Quantitative ptychographic bio-imaging in the water window

Max Rose,1,7 Tobias Senkbeil,2 Andreas R. von Gundlach,2 Susan Stuhr,2 Christoph Rumancev,2 Dmitry Dzhigaev,1 Ilya Besedin,3,4 Petr Skopintsev,5 Lars Loetgering,6 Jens Viefhaus,1 Axel Rosenhahn,2,8 and Ivan A. Vartanyants1,4,*

1Deutsches Elektronen-Synchrotron DESY, Hamburg, 22607, Germany
2Analytical Chemistry - Biointerfaces, Ruhr-University Bochum, Bochum, 44780, Germany
3National University for Science and Technology (MISIS), Moscow, 119049, Russia
4National Research Nuclear University MEPhI (Moscow Engineering Physics Institute), Moscow, 115409, Russia
5Paul Scherrer Institute, Villigen, 5232, Switzerland
6Institute for X-Optics, RheinAhrCampus Remagen, 53424 Remagen, Germany
7max.rose@desy.de
8axel.rosenhahn@rub.de
*ivan.vartanyants@desy.de

Abstract: Coherent X-ray ptychography is a tool for highly dose efficient lensless nano-imaging of biological samples. We have used partially coherent soft X-ray synchrotron radiation to obtain a quantitative image of a laterally extended, dried, and unstained fibroblast cell by ptychography. We used data with and without a beam stop that allowed us to measure coherent diffraction with a high-dynamic range of $10^6$. As a quantitative result, we obtained the refractive index values for two regions of the cell with respect to a reference area. Due to the photon energy in the water window we obtained an extremely high contrast of 53% at 71 nm half-period resolution. The dose applied in our experiment was $10^4$ Gy and is well below the radiation damage threshold. The concept for dynamic range improvement for low dynamic range detectors with a beam stop opens the path for high resolution nano-imaging of a variety of samples including cryo-preserved, hydrated and unstained biological cells.

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References and links
43. Recently it was claimed that although the ePIE algorithm exhibits improved reconstruction speed, it is easy to come across situations when the difficulties of experimental nature may render certain data more difficult to reconstruct. See, A. Maiden, D. Johnson and PENG LI, “Further improvements to the ptychographical iterative engine,” Optica 4, 736 (2017).
1. Introduction

Imaging of unstained cells and biological tissues with X-rays with an energy within the water window range offers quantitative analysis routes for biology. X-ray microscopy provides higher spatial resolution than conventional visible light microscopy due to the comparably short wavelength of X-rays. Opposed to high-resolution electron microscopy, the longer absorption length of X-rays allows imaging the interior of comparably thick samples in the µm-range. Zone plate based soft X-ray tomographic (SXT) microscopes opened with great success the window into the world of native-state cells [1]. However low transmission efficiency and aberrations of X-ray zone plates has stimulated the field of coherent X-ray diffractive imaging (CXDI) [2, 3]. X-ray ptychography is a scanning variant of CXDI to obtain large field of view representations of objects with high spatial resolution [4, 5]. Biological samples have been measured with CXDI in three dimensions (3D) [6] and by ptychography in a cryogenic environment with hard X-rays (8 keV) while the contrast between sample and ice is rather low [7, 8]. In order to gain element specific sensitivity it was suggested to combine the high-resolution imaging capabilities of ptychography with X-ray fluorescence microscopy using hard X-rays [9, 10]. Although X-rays between 6 keV and 13 keV provide less complicated experimental access to nano-structure determination, the low interaction coefficient with matter results in low contrast images for biological materials. The soft X-ray range offers unique imaging opportunities for high-contrast bio-imaging. The so-called water window energy range is located between the absorption edges of carbon (284 eV) and oxygen (532 eV) [11]. It provides a maximum contrast between biological and aqueous components. Remarkably high-contrast can be obtained without any additional contrast enhancing sample preparation like staining [12–14]. Thus a cell stays in its most natural state promising insight into its undisturbed nano-structure.

On the other hand, resolution in ptychography is limited by the maximum diffraction angle to which coherent scattering is detected. Covering the large dynamic range of the diffraction signal with most of the presently available detectors is a problem, especially for biological samples. Techniques like diffuser masks that reduce the dynamic range of the signal by one order of magnitude were tested only for hard X-rays and are difficult to use for soft X-rays [15]. A dynamic range improvement can also be expected from multiple exposures with a beam stop as demonstrated for hard X-rays [16, 17]. The ultimate limitation on resolution is imposed by radiation damage induced in the sample by the absorbed dose [18]. Better than 10 nm resolution (half-period) will require other imaging modalities for biological samples. Ultra-short X-ray pulses produced by X-ray free-electron lasers (XFEL) have been proposed to image small single particles beyond the radiation damage resolution limit [19]. The current progress in single particle imaging (SPI) shows that substantial effort has been undertaken to overcome the radiation damage resolution limit [20–22].

Previously we have published water window ptychographical reconstructions of fossil silicon dioxide skeleton structures and fabricated test samples with resolutions between 50 nm and 90 nm [23, 24]. Chemical element specific contrast was also obtained in another study to highlight the unique imaging opportunities in the water window [25]. We have tested high-dynamic range (HDR) ptychographic measurements using double exposures with and without beam stop on a test sample and achieved 18 nm resolution [26]. In this work we present the significant improvement of (HDR) measurements of a biological cell with soft X-rays using double exposures with and without beam stop. For high-resolution imaging (50 nm or better) of biological specimens with...
scattering signal that spans over multiple orders of magnitude the dynamic range extension by a beam stop is required when measuring with low dynamic range detectors.

2. Theory

2.1. Mode decomposition

For our ptychographic reconstruction we have implemented the multi modal probe expansion to extract the modes of the probe as described in [27]. This takes the partial coherence of X-rays into account for which better reconstruction results can be expected. Instead of a single probe function we now have a family of $l$ probe functions $P_l(r)$ that are independent modes that are treated under the framework of the coherent mode representation of partially, coherent, statistically stationary fields. We can write the cross-spectral density function as a sum of independent spatially coherent modes (we omit the frequency dependence, assuming narrow-bandwidth or quasi-monochromatic radiation)

$$W(r_1, r_2) = \sum_l \beta_l P_l^*(r_1) P_l(r_2),$$

(1)

where $\beta_l$ and $P_l(r)$ are eigenvalues and eigenfunctions of the Fredholm integral equation of the second kind as described by [27]. The non-negative and real eigenvalues $\beta_l$ are also called mode weights [29]. The interaction of the probe with the object at the $i$-th scan position is described by a multiplication and forms the exit surface wave (ESW)

$$\psi_{i,l} = P_l \cdot O_i,$$

(2)

The far-field diffraction pattern at the $i$-th scan position is then the sum over the squared moduli of the Fourier transformed ESW modes

$$I_i = \sum_l \beta_l |\mathcal{F}\{\psi_{i,l}\}|^2$$

$$= \sum_l \beta_l |\mathcal{F}\{P_l \cdot O_i\}|^2,$$

(3)

(4)

where $\mathcal{F}$ denotes the Fourier transform.

2.2. Ptychographic algorithm

In addition to the multi-modal expansion we introduce the sequential difference map (sDM) algorithm that was applied for the ptychography reconstruction. While several reconstruction algorithms have been proposed in the literature ranging from convex [30] to non-convex [31] reconstruction strategies, a comparison of these algorithms is beyond the scope of this paper. Benchmarks of various reconstruction algorithms for diffractive imaging can be found in [30, 32, 33]. Most prominent among the reconstruction strategies proposed for ptychographic imaging are alternating projection strategies such as the extended ptychographic iterative engine (ePIE) [34] and alternating reflection strategies such as the difference map (DM) [35]. While alternating projection algorithms are prone to stagnation in local minima, alternating reflection strategies have better convergence properties [32] and can be shown to find a global solution upon stagnation [35]. However, the original parallel implementation of DM used for ptychography was described to be slower in convergence than sequential algorithms [34]. We therefore implemented the sDM algorithm which we found to combine fast convergence and relaxed memory requirements. This is expected to find application as ptychographic datasets increase with better resolution or larger objects being scanned. The detailed description of the sDM algorithm and a comparison to conventional DM and ePIE algorithms is given in Appendix B.2.
3. Experiment

We used a fibroblast cell as a sample. Fibroblast cells are active in many living organisms and play an important role in extracellular matrix formation, tissue metabolism and wound healing for example. The extended size of several tens of micrometer of the whole cell and its fibrous cytoskeleton with fine filaments bundles of few tens of nanometers make fibroblasts a suited sample for ptychography. For our experiment the fibroblast cells (REF52WT) were cultivated on fibronectin coated Si$_3$N$_4$ membranes (75 nm thick, 1 mm × 1 mm window, Silson Ltd. Northampton) for 24 hours in Dulbecco’s Modified Eagle’s Medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (Gibco). The cells were fixated in 2% paraformaldehyde for 15 min. The water was subsequently exchanged against ethanol in decreasing ethanol/water concentrations and then critical point dried (Bal-Tec CPD 030).

The experiment was carried out at the soft X-ray beamline P04 of the PETRA III storage ring [36]. The APPLE-II type undulator was tuned to generate photons with energy $E_{ph} = 500$ eV ($\lambda = 2.48$ nm) that is close to the oxygen absorption edge. A spectral bandwidth of $\Delta E = 25$ meV was selected by an exit slit opening of 25 μm of the monochromator. For this spectral bandwidth the expected temporal coherence was about $\tau_c = 50\mu$m. Two mirrors focused the X-rays in vertical and horizontal direction. According to the beamline diagnostics we had a focused beam with full width at half maximum (FWHM) of 15 μm in both directions. The measurements were performed in the dedicated HORST (holographic roentgen scattering) vacuum chamber in the ptychographic mode [37]. We used our previously described method with non-redundant arrays (NRA) to measure coherence at the sample position [24, 38]. To select the coherent part of the X-ray beam we used a spatial filter in form of a pinhole with $d = 5\mu$m diameter. The photon flux behind the pinhole was about $1.39 \times 10^8$ Photons/s. Compared to our previous experiment with a twice smaller pinhole diameter [24], we obtained a similar photon flux at three times smaller X-ray bandwidth due to the newly added horizontal focusing mirror.

The geometry of the far-field diffraction experiment is shown in Fig. 1. The monochromatic X-ray beam incoming on the pinhole produced a well defined illumination on the sample at a distance $z_1 = 100\mu$m. Here the sample was located on the membrane facing the detector. The diffracted wave was measured by the detector at a distance $z_2 = 47$ cm in the far-field ($d^2/\lambda z_2 << 1$). For an area of $50 \times 50$ μm$^2$ on the sample we used $21 \times 21$ scan positions arranged on a rectangular grid with 2 μm step size in both directions to ensure a linear overlap of 60% between the illumination positions [39].

![Fig. 1. Experimental geometry inside the HORST chamber for the ptychographic imaging experiment. A beam stop is located on a Si$_3$N$_4$ membrane (transparent layer) in front of the detector for dynamic range improvement.](image)

The detector was a back-illuminated and Peltier cooled (~60 °C) CCD image sensor (DODX436-BN, Andor Technology Ltd., Belfast, UK). The square detector area of 27.6 mm × 27.6 mm
consisted of $N_{\text{det}} = 2048$ pixels in both directions with a pixel size of $p = 13.5 \mu m$. In our experimental conditions the sampling rate [40–42] of the diffraction intensities, i.e. the number of detector pixels per average speckle size in one dimension, was $\sigma = \lambda z_2 / (dp) = 17$.

We have used a double-exposure scheme to enhance the dynamic range (DR) of our detector. In total two scans were acquired with a fixed exposure time for each scan. The first scan consisted of short exposures (0.4 s) without beam stop (Fig. 2(a)). In this scan we reached $3.1 \mu m^{-1}$ spatial frequency which is defined in reciprocal space by the number of samples $N$ and the frequency sample $\Delta \nu$. The real space has the same number of samples $N$ and the pixel size $\Delta$. The reciprocity relation is $\Delta \nu \Delta = 1/N$ and $\Delta = 1/(N \Delta)$. The spatial frequency origin is located in the center of the diffraction pattern and thus the maximum frequency (Nyquist frequency) $u_{\text{max}} = u_{\text{Ny}} = \Delta u N / 2 = 1/(2 \Delta)$. The second scan consisted of longer exposures (3 s) with beam stop and slightly increased the beamline slits for a ten times higher photon flux (Fig. 2(b)). With beam stop we increased the maximum spatial frequency to $11 \mu m^{-1}$. The double exposure HDR data acquisition including the two scans took about 25 min. The significant overhead of 10 s per exposure was imposed by the detector readout rate and added about 2.5 h to the full data acquisition time. The stability for long exposures and large pycographic scans was explicitly addressed by the mechanical design of the HORST chamber and its sample holder. We have used a circular, semitransparent golden beam stop with a diameter of 1 mm and a thickness of 200 nm, sputtered on a 500 nm thick SiN membrane that was glued with all sides to a motorized metal frame (shown in Fig. 1). After background subtraction both scans were merged into a high-dynamic range (HDR) data set by scaling the data without beam stop with respect to the data with beam stop as described in Appendix A.1. The DR of the single exposure data set without beam stop was $2.3 \cdot 10^5$ and the enhanced DR of the acquired HDR data set was $1.7 \cdot 10^6$ as shown in Fig. 7.

As a conclusion the combination of two exposures per position extended the effective dynamic range of our detector. The benefit from using the beam stop is that signal up to the detector edges could be measured and we exploited the instrumental resolution capabilities of our experiment geometry.

![Fig. 2. Diffraction pattern of a single position of the fibroblast cell without beam stop a) and with semitransparent beam stop b). The white circles mark the effective signal cut-off with the corresponding spatial frequency. The diffraction patterns are shown on the same logarithmic scale.](image_url)
4. Results

4.1. Ptychographic image reconstruction

In order to obtain the full complex valued object function the phases of each diffraction pattern have to be retrieved. In ptychography this is achieved by an iterative algorithm and data redundancy from overlapping illumination positions on the object [4]. For the results shown here we used the sDM algorithm that allowed simultaneous reconstruction of the complex valued object and probe functions.

The ptychographic algorithm needs first input parameters to start the iterative process. We set the initial object function to random values between zero and one in amplitude and zero phase. The initial probe function was chosen to be a uniform circular amplitude distribution with a diameter of 5 μm matching the pinhole size. The height of the amplitude was normalized to the square root of the average flux incident on the detector plane. The phase of the first probe was set to zero. The remaining four probe modes were initialized with random values for the amplitude and zero phase. The algorithm processed the individual scan positions \( i \) in a random step order at each iteration. A full reconstruction went through different stages at which masks were used in succession to get the best reconstruction.

The feedback parameters for the sDM update rules were \( \beta_O = 0.5 \) and \( \beta_P = 0.1 \) for the object and probe updates, respectively. The first 20 iterations were performed by constraining the algorithm strictly to the data. For the next 50 iterations all defect detector pixels (good pixel mask) were not constrained but left freely evolving. Then 50 iterations followed where masked pixels in the beam stop area (beam stop mask) were also not constrained but left freely evolving in order to level any subtle inaccuracies introduced by the replacement with the short exposure data. In total the algorithm ran up to 4760 iterations until the calculated error norm did not change significantly anymore [43]. The same parameters were used on both the single exposure and double exposure HDR data. Here we present the reconstruction from the double exposure HDR dataset. The single exposure reconstruction is shown in Appendix B.3 for comparison. The reconstructed object is described by a complex valued function

\[
O(r) = e^{\frac{i}{2}A(z(r))} \cdot e^{-i\Delta \phi(r)},
\]

where \( r = (x, y) \) represents two-dimensional spatial coordinates. The first exponential term represents the object transmission \( T(r) = |O(r)| \) and the exponent of the second term is the object

![Fig. 3. Fibroblast cell images. a) Visible light image with the ptychography scan area marked by the white rectangle. b) Reconstructed fibroblast cell phase of the double exposure HDR data. Regions N (nucleus) and Nu (nucleolus) are quantitatively analyzed with respect to a reference area outside the cell marked by R. c) An enlarged view of the region shown by the black square in b) shows the cells fibrous cytoskeleton and dot structures. The red line denotes a profile used for contrast evaluation.](image)

phase $\Delta \varphi(r)$ that is obtained by X-rays propagating through the object. The projected object thickness is given by $\Delta z(r)$. Using the complex refractive index $n = 1 - \delta + i \beta$ yields the material absorption coefficient as $\mu = 2k\beta$ and the phase as $\Delta \varphi(r) = k\delta \Delta z(r)$ with the wavenumber $k = 2\pi / \lambda$ [44]. In the above description we assume that $\delta$ and $\beta$ are independent of the spatial coordinate $r$.

A visible light microscopy image of the fibroblast cell under investigation is shown in Fig. 3(a). The white square indicates the psychographically scanned region. Phase wrapping is a known problem for phase shifts larger than $2\pi$ and occurs also in our result. We used an automatic phase unwrapping algorithm based on sorting reliability following a noncontinuous path [45]. The unwrapped high-resolution phase image is shown in Fig. 3(b). An enlarged region that shows the details of fine features can be found in Fig. 3(c). The large oval nucleus (marked as N) is visible at the top of the fibroblast image. Inside the nucleus we see evidence of a protein and RNA rich nucleolus (a ribosome synthesis site, marked as Nu) with a size about 2 $\mu$m by 2 $\mu$m. Individual proteins or RNA inside of the nucleolus are not resolved in our experiment with a pixel size of $\Delta_{\text{min}} = 45.7$ nm.

In the enlarged view of the reconstructed cell image Fig. 3(c) we found irregular distributed dots with diameter between 225 nm and 360 nm, which suggest the presence of shedding vesicles that play an important role in exocytosis [46]. In addition, parts of the fibrous cytoskeleton are visible. As typical for eucaryotic cells those fibrous elements consist mainly of actin and vimentin, intermediate and micro-filament proteins. It is well known that individual filaments of a typical size of 10 nm form aligned bundles with a size between 50 nm and 100 nm [47]. Although we do not resolve single filaments we analyze the aligned bundles of these filaments (see Fig. 4). The visibility or contrast $C$ of image features with a periodic pattern can be described by

$$C = \frac{\varphi_{\text{max}} - \varphi_{\text{min}}}{\varphi_{\text{max}} + \varphi_{\text{min}}}.$$  

Here $\varphi_{\text{max}}$ and $\varphi_{\text{min}}$ denote the average maximum and minimum phase values denoted by the red and blue markers in Fig. 4 which shows the line profile from Fig. 3(c). We obtained a contrast of $C = 0.53$ at a spatial frequency of 7.1 $\mu$m$^{-1}$ and resolved filament bundles with an average width of 71 nm.

By using the HDR data we obtained high resolution with remarkably high contrast due to the water window photon energy. Using our improved algorithm and the coherent mode decomposition less artifacts were observed compared to the assumption of fully coherent X-rays. The evaluation of periodic image features show a direct measure of resolution. Besides visually identifying the eucaryotic cell components we extend our evaluation by a quantitative analysis in the next section.

![Fig. 4. Contrast profile line perpendicular to several aligned filament bundles along the red line in Fig. 3(c). Red and blue markers denote the maximum and minimum phase shift between the filament bundles.](image-url)
4.2. Quantitative analysis

For the quantitative analysis we used the relative phase shift to the reference that is an empty membrane region marked by R (see Fig. 3(b)). With respect to this region the phase values obtained in the regions N and Nu are calibrated. The transmission and phase values from regions N and Nu are plotted as histograms in Fig. 5(a) and 5(b). For a quantitative analysis we calculated the ratio of the refraction ($\delta$) and absorption ($\beta$) coefficient determined from the measured phase and transmission values by $\delta/\beta = \Delta\varphi/\ln T$ (shown in Fig. 5(c)). The mean values of the distributions are indicated by the dashed and solid black lines and their values are summarized in Table 1. Our tabulated results show that the nucleus has a larger transmission $T_{\text{mean}}$ compared to the nucleolus. We observe less phase shift $\Delta\varphi_{\text{mean}}$ for the nucleus compared to the nucleolus. This suggests a higher density of the nucleolus present in the measured cell that transmits less and induces a stronger phase shift to the X-rays. The experimentally determined values of $(\delta/\beta)_{\text{mean}}$ are very similar because the average molecular composition of both nucleus and nucleolus appears to be very similar (both distribution mean values indicated by black lines nearly overlap in Fig. 5(c)). The ratio of refraction and absorption of a typical protein composition $\text{C}_{94}\text{H}_{139}\text{N}_{24}\text{O}_{53}\text{S}$ with a density of $\rho = 1.35 \, \text{g/cm}^3$ was used earlier to estimate contrast in X-ray microscopy [14]. Here we refer to this generic protein to compare its $\delta/\beta = 3.67$ value at 500 eV to our measurements [48]. As we see from Fig. 5(c)) the generic protein value deviates slightly from our measurements which can have several reasons due to sample preparation and the nature of the projection image. Due to projection we integrate over the beam path and as such obtain an average over a variety of different cell structures. The quantitative values obtained by the reconstructed fibroblast image contain information that can be compared to typical molecular compositions.

<table>
<thead>
<tr>
<th>region</th>
<th>$T_{\text{mean}}$</th>
<th>$\Delta\varphi_{\text{mean}}$ [rad]</th>
<th>$(\delta/\beta)_{\text{mean}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>nucleus N</td>
<td>0.36</td>
<td>-4.01</td>
<td>3.95</td>
</tr>
<tr>
<td>nucleolus Nu</td>
<td>0.26</td>
<td>-5.21</td>
<td>3.91</td>
</tr>
</tbody>
</table>

Fig. 5. Histograms of quantitatively measured transmission a) and phase values b) from two regions. The ratio of the refraction and absorption coefficient is given in c). Mean values of the measurements are indicated by black dashed and solid lines and the literature value for a typical protein compound is indicated by the red line.

4.3. Resolution and dose

To quantify the obtained resolution for both reconstructions we evaluated the phase retrieval transfer function (PRTF) [49, 50]. It is the ratio of the reconstructed and measured modulus as a
function of spatial frequency that was adopted for psychography [51]. In order to take into account the decreasing signal to noise ratio for increasing spatial frequency the Wiener filtered PRTF (wPRTF) was used [52]. To obtain the wPRTF we multiplied the PRTF by a weighting function (Wiener filter [53]) that varies from one for high signal to zero where the signal approaches the noise level. We constructed the filter function from the measured data according to [52].

![Graph showing PRTF for psychographic fibroblast cell reconstructions](image)

**Fig. 6.** wPRTF for the psychographic fibroblast cell reconstructions. The reconstruction yielded 250 nm half-period resolution from single exposure data and 54 nm from HDR data.

Two wPRTF curves are shown in Fig. 6. The black curve corresponds to the low resolution reconstruction from the single exposure data (see Appendix B.3) which crosses the 0.5 threshold at 2 μm^{-1} or 250 nm half period resolution. This is an expected result because the data without beam stop did not contain sufficient signal beyond 3 μm^{-1} (see Fig. 2). The red curve corresponds to the HDR data set with beam stop and crosses the 0.5 threshold at 9.3 μm^{-1} which corresponds to a half-period resolution of 54 nm. With the beam stop enhanced HDR data we achieved a reconstruction close to the technical limit of 11 μm^{-1} imposed by the size of the detector (see Fig. 2).

The general limits in resolution are imposed by structural changes during the exposure as a consequence of radiation damage. Psychography and other coherent imaging techniques are by principle dose efficient because no lenses are used between the sample and detector [3]. We aimed for the minimum dose in agreement with our resolution demand (see Appendix A.2 for dose calculation). In our experiment the dose was estimated by adding up the doses with and without beam stop for both exposures to obtain the total dose per illumination position of $D_{total} = 9.5 \cdot 10^6 \text{ Gy}$ applied in the region of the cell nucleus. This dose value is far from the destructive regime. We did not observe changes in the diffraction patterns during the exposure and conclude that no radiation damage effects were induced. The absorbed dose on the sample in our experiment is similar to the reported dose values for the resolution range between 70 nm and 90 nm [18]. This confirms our resolution estimate from the contrast analysis (71 nm) of the periodic image features of the psychographic fibroblast cell reconstruction.

As a conclusion we have used different resolution estimates. With the PRTF approach we clearly see that the beam stop measurement for HDR adds a significant amount of data that enables high resolution for biological samples. The contrast analysis of periodic features was the most direct and unambiguous way to determine resolution. Finally, we confirmed our findings with the dose estimate and found that we performed the experiment at a configuration where the resolution limitations given by detector size, detector dynamic range and radiation dose are balanced very well for the HDR data.
5. Conclusion

A step towards the unique water window imaging opportunities with high-resolution ptychography was the focus of our work presented in this paper. We have described a ptychographic experiment on a large unstained and dried eucaryotic cell with a photon energy of 500 eV in the water window range. Quantitative phase images were obtained by double exposure measurements with and without beam stop to get the full diffraction pattern with low and high spatial frequency components. We successfully compensated for the low detector dynamic range and extended the measured signal by two orders of magnitude with a beam stop using two exposure times and beamline slit settings. As a result, we achieved a factor of 3.6 improvement in spatial resolution that is remarkable and larger than reported by other groups who have used beam stops in their experiments [16]. Using soft X-rays in the water window range was our key for high-contrast images of a dried fibroblast cell sample without staining. Together with improvements in the ptychographic reconstruction algorithm and coherent mode decomposition our reconstruction from HDR data resulted in a high-contrast (53%) phase image that demonstrates the important improvement of our beam stop approach for ptychographic imaging within the water window. A reference area in the image was used to calibrate the phase image. Thus quantitative values for the complex refractive index were obtained. The obtained resolution was estimated using PRTF and periodic feature contrast analysis that gave us values between 54 nm and 71 nm. With the HDR mode of our ptychographic measurements we enabled high resolution of biological specimen beyond the theoretical limitation (200 nm) of conventional optical far-field microscopy.

By using fast detectors dedicated for measurements at soft X-rays with intrinsic large dynamic range ($> 10^5$) and rapid image acquisition [54, 55] we expect further improvement in resolution for coherent imaging techniques in the water window energy range. High-resolution together with high-contrast meets a demand in biological nano-imaging. Our high-dynamic range strategy with multiple exposures can be combined with future HDR detectors to aim at the maximum resolution, down to the biological radiation damage resolution limit of 10 nm [18] for coherent water window studies of unstained cells.

Our results show that although quantitative pictures can be retrieved the biological interpretation is limited due to the projection nature of 2D X-ray imaging. The extension towards 3D imaging will improve the informative value but comes with the cost of mechanical complexity and the demand for fast imaging detectors in order to keep the data acquisition time within reasonable limits [56]. Quantitative ptychographic phase imaging requires least invasive preparation methods for relevant results which is the advantage of the water window that we will exploit in future studies. High-resolution and high-contrast studies, as we have presented in this work, will open water window X-ray ptychography to a wider range of samples including cryo-preserved, hydrated and unstained bio-samples.

A. Diffraction pattern processing

A.1. HDR data composition

The first processing step was to subtract the average dark signal by pixel in each diffraction pattern (ten dark frames were used). To reduce the data without violating the oversampling condition we binned the diffraction patterns by a factor $b = 3$ and cropped the images in a subsequent step to $N_{\text{crop}} = 630 \times 630$ pixels. With this the pixel size of the reconstruction is determined by $\Delta_{\text{min}} = \lambda z_2 / (bpN_{\text{crop}}) = 45.7$ nm. Pixel values that stayed below a threshold of ten analog-digital units (ADU) were indexed as background mask and were not used for the ptychographic reconstruction. Any defect detector pixels were indexed as good pixel mask. All pixels covered by the beam stop were indexed as beam stop mask.

For the HDR data set both measured data sets with and without beam stop were merged in the following way: We took the pixels of the data without beam stop that were indexed by the
beam stop mask and multiplied them by a scaling factor and then copied the values into the data set with beam stop. This was done for each pair of corresponding diffraction patterns. A global scaling factor of 75 for all diffraction pattern pairs was obtained from the ratio of signal from the beam stop data to the data without beam stop at a spatial frequency of 1 \( \mu m^{-1} \). The power spectral density (PSD, that is the angular averaged signal) for the over all pytchographic positions averaged diffraction dataset as a function of spatial frequency and half-period resolution is shown in Fig. 7. The blue curve represents the data without beam stop and the black curve the data with beam stop. The red curve represents the HDR data set with an extended dynamic range of 1.7\( \cdot 10^6 \) which is roughly two orders of magnitude better than from the single exposure data set without beam stop (red curve). The signal cut-off is located at 3.1 \( \mu m^{-1} \) (\( \Delta_{eff}^{\text{noBS}} = 161 \text{ nm} \)) and 11 \( \mu m^{-1} \) (\( \Delta_{eff}^{\text{BS}} = 45 \text{ nm} \)), for the data without and with beam stop, respectively. This means that the HDR data is not limited by the detector dynamic range but rather by the detector size in the used experiment geometry. Using the beam stop an improvement in resolution of 3.6 can be calculated using the ration of the cut-off resolution values mentioned above.

A.2. Dose estimate

The dose estimate was obtained by the following method. The summed photons per scan position around the reference region \( R \) for the short exposure without beam stop and the long exposure with beam stop were \( n_{R}^{\text{noBS}} = 2.65 \cdot 10^5 \) photons and \( n_{R}^{\text{BS}} = 8.32 \cdot 10^6 \) photons ( photon counts were estimated with the detector signal by 73 ADU/photon). In comparison the summed photons per scan position around the nucleus \( Nu \) for the short exposure without beam stop and the long exposure with beam stop were \( n_{Nu}^{\text{noBS}} = 6.48 \cdot 10^4 \) photons and \( n_{Nu}^{\text{BS}} = 6.26 \cdot 10^5 \) photons. The absorbed number of photons in the region \( Nu \) was then calculated by the difference to the reference \( R \) with \( n_{abs} = n_{R} - n_{Nu} \) for both exposures.

If we use the average phase of \( \phi_{\text{mean}} = -4 \text{ rad} \) from Table 1 to estimate the optical path length through the cell nucleus (see Eq. 5) we get \( |\phi_{\text{mean}}| \lambda/(2\pi\delta) = 1.5 \mu m \) (with \( \delta = 1.06 \cdot 10^{-3} \) and \( \lambda = 2.48 \text{ nm} \)). The approximate cylindric volume defined by the circular footprint of the X-rays was \( V_{cyl} = 2.9 \cdot 10^{-17} \text{ m}^3 \) and the corresponding mass \( m = 3.9 \cdot 10^{-14} \text{ kg} \) (using a density of \( \rho = 1.35 \text{ g/cm}^3 \)). The absorbed dose per position \( D_{pos} = E/m \) was calculated with the deposited energy of all absorbed photons \( E = n_{abs} E_{ph} \). In fact this dose per position has to be multiplied by a factor that takes into account the overlap of illumination positions on the sample. In our
experiment the overlap factor was determined from simple geometric considerations and the absorbed dose per position was estimated with $D = 6 \cdot D_{\text{pos}}$. The absorbed dose in the nucleus for the short exposure without beam stop was $D_{\text{Nu}}^{\text{BS}} = 2.42 \cdot 10^3 \text{ Gy}$ and for the long exposure with beam stop $D_{\text{Nu}}^{\text{BS}} = 9.3 \cdot 10^4 \text{ Gy}$. We find the short exposure added only about 2.6% to the dose per position in the nucleus region.

B. Ptychographic reconstruction

B.1. Probe reconstruction

We used the singular value decomposition (SVD) to retrieve independent (orthogonal) modes $P_i(r)$. Note that the mode weights $\beta_i$ of Eq. (1) are related to the singular values $\sigma_i$ from the SVD by $\beta_i = \sigma_i^2$. As result we obtained the following weights (sum normalized to unity): $\beta_1 = 0.741$, $\beta_2 = 0.132$, $\beta_3 = 0.067$, $\beta_4 = 0.033$ and $\beta_5 = 0.028$. The reconstructed five complex valued probe modes $P_i(r)$ with their energies $\varepsilon_i = \beta_i^2$ are shown in Fig. 8. We observe a dominant mode (Fig. 8(a)) with 74.1%. The two following modes (Fig. 8(b) and 8(c)) are clearly orthogonal to each other and much weaker with contributions of 13.2% and 6.7% respectively. The last two modes have contributions of less than 4% (Fig. 8(d) and 8(e)). They contain fringes that are resolved nearly to the pixel level. The number of modes was limited to five since the energy contribution of higher modes was less than one per cent. As soon as all modes originate from the partial coherent nature of the incoming X-ray beam we can calculate the degree of transverse coherence from the mode weights by $\zeta = \sum \beta_i^2 / (\sum \beta_i)^2 = 0.57$ [57]. This result means that the degree of coherence of the X-ray beam passing the 5 $\mu$m pinhole is 57%. The beam is not yet fully coherent at these experimental conditions.

Fig. 8. Probe modes and corresponding weights. Hue encodes the phase and brightness the modulus as indicated by the color wheel in the lower right corner.

The mode decomposition shows the partial coherent nature of the X-ray illumination. The retrieved degree of transverse coherence is similar to our previously reported results indicating high coherence behind the pinhole [24]. Note that we have used only the dominant mode in the ptychographic update rule of the object which works well for highly coherent X-ray illumination [58].

Besides the complex object function from Eq. 5 ptychography also retrieves the complex valued probe function that can be propagated numerically using Fresnel propagation equations. The modes were propagated individually and the sum of all probe mode intensities along the propagation axis is shown in Fig. 9(a). Here the plane of the object is located at $z = 0 \text{ mm}$ and
the pinhole is at \( z = -0.1 \text{ mm} \). Cross sections of the beam at both positions are shown in Fig. 9(b) and 9(c)). In the pinhole plane we observe a flat intensity distribution that shows the residual artifact due to a rectangular raster scan grid [59]. The diameter in the pinhole plane coincides with the pinhole diameter of 5 \( \mu \text{m} \). In the sample plane we observe the diffracted beam with its diffraction rings according to Fresnel diffraction from the pinhole. At this plane the beam is well confined as required for coherent imaging by the sampling criterion [42]. The short distance of 100\( \mu \text{m} \) between pinhole and sample could be retrieved by the propagation analysis.

Besides using ptychography for X-ray illumination characterization, the probe analysis is a tool for checking the results upon consistency with the experimental parameters. In our experiment we could verify the pinhole size and position as well as the pinhole to sample plane distance.

**Fig. 9.** Reconstructed and propagated illumination intensity as the sum over all modes a). Intensity distribution in the pinhole b) and the sample plane c).

### B.2. Comparison of algorithms

#### B.2.1. Difference map (DM)

The formulation of the DM algorithm in the context of ptychography is described as follows [59]. Find an object transmission function \( O_j \) and illumination \( P_j \) that comply with the ptychographic overlap constraint to form an exit surface wave (ESW) by multiplication

\[
\psi_j (r) = P_j (r - r_j) \cdot O_j (r), \ \forall j
\]

and additionally with the intensity constraint

\[
I_j = |F \{ \psi_j \} |^2, \ \forall j
\]

for all positions \( j \). The reasoning was (using functional derivatives) that applying in turns (not simultaneously) the update rules

\[
O^{n+1} (r) = \frac{\sum_j P_j (r - r_j) \psi_j^{n+1} (r)}{\sum_j |P_j (r - r_j)|^2}
\]
and
\[ p^{n+1} (r) = \frac{\sum_j O^n (r + r_j) \psi_j^{n+1} (r + r_j)}{\sum_j |O^n (r + r_j)|^2}, \]  
(10)

with \( n \) denoting the number of iteration and the \( \bar{P} \)-symbol the complex conjugate. The ESW is updated by the difference map (the updated ESW)
\[ \psi^{n+1}_j (r) = \psi^n_j + \pi_F \left[ 2\pi_O \left( \psi^n_j - \psi^n_j \right) - \pi_O \left( \psi^n_j \right) \right] \]  
(11)

that uses the overlap projector
\[ \pi_O \left[ \psi^n_j (r) \right] = P^n (r - r_j) \cdot O^n_j (r) \]  
(12)

and the Fourier magnitude projector
\[ \pi_F \left[ \psi^n_j \right] = \mathcal{F}^{-1} \left( \sqrt{I_j} \mathcal{F} \psi^n_j \right). \]  
(13)

Note that equations (9) and (10) are implemented in a parallel fashion, which means the object and probe depend on a weighted average of all updated ESWs. Further at each iteration a so-called state vector \( \Psi = (\psi_1, \psi_2, \ldots, \psi_j) \) containing the ESWs at all positions is required.

B.2.2. Extended ptychographic iterative engine (ePIE)

Besides DM another strategy was reported, the extended ptychographic iterative engine (ePIE) claimed superior reconstruction speed due to a sequential implementation [34]. The ePIE algorithm differs from the DM in the object update
\[ O^{n+1} (r) = O^n (r) + \beta_O \frac{p^n}{\max |p^n|^2} \left( \psi^{n+1}_j - \psi^n_j \right) \]  
(14)

and in the probe update
\[ p^{n+1} = p^n + \beta_P \frac{\bar{O^n}}{\max |O^n|^2} \left( \psi^{n+1}_j - \psi^n_j \right), \]  
(15)

where
\[ \psi^{n+1}_j = \pi_F \left[ p^n (r - r_j) \cdot O (r) \right] \]  
(16)

contains the same Fourier magnitude projector of equation (13) and the ESW at the \( j \)-th position
\[ \psi^n_j = p^n (r - r_j) \cdot O (r). \]  
(17)

The parameters \( \beta_P \) and \( \beta_O \) are feedback parameters typically chosen in the interval \([0, 1]\). It is seen that the ePIE differs significantly from the DM in two points:

- ePIE is sequential using its update functions at each position (equations (14) and (15)) while DM is parallel using a weighted average of all positions in its update function (equations (9) and (10))
- ePIE doesn’t require a state vector \( \Psi \) and thus occupies less memory in the computer
B.2.3. Sequential difference map (sDM)

Recently it was claimed that although the ePIE algorithm exhibits improved reconstruction speed, it is not difficult to come across situations where ePIE fails to solve the ptychographic problem and getting stuck in local minima [43]. From this point of view our idea was to maintain DM’s ability to climb out of local minima while enabling a sequential implementation known from ePIE. This is fairly straightforward by adjusting the update rule of the DM as follows. Therefore we implemented the sequential difference map (sDM). The updated object and probe are given sequentially as in the ePIE update, i.e.

\[ O^{n+1}(r) = O^n(r) + \beta_O \frac{P^n}{\max|P^n|} \left( \psi^{n+1}_j - \psi^n_j \right) \]  

(18)

and

\[ P^{n+1} = P^n + \beta_P \frac{O^n}{\max|O^n|} \left( \psi^{n+1}_j - \psi^n_j \right). \]  

(19)

However, the updated ESW is given by

\[ \psi^{n+1}_j = \pi_M \left[ P^n O^n_j \right] + \pi_M \left[ 2P^n O^n_j - \pi_M \left( P^n O^n_j \right) \right] - P^n O^n_j, \]  

(20)

which is similar to the difference map update rule. The equations (18) to (20) are termed sequential difference map (sDM). If the algorithm finds a point satisfying \( \psi^{n+1}_j = \pi_M \left[ P^n O^n_j \right] \), for all \( j \), then it follows that a solution is found that satisfies both the Fourier magnitude and overlap constraint. Hence a suitable error metric is given by

\[ e \sim \sum_j \left( |\psi^{n+1}_j| - \left| \pi_M \left[ P^n O^n_j \right] \right| \right)^2. \]  

(21)

Note that the sDM has the following attributes:

- sDM is sequential
- no state vector \( \Psi \) is required in sDM and requires less memory than the DM algorithm.

Further we observed that sDM yields reconstruction less prone to local minima as compared to ePIE.

B.3. Reconstruction from single exposure data

The single exposure data without beam stop shown in Fig. 2(a) were reconstructed in order to demonstrate the limited resolution due to the low dynamic range of the detector as compared to the double exposure HDR data. The ptychographic unwrapped phase image reconstructions are shown in Fig. 10. While comparing this reconstruction to the HDR data it is evident that the reconstruction quality was improved substantially. In practice, the improvement is not only seen in resolution but also by reduced image noise. The artifacts in Fig. 10(a) are the consequence of the phase unwrapping of very noisy image data. In addition to the noise also the raster grid artifact [59] was visible in the reconstruction of Fig. 10(a). Such artifacts are not present in the HDR reconstruction for which the phase unwrapping works reliably. Also the enlarged region shown here is seen to be corrupted by noise that made a contrast analysis impossible. Whereas in the HDR reconstruction of Fig. 3(b) fine details can be clearly resolved.
Fig. 10. Reconstructed fibroblast cell phase of the single exposure data without beam stop a). Unwrapping artifacts arise due to the low SNR. The magnified view without unwrapping artifacts show only low resolution without the cell details b).

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