Digital In-line Holography with femtosecond VUV radiation provided by the free-electron laser FLASH

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Abstract: Femtosecond vacuum ultraviolet (VUV) radiation provided by the free-electron laser FLASH was used for digital in-line holographic microscopy and applied to image particles, diatoms and critical point dried fibroblast cells. To realize the classical in-line Gabor geometry, a 1 µm pinhole was used as spatial filter to generate a divergent light cone with excellent pointing stability. At a fundamental wavelength of 8 nm test objects such as particles and diatoms were imaged at a spatial resolution of 620 nm. In order to demonstrate the applicability to biologically relevant systems, critical point dried rat embryonic fibroblast cells were for the first time imaged with free-electron laser radiation.

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Due to their specific interaction with matter x-rays are a versatile probe to study biological objects. This unique property has been utilized in soft x-ray microscopy which is a useful tool to study non periodic biological objects with high spatial resolution [1-3]. In contrast to fluorescence microscopy, no staining is required as intrinsic material contrast is high due to the strong interaction of x-rays with matter. The strong interaction is on the other hand a curse as radiation damage might occur and change structures during the measurement [1]. Especially when the dose needed to achieve a desired resolution (Rose criterion) is of the naturally limited [4]. Theory predicts that one way to overcome this limitation is single pulse imaging with intense x-ray pulses of some femtoseconds duration [5, 6]. These ultrashort vacuum ultraviolet (VUV) or x-ray pulses are provided by free-electron lasers (FEL) such as FLASH (Free-electron LASer in Hamburg) [7, 8] which creates photons using the SASE (self amplified spontaneous emission) principle [9]. The first realization of coherent diffraction imaging using a single femtosecond photon pulse was shown in 2006 by Chapman et al. at FLASH with a spatial resolution of 62 nm [10]. The recorded diffraction pattern contained information about the test object as the short pulses were able to scatter (or diffract) from the object before it exploded. For the future such experiments can be anticipated at even shorter wavelength with the currently constructed x-ray free-electron lasers in the USA, Japan, and Germany [11-13]. For these new FEL sources, microscopy techniques are desired which exploit the high peak brilliance and coherence provided in order to study biological samples. Coherent diffraction imaging is such a technique and capable to image biological specimen with high spatial resolution [14]. Digital in-line holography is another, fully coherent, lensless microscopy technique which in contrast to diffraction microscopy contains an intrinsic
reference wave and images can be reconstructed without any phase retrieval [15]. The basic concept of an in-line holographic microscope goes back to the initial idea of D. Gabor [16] to use a divergent light cone as coherent projection microscope. While the original concept was invented to enhance the resolution of electron microscopes, also VUV and soft x-ray radiation can be used for in-line holographic microscopy [17-21]. New approaches even combine holography with coherent diffraction imaging to overcome resolution limitations set by the pinhole radius [22]. One ultimate vision is to utilize the intrinsic three dimensionality of in-line holography, which is applied e.g. for biological tracking applications with visible laser light [23, 24]. Holographic approaches with a spatially separated reference wave have also been used with x-rays in the Fourier Transform [25, 26] and Off-axis [27] geometry in order to avoid the twin image. Besides Holography, promising non iterative reconstruction methods receive increasing interest, which have great potential for high resolution x-ray imaging [28, 29]. Recent results on the time-delayed interference of femtosecond radiation from FLASH in the "dusty mirror geometry" already suggest the applicability of digital in-line holography with the short pulses of free-electron lasers [30]. So far, femtosecond VUV and soft x-ray pulses produced by high-harmonic generation have been used for digital in-line holography with a best spatial resolution of 800 nm [31-33]. The goal of this study is to demonstrate the feasibility of in-line holographic microscopy with femtosecond FEL radiation and to present the first results obtained for particle samples, diatoms and dried fibroblast cells. The obtained reconstructions have the highest resolution ever achieved with digital in-line holography using VUV femtosecond pulses and are the first in-line holographic microscopy results ever measured at a VUV free electron laser.

2. Experiment

![Fig. 1. Schematic drawing of the experimental setup used at FLASH for holographic microscopy.](image)

The experiments were carried out at the PG0 beamline at FLASH, the free-electron laser at DESY (Hamburg, Germany) [34]. Figure 1 shows a sketch of the experimental setup. Femtosecond x-ray pulses produced by FLASH enter the plane grating monochromator in specular reflection providing the zeroth order at the fundamental photon wavelength of 8 nm. For our experiments, FLASH provided pulse trains consisting of 21 bunches with a repetition rate of 5 Hz and a mean energy per pulse of 10 µJ, which corresponds to $4 \times 10^{11}$ photons per pulse at the source. As the zeroth order is used, the intrinsic bandwidth of the photon pulses is approximately 0.7% [34]. Higher orders contribute less than 0.5% to the photon intensity [34] and their contribution to the holograms can safely be neglected. As schematically shown in Fig. 1, the holographic microscope is a direct realization of Gabor’s initial idea [16] and consists of a 1 µm pinhole which produces a divergent photon beam, sample and detector [19, 20]. Both, pinhole and sample were mounted on xyz-translation stages to allow the alignment of the pinhole position with respect to the focal spot of the beamline. It has also the possibility to vary the pinhole-sample distance and lateral position during the experiment. The focus of the PG0 beamline results as combination of the focusing of the toroidal M1 mirror in the
monochromator unit [35] and a second toroidal refocusing mirror M3_{PG0} (Fig. 1) with a short focal length (50 cm, deflection angle 8°) located 8.3 m downstream of the monochromator grating just in front of the experiment. By scanning the pinhole, the size of the photon beam at the pinhole position – some mm out of the focus of the beamline – was determined to be 85 µm in the horizontal and 330 µm in the vertical direction. Using the transmission function of the PG0 beamline [34] and considering the geometrical overillumination of the pinhole, the total flux emerging from the pinhole for holographic imaging can be estimated to be in the order of $0.16 \, \text{nJ}$ or $6 \times 10^6$ photons per pulse. The sample is positioned in this light cone by the three axis translation stages and a distance to the pinhole between 2 mm and 8 mm is adjusted. The resulting scattering pattern is recorded by a CCD detector (Roper Scientific PI-SX 1300, back-thinned, back-illuminated, 20 µm pixel size, 16 bit, thermo-electric cooling down to -50°C) in a distance of 975 mm downstream of the pinhole. The digital holograms were numerically reconstructed by the Kreuzer implementation of the Kirchhoff-Helmholtz transformation [36]. It allows to directly reconstructing the hologram in different z-planes until the one in focus is found without any phase retrieval:

$$
K(r) = \int_{\text{screen}} d^2\xi |I(\xi)| \exp \left( \frac{ikr\xi}{k} \right)
$$

The integration extends over the two-dimensional surface of the screen with coordinates $\xi=(X,Y,L)$ where L is the distance from the source (pinhole) to the center of the screen (CCD chip) and $I(\xi)$ is the measured hologram. The wave front $K(r)$ can be reconstructed at various distances from the source in the vicinity of the object until a plane in focus is found. For the numerical implementation of the transform a fast algorithm is used that evaluates $K(r)$ without any approximations. A more detailed description about reconstruction and resolution of digital in-line holographic microscopy can be found in [36]. From the reconstruction distance, the field of view can be calculated by dividing the CCD chip size by the magnification resulting from the projection geometry. For all images, the fully illuminated part of the hologram is shown in order to visualize the interference fringes. The reconstructed images are mostly subparts of the full reconstruction to allow a more detailed perception of details within the objects of interest.

In order to characterize the imaging properties of femtosecond in-line holography, three different types of samples have been used in this work: Spherical particles, dried diatoms and fibroblast cells. To prepare the particle samples, a suspension containing silicon oxide particles with a diameter of 1 µm (purchased from Merck) was diluted and dispersed on a polymer window. Subsequent air drying yielded samples with particle agglomerates with different numbers of beads in each unit. Diatom samples were prepared by suspending powder of dried diatoms in water and dispersion on polymer windows followed by air drying. REF.52WT fibroblast cells were cultivated on fibronectin coated Si_3N_4 membranes for 24 hours in Dulbecco’s Modified Essential Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), all purchased at Gibco. After fixation in paraformaldehyde, the cell water was slowly exchanged against ethanol by six different ethanol/water concentrations and the cells were finally critical point dried (Bal-Tec CPD 030). For comparison, all samples were additionally characterized by optical microscopy (Nikon TE2000).
3. Results

![Fig. 2](image)

Fig. 2. Stability of the Airy disk produced by a 1 µm pinhole as spatial filter. Each picture in (a) was recorded with a single pulse train consisting of 21 pulses produced by FLASH with a mean pulse power of 10 µJ. The intensity varies by a factor of 3 as shown in the line scans (b) horizontally through the images (the position of the scan is indicated in the last image of the series (a)). The two arrows in (b) are the positions used to determine the first minima of the Airy pattern.

As the single pulses of FLASH are created by the SASE process within the undulator system initially by spontaneous emission, both the spatial position and the total intensity of the VUV pulses can vary. Using the fundamental wavelength of 8 nm we characterized the intensity fluctuation of the Airy pattern. For this purpose, a single pulse train consisting of 21 pulses with a mean pulse power of 10 µJ was focused on the 1 µm pinhole. The sequence of eight images in Fig. 2(a) reveals that the fluctuations of the photon beam do not affect the pointing stability of the Airy disk as its position on the detector remains the same for different pulse trains. This means that using a pinhole as spatial filter avoids any influence of the natural pulse to pulse position variations of the FEL on the holographic experiment and leads to an excellent pointing stability of the divergent source. The images are not saturated to full scale, which means that the intensity variations visible in the images are real count rate differences. This becomes more obvious using the intensity profiles shown in Fig. 2(b) measured horizontally across the image as indicated by the white line in the last of the eight pictures in Fig. 2(a). It reveals that the intensity of the Airy pattern varies by a factor of 3. These observed intensity variations are the convolution of fluctuations in the spatial position and pulse intensity changes. As indicated in the intensity profiles in Fig. 2(b) by the two arrows, the central maximum of the Airy pattern has a width in the horizontal axis of approximately 15.5 mm on the 26 mm large CCD chip. This illuminated area determines the effective numerical aperture [19] of the setup as 0.008 which sets a theoretical resolution limit of 614 nm.
Fig. 3. Holographic imaging of silicon oxide particles dispersed on a polymer window. VUV radiation from FLASH with a wavelength of 8 nm was used. (a) Hologram, (b) reconstruction, (c) magnified section of (b), and (d) profile through image along the line indicated in (b). Image sizes: (b) 40x40 µm$^2$ and (c) 32x20 µm$^2$.

Figure 3 shows holographic microscopy pictures of silicon oxide particles with a diameter of 1 µm. The 8 nm VUV radiation provided by FLASH was used without monochromatization. This means that the longitudinal coherence is restricted by the intrinsic bandwidth of the fundamental FLASH pulse of 0.7% [34]. The hologram 3(a) consists of the sum of five holograms. Each single hologram was acquired for 60 s and consists of 300 pulse trains. The reconstruction 3(b) shows that the little agglomerates of silicon oxide particles can be visualized and single particles can well be separated from each other. The 10% to 90% contrast change is measured along the line in Fig. 3(b) and quantified by the profile shown in Fig. 3(d), yielding a resolution of 620 nm. According to the theoretical consideration described above, we achieved the theoretical, NA-limited resolution. This experimental resolution of 620 nm is the highest yet reported with digital VUV in-line holography. Although the obtained resolution is not yet satisfying compared to the one obtainable with x-ray microscopy or diffraction microscopy, the fact that no zone plates are required and reconstruction can be done without phase retrieval as the phase is intrinsically present in the images are of great practical advantage.

Fig. 4. (a) Hologram of a diatom recorded at 8 nm photon wavelength. (b) Reconstruction of the hologram (a), image size 24x24 µm$^2$.
As a more complex object a dried diatom was used. In Fig. 4(a) we show the hologram which was again recorded at a photon wavelength of 8 nm. It contains the sum of five 60 s exposures. The reconstruction 4(b) shows that the structures of the object, as e.g. the four parallel features in the upper right corner extending away from the diatom, can be well reconstructed from the hologram. The resolution was determined by line profiles to be 620 nm, which corresponds well with the experimental geometry which was the same as for the above described imaging of the silicon oxide particles. Obviously, the resolution matches the theoretical predictions in both cases (Fig. 3 and Fig. 4), which means that the coherence provided by the free-electron laser is sufficient for holographic microscopy. It is important to note that the short pulse length does not limit the performance of the holographic imaging and NA limited resolution is achieved.

The two aforementioned samples, silicon oxide particles and diatoms, consist mainly of silicon and provide a high contrast at the used wavelength. Biological samples such as cells consist mainly of carbon. To demonstrate the applicability of holographic microscopy with VUV femtosecond radiation towards biologically relevant material, a critical point dried rat embryonic fibroblast (REF52WT) cell was imaged. Such fibroblast cells are responsible to form connective tissue and are often used as model system for cell adhesion studies [37]. In Fig. 5(a) the hologram recorded at a photon wavelength of 8 nm is shown and the distinct interference fringes already indicate significant scattering of the FEL radiation from the cell. The hologram again consists of five 60 s exposures. Figure 5(b) shows the reconstruction of the hologram 5(a), 5(c) a reflection DIC micrograph of the whole cell and Fig. 5(d) shows a phase contrast microscopy picture with the same field of view as the reconstruction 5(b). As the numerical aperture of the holography setup was only 0.008, the reconstruction 5(b) contains information of about ~100 µm sample thickness and therefore a projection of the whole nucleus. To have a better comparison between the two images we show in Fig. 5(d) a phase contrast microscopy image with a numerical aperture of only 0.45 which also provides the depth information of the whole nucleus rather than only a thin section which would be the case for higher NA. The comparison of images in Fig. 5(b) and 5(d) reveals that the shape of the nucleus and many small intracellular structures can be correlated with the optical
microscopy pictures. The general appearance and contrast caused by the cell nucleus agrees well with our former results obtained at slightly higher photon energy using synchrotron radiation [20]. Observable contrast differences between the optical microscopy picture and the reconstruction are also in agreement with this former study and reproduce the result that for some intracellular structures the interaction of VUV and soft x-rays causes different material contrast compared to visible light. The cell nucleus shows the strongest contrast and a transmission of 6%. The cell area surrounding the nucleus has a higher transmission by a factor of 2 (14%), which goes along with three dimensional appearance in the reflection DIC image in Fig. 5(c) that suggest a higher thickness in the nuclear region compared to the cell periphery. Some very dense nucleoli inside of the nucleus attenuate the VUV radiation down to 0.8%, which is already close to the background noise. Although these numbers have to be treated with caution due to possible radiation damage and potential artifacts from critical point drying, they are the first ones obtained for the interaction of free electron laser radiation with cell material.

4. Discussion and conclusion

The results of this study demonstrate the possibility to use femtosecond FEL radiation for digital in-line holographic microscopy. The intrinsic fluctuation and spatial shifts of FEL pulse trains provided by the SASE process can effectively be filtered by the use of pinholes which provides access to divergent wavefronts with excellent pointing stability. Using silicon oxide particles and fossil diatom samples we demonstrated that NA limited resolution can be achieved with digital in-line holographic microscopy using femtosecond FEL radiation. The microscopy images obtained for adherent cell samples are the first ones where free-electron laser radiation was applied to biologically relevant samples. One major challenge to achieve the goal of single pulse imaging with digital in-line holography is the formation of highly divergent light cones which contain the full photon flux provided by the free-electron laser. Due to spatial filtering in the described experiments only $10^{-5}$ of the total beam intensity provided by PG0 was used and 6300 pulses were required for a single exposure. This means that the creation of a divergent light cone e.g. by using zone plates which contains 10% of the intensity of a single pulse will already be sufficient for single pulse digital in-line holography. The realization of this challenge with photons in the water window to image biological samples using higher harmonics of FLASH or the currently developed XFELs is a sensible continuation of the described work.

Contribution of Authors

A.R., F.S., R.B., D.S., T.N., and T.W. conceived the experiment; samples were prepared by T.N., D.S., C.C. and R.B. The chamber used in the experiment was designed and built by T.N., D.S. and T.W. A.R. reconstructed and analyzed the data. The FLASH free-electron laser and the PG0 beamline were optimized by M.V.Y, E.A.S., S.S., E.S., K.H., B.F., N.G., R.T. and M.M.; all authors have contributed to the design, assembly and commissioning of the experiment, associated apparatus and software; the experiment was carried out by the Heidelberg and Remagen groups.

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