Howells et al., 2009; Schneider, 1998; Schneider et al., 1995). Allowing more than several hundred of different X-ray image projection views taken at ~30 nm (half-pitch) resolution, these experimental results opened the door for nanoscale tomography of whole cryogenic cells (Schneider et al., 2002, 2010; Weiß et al., 2000).

1.1. Description of the cryo X-ray microscope at HZB

The cryo full-field transmission X-ray microscope (TXM) is installed at the undulator U41 at the BESSY II electron storage ring. To monochromatize the undulator radiation, a focusing spherical grating monochromator (FSGM) is used. The monochromatized radiation is collected by an elliptically shaped, rotational symmetric condenser mirror which focuses the radiation on the sample. At first, we describe the performance of the monochromator beamline and afterwards the characteristics of the cryo TXM.

![Fig. 1. Attenuation length for water and protein in the soft X-ray photon energy range illustrating the “water window” where organic matter absorbs X-ray photons an order of magnitude stronger. Note that the X-ray absorption by heavy metals like gold (Au) or platinum (Pt) is significantly larger which makes nanoparticles out of these materials well suited as markers.](image)

1.2. Monochromator

The U41 X-ray microscopy beamline was designed to produce a real monochromatic image of the X-ray source as an intermediate source for the X-ray microscope. It must therefore allow for a setting of the photon energy without an accompanying change in the location of the focal position. An additional design goal was to adapt the divergence of the beam to the entrance diameter of the TXM condenser. This is about 2 mm and has to be illuminated homogeneously. The beamline consists of a spherical grating monochromator with variable deflection angle operated in outside diffraction order. Under the given spatial restrictions, this design allows for the strongest demagnification ratio.

The optical setup of the monochromator beamline is shown in Fig. 2. The toroidal mirror focuses the undulator beam horizontally onto the exit slit and produces a virtual vertical focus about 153 m downstream of the grating. The spherical grating is, therefore, operated in converging light. It disperses the undulator beam and forms a real vertical image at the exit slit. The focusing power in vertical, dispersive direction is distributed on two optical elements. The deflection angle at the grating is selected by means of a plane mirror. The mirror is mounted in an SX700-geometry (Riener and Torge, 1983) and selects the deflection angle according to the focusing condition for spherical gratings with fixed entrance and exit arm lengths. Gratings operating in outside diffraction order amplify the influence of surface errors of optical elements located in front of them onto the beamline performance. The plane mirror M2 is therefore mounted downstream of the grating. The exit slit selects the bandwidth of the monochromator.

Fig. 3 shows the measured flux through a 10 μm slit in the photon energy range 0.2–1.5 keV as well as a spectrum obtained with nitrogen filled in an ionization chamber to demonstrate the spectral resolution. With approx. \(10^{12}\) photons/s at a storage ring current of 100 mA after the exit slit and a spectral resolution below 0.1 eV, the performance of the U41-FSGM is well suited for high resolution imaging. The full width at half maximum (FWHM) values of the X-ray beam at the condenser position in 9 m distance from the exit slit are 1.6 mm in horizontal and 4 mm in vertical direction. These values are nearly constant between 0.25 and 1 keV photon energy and drop towards 1.8 keV to 1.1 mm in vertical and 2.2 mm in horizontal direction. The current capillary condenser with 1.6 mm diameter is mounted in vertical direction and overfilled in horizontal direction.

As the number of zones in the zone plate objective is always significantly lower than the spectral resolution of \(E/\Delta E \approx 5.500\), chromatic aberrations are avoided. In addition, the high spectral resolution permits near edge X-ray absorption spectroscopy (NEXAFS) studies in the soft X-ray region below 1 keV (Guttmann et al., 2012). With NEXAFS-TXM it is possible to determine the electronic binding states of molecules. This method is mainly limited by radiation damage for organic matter. An X-ray dose of more than \(10^{9}–10^{11}\) Gy already causes changes of the binding states at the molecular level and cannot be increased by applying cryogenic techniques (Beetz and Jacobsen, 2003).

1.3. Cryo full-field X-ray microscope

Driven by severe limitations of previous TXM set-ups which are restricted to small (diameter ≤ 8 μm) glass tube sample holders for tomography applications, the microscopy group at HZB developed a new full-field X-ray microscope based on an advanced X-ray condenser and a high precision cryo tomography stage (X-ray optical set-up shown in Fig. 4). The separation of monochromatization and object illumination is the key to the new optical concept. A conventional grating monochromator (FSGM) provides the high spectral resolution while a single-bounce ellipsoidal glass capillary...
employed as an achromatic condenser illuminates the object. This optical concept works ideally in combination with an undulator source providing the required collimated X-ray beam and high photon flux.

The single-bounce ellipsoidal glass capillary condenser was fabricated and evaluated by optical measurements during and after fabrication by XRADIA Inc. The resulting slope error of 80 μrad is well below the limit calculated by ray tracing. The X-ray performance of the capillary was tested with the TXM at the U41-FSGM beamline at a photon energy of 510 eV. Firstly, the capillary was adjusted to get the smallest possible focus. Fig. 5 shows an X-ray image of the focus which is in size 620×990 nm (FWHM-values) (Guttmann et al., 2009). To obtain a large homogeneously illuminated object field, the condenser is helically scanned. To analyze the performance of the new condenser, quantitative measurements were performed to measure the efficiency of the capillary. The focusing efficiency of the capillary was measured to be 80% for a photon energy of 510 eV. Note that this value exceeds the efficiency of zone plate condensers by an order of magnitude.

The new TXM also significantly improves the possibilities to study cryogenic samples. The new optical concept does not require a pinhole close to the sample plane – which is needed when a zone plate condenser is used – and gives space for the rotation of samples on flat holders (see Fig. 6). Taking advantage of the developments over the last decades in cryo electron tomography, the sample stage of the TXM is an adaption of a state-of-the-art TEM stage (CompuStage) from FEI Company. Therefore, the new microscope provides the same excellent mechanical accuracy and temperature stability known from cryo TEM, a tilt range of ±80° and lateral travel ranges of ±1 mm with a bidirectional reproducibility of <15 nm. The translation axes are mounted with respect to the rotation axis in order to make it possible to establish eucentricity for any part of the specimen, i.e. bring any point of the sample onto the spatially fixed tilt axis. Together with the high photon flux and the fully automated operation of the microscope’s control software (see Fig. 7), the acquisition time for a full tomographic tilt series is about 1 h but can be reduced to less than 30 min by optimizing all control procedures. The total exposure time to X-rays is 2–20 min depending on the thickness of the specimen.

1.4. Resolving power

The resolving power of a microscope depends on several parameters like the numerical aperture of the objective, the wavelength...
and the condenser illumination. For X-rays, no immersion oils exist, therefore, under ideal conditions the resolution of X-ray microscopes is determined by the latter parameters. An additional parameter characterizing the performance of a microscope is the image contrast. In order to separate the contrast issue from the pure resolution performance of an X-ray microscope, we use test patterns to measure the resolving power.

The resolving power of the TXM was determined in the 1st and 3rd order of diffraction of the zone plate objective by using a high quality Siemens-star pattern. The test star consists out of 85 nm thick gold structures with 20–100 nm half-pitch. Fig. 8 shows an X-ray micrograph of the Siemens-star taken at 510 eV photon energy using a gold zone plate with 25 nm outermost zone width (Rehbein et al., 2011). This measurement clearly shows that 20 nm lines and spaces can be resolved in 1st and 3rd order of diffraction.

The resolving power of an X-ray microscope can be increased by either using zone plates with smaller outermost zone width and/or by applying high orders of diffraction. Due to the high spectral resolution which avoids chromatic aberrations, the HZB TXM permits to use higher orders of diffraction of the zone plate objective. The X-ray optical set-up for imaging in the third-order is illustrated in Fig. 9 (Rehbein et al., 2009). For the experiments, a gold zone plate objective with 20 nm outermost zone width was used. Note that the numerical aperture of the zone plate applied in the 3rd order corresponds to a zone plate with an outermost zone width of 6.7 nm used in 1st order of diffraction. The higher spatial resolution of the microscope in the 3rd order was confirmed by imaging a Cr/B₄C multilayer test structure with five different periods (half-pitch of the structures are 20.6, 17.5, 14.3, 11.0 and 7.8 nm). In Fig. 10, an X-ray micrograph of the multilayer cross section imaged at 700 eV photon energy is shown (Rehbein et al., 2011). In Fig. 10 (right), intensity profiles perpendicular to the multilayer structures are plotted. To improve the signal-to-noise ratio, intensity line profiles were integrated over several pixels parallel to the multilayer structures. All profiles show clearly the modulation of the 20.6 nm wide lines down to the 11 nm wide lines, which is the highest spatial resolution reported by high-order imaging. Sub-10 nm structures were not resolved which might be prevented by vibrations of the X-ray optical components or the specimen holder itself.

1.5. Contrast transfer function

In the HZB TXM optical set-up, the aperture of the condenser and the zone plate objective are not matched. The glass capillary condenser matches the 58 nm zone structures of the zone plate objective at 510 eV photon energy. As a result the TXM operates under partially-coherent object illumination which was taken into account when calculating the theoretical contrast transfer function (CTF). The CTF was determined for the 1st order and 3rd order of diffraction of the zone plate objective. In Fig. 8, the X-ray micrographs used for the experimental CTF are shown. Larger lines and spaces in the range of 55–90 nm of the gold Siemens-star were
used to measure the object contrast. We determined 70% object contrast which corresponds to a gold bar thickness of 73 nm.

The theoretical and experimental CTF’s for imaging in the 1st and 3rd order are plotted in Fig. 11 (Rehbein et al., 2011). The plots in Fig. 11 show that the calculated and measured CTF for imaging using the 1st order of diffraction are in good agreement. The slight mismatch between the measured and calculated data for structures below 30 nm might be due to defects in these very narrow features.

For imaging in the 3rd order of diffraction, the measured CTF values are significantly lower (approx. 24%) than the theoretically expected object contrast of 70%. This result can only be explained by stray light superimposed to the sample image. We assume that the stray light is caused by scattered radiation of other diffraction orders than the 3rd order of diffraction. However, in the measured range the CTF seems to be more constant which is in agreement with theory. The calculated CTFs in Fig. 11 explain, why much finer features can be resolved in 3rd order of diffraction compared to the 1st order of diffraction as demonstrated with the multilayer lamella in the previous section: Although the measured contrast is on a low level of about 24%, 3rd order imaging is already useful for imaging structures below the cut-off frequency of 1st order.
zimaging as shown by the multilayer test structures (see Fig. 10). For biological applications, future work will concentrate on combining high image contrast with high spatial resolution. Steps towards a better alignment and performance of the optical components in the microscope as well as the fabrication of high resolution zone plates for use in first order of diffraction are ongoing. Future steps towards 3-D X-ray imaging with 10 nm require the combination of high spatial resolution and high contrast transfer. With lens-based X-ray microscopy, the realization of zone plates based on volume diffraction is the key to achieve this goal (Schneider et al., 2008).

1.6. Properties and unique features of the HZB cryo TXM

Table 1 summarizes the main parameters of the TXM at the U41 beamline at BESSY II. Compared to other soft X-ray full-field microscopes (for example XM-1 and XM-2 in Berkeley), the HZB TXM provides 10-fold better spectral resolution which permits NEXAFS studies. In addition, it allows using flat samples for high-tilt tomography provided long distance X-ray objectives (with large zone numbers) are used. TEM based sample holders were already implemented in an early version of a cryo STXM (Maser et al., 2000; Wang et al., 2000). This microscope provided similar spectral resolution, but has the disadvantage of a much slower data acquisition for tomography of biological cells. Currently, to our knowledge no soft X-ray cryo STXM is operational for tomography applications.

Compared to bending magnet TXMs with their glass tube sample holder, the handling of cryogenic samples in the HZB TXM is much more flexible. Currently, the HZB cryo TXM is unique for the investigation of adherent cryogenic cells. In the near future, commercial TXM’s designed by XRADIA Inc. will be installed at other synchrotron sources (for example at ALBA electron storage ring) which are based on the optical concept with glass capillary condenser and monochromator beamline. Similar types of microscopes are already working in the hard X-ray region, but so far no cryo tomography capability is implemented.

1.7. Correlative 3-D microscopy

Fluorescence microscopy is an established technique in biophysical investigations of cells and cell nuclei, whereas 3-D X-ray microscopy is a relatively new approach with great potential which
enables imaging of whole hydrated cells. Conventional optical fluorescence images are diffraction-limited to \( \lambda/2 = 200 \text{ nm} \), whereas current X-ray images can achieve a nearly 10-fold improvement in spatial resolution. The interaction of X-rays is element specific; therefore, X-ray nano-tomography can be used to quantify the packing density of organic material. However, different proteins or molecular structures cannot be distinguished directly in X-ray microscope images. This problem is solved by the availability of specific fluorescent probes detectable by fluorescence microscopy. Thus the two imaging modalities are complementary. Since fluorescence and X-ray microscopy permit analysis of whole cells, it is possible to investigate the same cell in both microscopes (see Fig. 12). These correlative studies are ideally suited for X-ray microscopy because of its ability to image whole cells in 3-D. This enables high throughput imaging of objects larger than a few hundred nanometer, which would otherwise be extremely time-consuming to locate and then serially reconstruct using correlative fluorescence and cryo electron microscopy of thin cell sections.

Radiation damage requires to image cells under cryogenic conditions in the X-ray microscope. On the other hand light microscopes work ideally at room temperatures allowing to increase the spatial resolution with immersion oils. However, experiments with nanoparticles showed that their position changes during the transition from the wet phase to cryogenic temperatures. Even cells can alter their shape and/or position during this short transition time. Therefore, the correlative approach has to be performed in both microscopes at cryogenic temperatures. Liquids like cryogenic ethane were discussed in the literature to serve as an immersion oil at low temperatures (Le Gros et al., 2009). However, such an approach requires an external special cryo light microscope. For high throughput correlative microscopy we decided to develop an in-vacuum light microscope which can be moved onto the optical axis of the TXM by replacing the condenser stage. With this approach, the cryogenic cells can be directly inspected in the TXM in bright field, DIC or fluorescence mode. This set-up of the incorporated fluorescence light microscope is illustrated in Fig. 13.

A 3-D sketch of the light microscope and the X-ray condenser (capillary) set-up in the vacuum chamber is shown in Fig. 14. The capillary condenser is mounted on a six axis micro positioning system (SpaceFab from MiCos GmbH) which itself is mounted on a linear stage for larger movement in the X-ray beam direction. The illumination (Zeiss HXP 120) for the fluorescence light microscope is coupled via a flexible light guide through a vacuum window on a collimator lens already inside the vacuum chamber. A mirror redirects the light to the filter cube (bright field, DIC, GFP fluorescence (Filter set 38 from Zeiss, GFP, exc 470, emm 525), rhodamine fluorescence (Filter set 43 from Zeiss, Cy 3, exc 545, emm 605)) which is movable perpendicular to the X-ray optical axis. A second mirror redirects the light to the objective. This mirror together with the objective (Zeiss LD EC Epiplan Neofluar 100×/0.75 DIC) and the DIC prism is placed on a stage which can be adjusted perpendicular.
(in y-direction and x-direction) to the optical axis by moving on a bevel. The DIC-prism can be motor driven adjusted. The objective together with the DIC prism can be moved along the optical axis independently to the mirror, which redirects the beam back through the filter cube to a lens tube. At the exit of the lens tube towards the detector, the optical path contains a glass window which seals the vacuum chamber of the TXM. Outside the vacuum a CCD camera (Leica DFC360FX, a high-sensitive, monochrome high-speed camera for fluorescence microscopy) records the light microscope images. The mechanical set-up of this light microscope was built by MiCos GmbH.

The operating modes for the light microscope and the X-ray microscope are shown in Fig. 15. To avoid collisions, switching from the light microscope working position requires the retraction of the light microscope objective along the X-ray optical axis, thereafter movement of the objective stage on the bevel to the “parking” position. Finally, to obtain the X-ray condenser in operating position, the capillary stage has to be moved along the X-ray optical axis towards the sample.

First correlative images of plunge frozen algae are shown in Fig. 16. As these algae are already self–fluorescent, they are suited test specimens. Images in different operation modes of the light microscope are shown: bright field (BF), differential interference contrast (DIC), green fluorescent (GFP) and red fluorescent (Rho). As the specimen is faced towards the reflected light microscope objective, the grid bars which appear in the transmission X-ray images are not visible in the light microscope images. They are located in a different focal plane and hidden by the holy carbon support film well visible in the BF and DIC images. Note that different structures within the samples are visible in different fluorescence channels. To adapt the light microscope images to the higher magnification X-ray images only the corresponding part of the light microscope images are shown. To visualize the whole sample, the upper and lower X-ray micrographs in Fig. 16 are tiled images to obtain the required large image field. Other correlative microscopy images using the HZB set-up are presented in this issue by Hagen et al. (2012) and Chichón et al. (2012). In summary, with correlative microscopy we expect to develop a widely applicable
technique that, for example as applied to nuclear structure, will yield significant new insights.

2. Discussion and future developments

We have presented the HZB TXM which is the first full-field X-ray microscope of its kind allowing studies of adherent cells under cryogenic conditions. As shown in the literature and in this issue X-ray microscopy can visualize the ultrastructure in thick eukaryotic cells with natural element-specific contrast in their natural hydrated environment (Chichón et al., 2012; Hagen et al., 2012; Müller et al., 2012; Schneider et al., 2010). The achievable resolution of cryo X-ray tomography is currently within a factor of 3–4 of cryo electron tomography (Carrascosa et al., 2009), but not limited by thin sections.

Its current 2-D resolution of close to 10 nm (half-pitch) is not yet transferred into X-ray tomograms of cells which are approximately 3-fold lower in spatial resolution. At water window wavelengths full-field X-ray microscopy mainly suffers from the limited depth of focus which is already about one micrometer for zone plates with an outermost zone width of 25 nm. Theoretically, we can expect to reconstruct sub-volumes within cells on the order of the depth of focus with high spatial resolution (Bertilson et al., 2011). Therefore, further developments of this technique have to overcome the simple geometrical optical approach for the tomographic reconstruction. In particular, more advanced methods taking into account the partially coherent illumination have to be developed to fully exploit the resolving power of high resolution zone plates. One of the challenging parts on this way is the contrast reversal for defocused object features which could already be seen in Talbot type experiments with grating test structures. In addition, we cannot increase the total X-ray dose required for higher resolution by another order of magnitude.

Which alternative methods exist for 3-D X-ray imaging? In this paper, we cannot give a complete overview about all alternative approaches without lenses. In principle, they can be divided into two groups: Coherent Diffraction Imaging (CDI) and X-ray holography. One of the most popular approaches is CDI (see also review Chapman and Nugent (2010)). CDI is a lens-less technique where the object is directly illuminated with a spatially coherent X-ray beam. In principle, lens-less X-ray imaging techniques could overcome the depth of focus problem. In CDI, the diffracted light is collected by a CCD detector. It does not require an inefficient X-ray objective which is an advantage. However, the CDI approach transfers the problem towards the phase problem, because the detector only records the diffracted intensities but not the required amplitudes and phases. Real space and reciprocal space are linked by Fourier transforms like in conventional X-ray microscopy where the objective is performing this transformation. However, in CDI the phase is lost and needs to be recovered with the computer. As a consequence, real space constraints to restrict the imaged object to a confined region have to be introduced. In other words, the object features have to be within a well defined rather small isolated area, the so-called “support” region. In practice, it is difficult to prepare flat grown adherent cryogenic cells to fulfill this criterion. To our knowledge up until now no high resolution CDI reconstructions from cryogenic cells were presented. In the literature, the CDI work concentrated so far on test pattern or dried samples (Nelson et al., 2010). Similar to TXM tomography for 3-D imaging with CDI the object has to be tilted.

Another potentially lens-less approach is X-ray holography. This method requires coherent radiation and a reference wave to form the interference pattern with the diffracted wave field. Ideally holography requires well defined wave fields. However, clean laser modes like the TEM$_{00}$ mode are not existing in the X-ray wavelength region. A lens-less Fourier transform holography set-up was realized by fabricating a tiny pinhole close to the object which serves as spatial filter and source for the reference wave (Eisebitt et al., 2004). In this case the pinhole determines the obtainable spatial resolution. The advantage of this compact set-up is also its main problem, because only a tiny fraction of the X-ray beam is used. To increase the usable photon flux, holography set-ups employing zone plates or mirrors were realized (Gorniak et al.,

![Image](image1.png)

Fig.14. 3-D sketch of the incorporated light microscope and the X-ray condenser set-up in the vacuum chamber of the cryo TXM.

![Image](image2.png)

Fig.15. Working positions for the light microscope (left) and the X-ray microscope (right).
Without going into detail, here the main limitation comes from imperfections of the X-ray optics and the extreme difficulty to develop suitable spatial filters to clean up the distorted reference wave field.

Scanning transmission X-ray microscopes (STXM’s) could reduce the X-ray dose, because the X-ray objective is upstream of the object. A soft X-ray cryo STXM with cryo TEM holder was developed by Maser et al. (2000), Wang et al. (2000). The advantage of a lower dose comes with the problem of a much longer exposure time, because the image is formed sequentially pixel by pixel which takes for large pixel numbers (comparable to TXM images) approximately two orders of magnitude longer than with the TXM. Tomography in the conventional STXM also requires to tilt the object; therefore, the limited depth of focus causes the same reconstruction problems as in the TXM.

An approach to overcome the resolution limit of the objective in a STXM is ptychography which makes use of an X-ray lens to illuminate a small well-defined sub-region of the sample with coherent radiation (Rodenburg et al., 2007). In order to be able to surpass the resolving power of the lens, the diffraction pattern has to be recorded by a CCD camera. The specimen is scanned with overlapping sub-regions and their exact relative position is measured with nanometer accuracy. Instead of a single measurement as in CDI, the diffraction-pattern phase problem is solved by the higher degree of redundancy with the overlapping information in the recorded interference pattern. By comparison, lens-based microscopes image a point in the object plane into a point in the image plane. In other words X-ray imaging of a two-dimensional object with a zone plate can be described by a 2-fold Fourier transform of the amplitude transmission of the object plane to the image plane including a low-pass spatial frequency filter. In ptychography, the coherent radiation emerging from the lens carrying wave field distortions originating from the lens itself interferes with the diffracted light of the object in the detector plane. As ptychography requires the back transformation from reciprocal into real space, the distortions – caused mainly by lens imperfections – in the coherent beam illuminating the object might cause a problem for the detection of weakly scattering object structures. As applied to biological samples, this method is still under development. Recent 3-D ptychography bone studies have not surpassed conventional TXM or STXM results in terms of the achievable spatial resolution and in addition they require much longer exposure times (Dierolf et al., 2010).

Which possibilities exist for improving lens-based 3-D X-ray imaging? The most obvious approach is imaging at higher photon energies. As the depth of focus scales linearly with the photon energy this approach is directly improving the situation for tomography. In addition, unique opportunities are opening up as elements like phosphorus or sulfur distributions in cells can be visualized. Phase contrast microscopy will be the key technique for imaging at the same dose level as required for soft X-rays (Schmahl et al., 1994). In addition, X-ray induced X-ray fluorescence (XRF) measurements in a STXM are feasible which is a powerful method to study the distribution of trace elements in biomedical and environmental specimens (Kirz et al., 1995; Sakdinawat and Attwood, 2010). All these imaging techniques in the multi-keV or hard X-ray photon energy range require advanced high efficient X-ray optics.

As shown in this paper, one of the major task for the upcoming years will be the development of advanced X-ray optical set-ups suppressing background X-ray light and to overcome the depth of focus limitation in tomography. As X-ray optics providing a spatial resolution of 10 nm already provide a small depth of focus of ≤100 nm in the water window, 3-D X-ray optical sectioning by deconvolution might be another solution. Ideally, this has to be paired with phase contrast to further increase the contrast of weakly absorbing cell features. Currently, no straightforward algorithm exists to handle optical stacks taken under these partially coherent illumination conditions.

An X-ray optical design which could give 3-D cell information without any further image processing would be the confocal STXM.
The confocal STXM requires two objectives, one illuminating locally the sample and another objective downstream the sample collecting the transmitted X-rays. As in confocal light microscopy, a small pinhole between this objective and the detector has the function to remove defocused object planes. Similar to optical sectioning in the TXM, phase contrast is essential in the confocal STXM to increase the object contrast. The realization of the confocal STXM relies also on the development of high quality X-ray optics and requires X-ray sources providing a high degree of spatial coherence.

To obtain images with a given signal-to-noise ratio, all the discussed lens-based or lens-less transmission X-ray imaging approaches require photon densities which scale inversely with the 4th power of the desired spatial resolution (Kirz et al., 1995; Schneider, 1998). Therefore, they require all within an order of magnitude the same X-ray dose which also scales inversely with the 4th power of the resolution (Kirz et al., 1995; Schneider, 1998). For the detection of secondary signals, e.g., X-ray fluorescence photons or photoelectrons, the scaling law is less drastic. It scales inversely with the 3rd power of resolution and has potentially the advantage of providing cell images at orders of magnitude lower X-ray doses. From this point of view, which would be the ideal low-dose 3-D X-ray microscope? The very successful dual-beam focused ion beam (FIB)/SEM slice-and-view technique with SEM based image formation suffers from the weak interaction of electrons with matter. X-ray imaging methods are relatively new and much less developed compared to traditional chemical bonds. At the moment, many different X-ray imaging techniques both with and without lenses are under development.

In summary, in the nano-ages new tools for the analysis of complex biological structures are essential. So far the nano-world has been mainly inspected by electron microscopy using a variety of different methods to utilize the image contrast formed by the interaction of electrons with matter. X-ray imaging methods are relatively new and much developed compared to traditional microscopy techniques. However, they provide at least the same variety of interactions with matter to detect specific elements or chemical bonds. At the moment, many different X-ray imaging techniques both with and without lenses are under development. Currently, the most successful technique for 3-D X-ray imaging of adherent cells is the cryo TXM. So far basically all X-ray cell studies are based on high quality X-ray optics and advanced X-ray optical set-ups. In particular, correlative fluorescence and X-ray microscopy open up new possibilities for structural biology. In addition, the pre-inspection of the cryogenic samples with an external cryo light microscope would severely increase the efficiency of the TXM beamtime. The main future goal is the development of novel methods for 3-D X-ray imaging towards 10 nm spatial resolution to make use of the unique interactions of X-rays with cells.

Acknowledgments

The authors gratefully acknowledge the valuable support by S. Hein for the TXM software development and B. Niemann during the TXM design phase. This work was performed in part by the Human Frontier Science Program Research Grant Ref. RGP0053/2005-C, the German Federal Ministry of Education and Research under contract number 05K4BY1/7, the 7th framework program (Grant agreement 226716, WP23 NANOFOX) and BioStruct-X (grant agreement number 283570).

References