I. INTRODUCTION

X-ray microscopy in the water-window region (λ = 2.3–4.4 nm) utilizes the natural contrast between water and carbon-based substances, e.g., proteins.1,2 This, in combination with the possibility to study hydrated samples several micrometers thick makes it an attractive technique for high-spatial-resolution imaging (typically 30 nm) of biological samples. A common denominator of current operational x-ray microscopes is that they rely on a high-brilliance synchrotron radiation source. In the present article we describe the design of a compact x-ray microscope with sufficient stability, ease-of-use, and reliability to become a routine tool outside the synchrotron community.

Although x-ray microscopy was suggested not long after Röntgen discovered the x rays it was the more recent development of efficient x-ray optics1–3 and high-brightness synchrotron radiation sources4 that laid the practical foundation for x-ray microscopy. Several different x-ray microscopy techniques, e.g., full-field transmission, scanning, and dark field, have been demonstrated.4,5 During the last decade a few microscopes worldwide have reached the stability, user-friendliness, and reliability to allow application-motivated research. This has resulted in a rapidly increasing number of x-ray microscopy applications in biological, environmental, and soil sciences, all motivated by the high-resolution imaging capability of unstained thick hydrated objects, as well as in materials and surface science.4 Many of these applications require careful sample preparation and parallel investigations by several different instruments now available in the application scientists’ home laboratory. The impact of x-ray microscopy would be even larger should such microscopes be easily accessible also inside the home laboratory. However, to date only a few attempts have been made to develop compact (nonsynchrotron-based) water-window transmission x-ray microscopes.6–8 Suitable for this. Only one concept (Ref. 8) has demonstrated suboptical spatial resolution with reasonable exposure times. This instrument is based on a liquid-jet laser-plasma9 source in combination with multilayer and diffractive x-ray optics.

In the present article we discuss the design of an application-oriented microscope based on the principles of Ref. 8. The major improvements are stability, ease-of-use, and reliability. The improved mechanical stability allows long exposure times without thermal drift making this the first compact microscope with potential for synchrotron-like image quality, despite the lower flux of the laser-plasma source. In addition, a higher resolution than previously shown is demonstrated. Equally important for application-oriented work are a user-friendly and reliable design allowing laypersoners to operate the microscope and that many samples may be imaged consecutively. For this purpose the microscope is computer controlled and a fast loading system has been developed, resulting in an approximate time of 5 min to load a sample and record an image. Below we describe the improved microscope and discuss its imaging properties.

II. MICROSCOPE DESIGN

The basic arrangement of the microscope is illustrated in Fig. 1. The light source is a regenerative droplet-target laser-plasma x-ray source, which has been thoroughly described in previous papers.9,10 Operating with ethanol as the target liquid results in strong line emission from highly ionized carbon and oxygen atoms in the water window. A spherical multilayer mirror designed for normal-incidence reflectivity at λ = 3.37 nm serves as condenser optics.11 The multilayer period of this mirror is designed to match the strong emission line (1 s-2p in C VI) from carbon at λ = 3.37 nm. Using a 100 Hz Nd:YAG laser (Coherent Infinity) with 100 mJ/pulse in 3 ns and operating at λ = 532 nm to excite the plasma results in ~1011 photons/μsr pulse) from this line. With a magnification of 1.8× and a source size of ~25 μm [full width at half maximum (FWHM)] this results in a reasonably uniform illumination over the entire 20 μm diameter.
field of view. A nickel phase zone plate, with 30 nm outermost zones and an absolute efficiency of 7.3% at $\lambda = 3.37$ nm, is located $\sim 500 \ \mu m$ above the object plane. This zone plate magnifies the image 1000$^\times$ onto a thinned, back-illuminated charge coupled device (CCD) camera (Photometrics series 300 with a Site 003B chip). With a pixel size of 24 $\mu m$, each pixel corresponds to 24 nm and the resolution is therefore limited to $\sim 50$ nm at this magnification. The ultimate resolution of the zone plate is $\sim 30$ nm and can be achieved by increasing the magnification. However, this will increase the exposure time and reduce the field of view.

Below we discuss the design of a microscope based on the above arrangement for spatial stability, ease of use, and reliability. In addition, the alignment procedure for optimal microscope performance is described. First it should be noted that the microscope is vertically mounted, i.e., the optical axis is vertical as in an ordinary optical microscope (cf. Fig. 1). This is different from synchrotron-based microscopes, which are limited to a horizontal axis due to the nature of the light source. Besides the benefit of following the concept of established techniques such as optical and electron microscopy, the vertical arrangement also relaxes design requirements as regards gravity effects on the sample. The microscope is constructed on a 5 cm thick vertical aluminum bar on which three different vacuum modules, separated by vacuum valves, are mounted: one for the source and condenser, one where the sample and zone plate are located, and finally one for the CCD detector. Each module is equipped with a turbodrag vacuum pump, a 500 l/s (Pfeiffer TMH521) for the source and two 40 l/s (Pfeiffer THM071) for the sample and the detector, respectively. This allows fast venting of each module, which is especially important for the sample module (see below). The detector module is connected via flexible bellows to the sample module allowing rapid change of magnification by raising or lowering the CCD. The source/condenser module is equipped with a large window and an x-ray sensitive diode to allow continuous monitoring of the source performance. A central stop is mounted 2 cm above the plasma source. The purpose of this is to prevent direct plasma light from reaching the sample and to block a central part of the zero-order radiation from the zone plate, thereby creating an image field. The condenser mirror is positioned on a five-axis motorized stage (New Focus, Picomotor 8321) approximately 260 mm below the source. A shutter (Prontor, 50 mm), positioned between the condenser and the plasma, controls the exposure time and protects the mirror while not exposing.

In the present version of the microscope the sample is positioned in a combined sample/zone-plate holder in vacuum. Compared to methods used at synchrotron microscopes, where the sample is in air between thin vacuum windows, this gives a $\sim 200\%$ gain in photon flux which is important when not using high-power synchrotron sources. With the modular design the typical time to exchange a sample is only 5 min. The modular design is also very flexible and allows for future insertion of, e.g., a cryosample holder. Unfortunately the design of a future environmental chamber for the sample is more complicated with this approach. However, Kaznacheyev et al. have demonstrated such chambers for use inside a vacuum.

The sample/zone-plate holder is the key component of the microscope. During exposure, the relative positions of the zone plate and the sample must be fixed within the resolution, i.e., 30 nm. This is achieved with the design depicted in Fig. 2. The holder is positioned in the microscope with a three ball-bearing arrangement as illustrated in Fig. 2(b). With this design it is possible to remove and reposition the holder with a few micrometer accuracy. The holder can also be inserted in an ordinary inverted microscope (Olympus IMT-2) for alignment purposes. Here the sample (or a pinhole, see below) can be correctly aligned to the zone plate in the x, y, and z directions by computer-controlled picomotors (New Focus). The zone plate is inserted in the holder from above with a similar three-ball-bearing arrangement and the sample holder from below, see Fig. 2. A few cm below the object plane a thin metal filter is positioned (typically 300 nm of titanium). This filter prevents scattered laser light from
reaching the CCD camera. It can be removed with a lever from outside for alignment purposes.

Below the alignment procedure of the microscope is described. The laser-plasma light source in our microscope differs from the beam-like synchrotron light source. With our 4-\(\pi\)-steradian-emitting source, spatially defined only by the overlapping volume between the target material and the laser-beam focus, we have put extra effort into the design of a straightforward alignment procedure. One factor adding to the simple alignment is the spherical-normal-incidence condenser mirror. This type of condenser was partly chosen for its good optical properties. Numerical simulations have indicated an acceptable deviation of \(\pm 1\) mm from the optical axis before any significant aberrations occur.\(^{11}\) In order to establish an optical axis, a small (50 \(\mu\)m) pinhole is positioned in the sample holder and aligned to the zone plate in the inverted microscope. After removing the zone plate, the holder is inserted in the x-ray microscope. By moving down the CCD to a small magnification (200\(\times\)) it is possible to record an image of the entire condenser mirror. If the direct-light blocker (normally positioned 2 cm above the plasma to remove direct light from the plasma to reach the CCD) is removed, reflected light from the mirror passing through the pinhole and direct light from the plasma could be recorded simultaneously. In order to be aligned on the optical axis the direct light from the plasma should be positioned in the center of the condenser image. This alignment procedure is somewhat tedious but, fortunately, not necessary to perform unless the condenser mirror has been removed.

The focus of the condenser in the \(z\) direction, i.e., the direction of the optical axis, is also aligned with the 50 \(\mu\)m pinhole. With the laser running at low power and the 300 nm Ti filter removed, the 532 nm laser light scattered from the target material and reflected in the condenser can be used for this alignment. This is done in two ways. The pinhole and the size of the focused spot are viewed in a stereo microscope (Nikon SMZ 2T) and the image of the mirror passing through the pinhole is studied on a white screen 10 cm above the pinhole. With these two methods the focus can be found within \(\pm 500\) \(\mu\)m. This alignment procedure is also only necessary when the mirror has been removed.

Assuming that the above-discussed alignments have been performed, recording of an image follows this scheme: First the microscope is evacuated with a pinhole inserted in the object holder and no zone plate. The condenser is fine-tuned so the laser light reflected from it passes through the pinhole. When doing this, the thin metal filter is removed. This alignment procedure can often be omitted if the mirror has been removed. After venting the sample compartment, the holder is removed and positioned in the inverted microscope. The zone plate and a sample holder with several samples are inserted. One sample is selected and aligned to the zone plate. The working distance (between the sample and the zone plate) is correctly adjusted using a length gauge (Heidenhain, MT 12B) mounted on the inverted microscope. After reinsertion of the holder, the thin metal filter, and evacuation of the chamber the microscope is ready for imaging.

A fully automated system is desirable for ease-of-use, so the microscope user can focus on the application, not on running the instrument. For this purpose a LabView-equipped computer controls most of the operation of the microscope. We are continuously incorporating more and more functions in a single program, which will finally control all functions of the microscope. This would result in a fully automated operation with only a few buttons for the different procedures, e.g., start, record image, vent sample chamber. Such a system is important not only from the point of operability but also from an instrument protection aspect. Performing procedures in the wrong order, when dealing with fragile x-ray-optical components and different vacuum compartments, may result in a disaster.

\section*{III. Experiments}

With the design and alignment procedures described above we have accomplished the building of a mechanically rigid compact x-ray microscope with spatially stable illumination. We have performed tests where the illumination is spatially stable within the image field (20 \(\mu\)m) for several days of operation. Such long-term stability is of outmost importance to allow application-oriented experiments. In this section the overall performance of the microscope is demonstrated on different samples. All images are recorded with 1000\(\times\) magnification.

A good test sample is the hyperbolic grating. Such a grating is designed to have a uniform distribution of spatial frequencies, which makes it perfect for evaluation of the imaging properties of our system. Moreover, it can be treated one-dimensionally, i.e., integration of several pixel rows can be performed thereby increasing signal-to-noise ratio. Such a grating has previously been used in x-ray microscopy for resolution evaluation.\(^{13}\) We have manufactured such gratings in nickel with a nanolithography technique previously used to manufacture x-ray optics.\(^{14}\) A radiation hardened trilayer resist system with a plating base is prepared on a thin silicon membrane substrate. Subsequent steps of electron beam lithography, reactive ion etching, and electroplating then structures the final grating on the substrate. The size of the grating is 10\(\times\)10 \(\mu\)m with lines ranging from 0.5 \(\mu\)m down to 30 nm. Figure 3 shows a 5 min exposure. Unfortunately, the silicon substrate on which the grating is made seems to be much thicker than the expected 170 nm. This results in a low transmission of x rays and, thus, a bad signal-to-noise ratio.
Clearly, with such large absorption unreasonably long exposure times are required with the compact x-ray microscope.

Figure 4 shows a 5 min exposure image of a 100 nm period (50% duty cycle) test grating made of gold on a silicon substrate. The image shows excellent modulation and gives an indication of our resolution. However, reliable quantitative determination of resolution requires knowledge of the height of the structures, a parameter that has not yet been measured. It is nevertheless clear from these first tests that the new microscope has an improved resolution and improved signal-to-noise ratio compared to the previous version of the microscope.

We have also imaged 80 nm colloidal gold particles distributed on a 50 nm thick silicon nitride window. Colloidal gold conjugated antibodies have important applications as markers for specific proteins, as demonstrated in electron microscopy and now also in x-ray microscopy. Figure 5 shows a 5 min long x-ray microscope image (a) with a comparative scanning electron microscope image (b). The pixel size in the x-ray image is 24 nm and the average photon number in each bright pixel is approximately 1000. This photon number and corresponding signal-to-noise ratio is similar to that of synchrotron-based x-ray microscopes. The contrast is still somewhat low, probably due to a scattered x-ray background. With background reduction and image processing techniques, we expect colloidal-gold-based protein-specific biological imaging will be possible with the compact x-ray microscope.

IV. DISCUSSION

In this article we have discussed the design and operation of an application-oriented, spatially stable, compact x-ray microscope. This design has improved resolution and signal-to-noise ratio, thereby approaching synchrotron-like image quality albeit at 5 min exposure time (the typical exposure times for synchrotron based instruments is ~10 s). Although the microscope is operational at the present performance level, improvements are always welcome. Two of the most important improvements, shorter exposure time and lower operating wavelength, are discussed below.

Improved condenser mirror performance is projected to have the largest impact on the exposure time. The present mirror consists of 200 bilayers of W/B$_{5}$C. The average reflectivity at $\lambda = 3.37$ nm is ~0.5% on the 58 mm diameter mirror, but in some areas the reflectivity is as high as 3%. Measurements performed with our compact reflectometer have revealed that this is due to slight nonuniformity of the mirror $d$ spacing resulting in a small spectral mismatch with our line-emitting fixed-wavelength source. Thus improved fabrication techniques for uniform $d$ spacing would result in increased photon flux at the sample. Furthermore, recent advances in Cr/Sc multilayer fabrication have demonstrated 5.5% reflectivity at our operating wavelength. With a uniformly deposited Cr/Sc mirror we expect to gain a factor of 10 in average reflectivity. In addition, the focusing properties of the present condenser mirror fall below theoretical expectations. Only ~15% of the reflected light is focused to the expected spot of ~30 $\mu$m. The reason for this is not fully investigated yet but we believe it is due to a midspatial frequency waviness in the substrate or the multilayers (flare).

Improved deposition technology is expected to reduce the flare a factor of 2 to 3.

The most straightforward way to shorten exposure times is to increase the average laser power. However, this is not easily realized. The laser used today (Coherent, Infinity: 100 Hz and 10 W $\lambda = 532$ nm) is one of the most powerful, commercially available Nd:YAG lasers with a pulse width <10 ns. New and more powerful laser designs are being developed in the growing field of extreme-ultraviolet (EUV) lithography. Prototypes available today have demonstrated >500 W average laser power at $\lambda = 1064$ nm and 2000 Hz. One such module would be likely to reduce our exposure times a factor of >10. Furthermore, recent experiments matching target geometry with laser pulse width indicate an improvement in x-ray flux by a factor of 2 to 3. Thus, in summary, improvements in the condenser mirror and the source indicate that compact x-ray microscopy with exposure times of seconds should be possible in the future.

Future improvements of the sample handling include incorporating an environmental chamber to allow imaging of hydrated samples. Two 50 nm thick silicon nitride foils and ~5 $\mu$m of water will lower the transmission and increase the exposure time approximately ten times. With the present performance of the microscope this leads to impractically long exposure times. One solution to this problem would be moving from the current operating wavelength $\lambda = 3.37$ nm to $\lambda = 2.48$ nm, where the transmission through water is much higher. This also allows imaging of thicker objects. We have previously demonstrated a debris-free x-ray source for this wavelength, and our ongoing collaboration with the Department of Physics, Linköping University, Sweden is aiming at the manufacture of condenser mirrors for $\lambda = 2.48$ nm. Another solution would be the incorporation of a cryosample holder in the x-ray microscope, following the well-known cryotechnique for electron microscopy. With this method it should be possible to image hydrated, shock-

FIG. 4. A 5 min x-ray exposure of a grating with 50 nm structures.

FIG. 5. 80 nm gold spheres on silicon nitride imaged by compact x-ray microscopy (a) and electron microscopy (b).
frozen cells without the need of any foils. Several different approaches to cryo-x-ray microscopy on synchrotron-based instruments have been demonstrated.22,23

Given the attractive features in x-ray microscopy, such as high resolution, intrinsic contrast, and the possibility to image thick, hydrated samples, and some of the improvements discussed above, a compact full-field transmission x-ray microscope has the potential to become one routine tool among other tools in the application-oriented laboratory in the future.

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