Hard X-ray imaging of bacterial cells: nano-diffraction and ptychographic reconstruction


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Abstract: Ptychographic coherent X-ray diffractive imaging (PCDI) has been combined with nano-focus X-ray diffraction to study the structure and density distribution of unstained and unsliced bacterial cells, using a hard X-ray beam of 6.2keV photon energy, focused to about 90nm by a Fresnel zone plate lens. While PCDI provides images of the bacteria with quantitative contrast in real space with a resolution well below the beam size at the sample, spatially resolved small angle X-ray scattering using the same Fresnel zone plate (cellular nano-diffraction) provides structural information at highest resolution in reciprocal space up to 2nm⁻¹. We show how the real and reciprocal space approach can be used synergistically on the same sample and with the same setup. In addition, we present 3D hard X-ray imaging of unstained bacterial cells by a combination of ptychography and tomography.

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References and links

1. Introduction

To understand the important biophysical problem of DNA compactification in bacterial cells, ideally the three-dimensional density distribution at the sub-cellular level needs to be imaged with quantitative contrast values. Indeed, a simple unknown quantity relevant to the understanding of DNA compactification in bacterial nucleoids is the local mass density. This important question cannot be solved by electron microscopy or fluorescence microscopy studies, not for reason of resolution but of contrast. More generally, the problem of mapping out the native three-dimensional density distribution in biological cells, without staining and slicing, is a formidable task. Density contrast can provide useful insight into many biophysical and biological problems by bridging previously disjunct pieces of information: (a) averaged densities derived from centrifuging separated and purified organelles, which is one of the most common tools in biochemistry and molecular biology, and (b) the three-dimensional (3D) structure derived from imaging techniques such as confocal fluorescence microscopy or electron tomography.

To this end, we want to advocate the use of coherent X-ray imaging and tomography to shed light on this unsolved issue, since these techniques offer a unique potential for quantitative three-dimensional determination of density in unstained and unsliced biological cells and tissues. In particular, as discussed in [1], electron density can be derived from hard X-ray imaging independently of local stoichiometry for nearly all biologically relevant elements, if the photon energy is higher than the corresponding absorption edges.

The bacterium addressed in this work, *Deinococcus radiodurans* [2, 3] is famous for its ex-
extraordinary resistance to high doses of ionizing radiation, which has been tentatively linked to the structural organization of DNA in the nucleoid. More precisely, some of the DNA repair mechanisms have been postulated to rely on the particular structural arrangement of its nucleoid [4–6]. Determination of the mesoscopic density in the nucleoid would help to test these ideas and eventually to discriminate different models of DNA packing. Towards this goal, we have previously shown that the projected electron density of unstained and unsliced freeze-dried cells of the bacterium *Deinococcus radiodurans* can be derived from coherent X-ray diffraction imaging (CDI) based on ptychographic phase retrieval [1]. Ptychographic phasing was developed in the field of electron microscopy and was introduced in X-ray microscopy by Rodenburg and co-workers [7, 8]. In contrast to conventional CDI [9], Fresnel CDI [10] and Keyhole CDI [11], where the solution of the ‘Phase-Problem’ by iterative algorithms such as the ‘ER’ or ‘HIO’ [12] is mainly based on the support constraint, ptychography takes advantage of imposing redundancy in the data by combining data sets recorded by scanning the sample with partial overlap through the illuminating beam. Generalizing this approach, it was shown that this so-called overlap constraint is powerful enough not only to solve for the unknown sample, but also for the unknown illumination function (so-called probe) [13]. Ptychography has been shown to yield quantitative contrast for weakly scattering specimens such as biological cells [1] and has been used for 3D reconstruction of mineralized specimens [14], stained biological cells [15] and SiO2 microspheres [16]. In this work we want to extend our previous ptychographic study of *Deinococcus radiodurans* to higher resolution on the 50nm scale, by using nano-focused undulator radiation with increased flux density. Furthermore, the present work aims at an extension from two-dimensional (2D) maps to three-dimensional (3D) density maps, by a combination of ptychography and tomography. This is significantly more challenging for biological cells with typically much smaller variations in the index of refraction than for mineralized specimens with high contrast [14] and stained biological cells [15].

We would like to complement ptychography with a higher resolution structural analysis in reciprocal space, i.e. by nano-beam diffraction. Note that notwithstanding impressive progress, coherent imaging of cellular samples [17–19] has always been limited to moderate resolution, not reaching resolution of typical diffraction experiments on soft matter samples. Here we combine coherent imaging with nano-beam diffraction for structure analysis of biological cells. By simple experimental parameters (slit opening, detector distance, beamstop, defocus position), we can switch between a setting optimised for high resolution diffraction, and a setting optimised for coherent imaging, on the same cell (see schematic in Fig. 1). Compared to a standard nano-diffraction experiment the following advantages apply for a combined approach: (i) the illuminating beam (probe function) and corresponding interaction volume can be deduced quantitatively from ptychographic inversion instead of simple knife edge scans, (ii) correlative microscopy images of projected density and diffraction observables are obtained, (iii) one can easily and quickly determine where exactly the beam is positioned on the cell with a full field (defocus) image, before more lengthy scanning diffraction maps are recorded. Compared on the other hand to a pure coherent imaging study, analysis in reciprocal space can yield higher resolution by averaging over the illuminated spot, and by circumventing the stringent requirements (oversampling, signal-to-noise, coherence) of full phase reconstruction. Apart from density as a contrast value, many more structural parameters (inter-molecular spacings, short range correlations, lattice constants, form factors) can reasonably be interpreted by a local average on length scales of 10 – 1000nm. Fourier components corresponding to much smaller structures can thus be measured, very much like in a standard small angle X-ray diffraction (SAXS) experiment [20, 21], but now scaled down to a sample volume corresponding to a specific part of a cell. Nano-diffraction of biological cells will thus result in a 2D image with a resolution determined by the focal size, with each pixel encoding different observables, in particular...
diffraction observables. This concept of scanning SAXS has been introduced first on the micron scale, with seminal work in the field of biological materials by Fratzl and co-workers [22–25] (see also [26,27]), and can now with further progress in X-ray focusing be easily extended to the nano-scale. The pixel of the real space image can encode: phase shift (i.e. projected density), ion concentration from fluorescence yield, small-angle scattering intensity extracted from the far-field pattern (darkfield), or other structural parameters derived from the full 2D diffraction pattern, e.g. fibre bundle orientation and spacing. Beyond a simple contrast value, for each pixel a fully quantitative diffraction pattern is available at much higher signal-to-noise than typically in CDI, since oversampling and coherence constraints can be relaxed. In contrast to electron microscopy, no sectioning of the cells is necessary for X-ray imaging.

After this introduction, the experimental details are presented, including a brief account of sample preparation, the used instrumentation at the beamline, as well as the experimental parameters of ptychographic imaging, tomography, including details on dose estimation, and cellular nano-diffraction. Next, results are presented, first for ptychography obtained from a test pattern to retrieve the probe function followed by the cellular samples, then the cellular tomography, and finally the nano-diffraction data. The paper closes with some conclusions and a brief outlook.

2. Experiments

2.1. Sample preparation

Cells of the Deinococcus radiodurans wild-type strain were cultivated from freeze-dried cultures (DSM No. 20539 by the German Collection of Microorganisms and Cell Cultures) for one day at 30°C on petri dishes covered with nutrient medium (corynebacterium agar: 10 g/l casein peptone, 5 g/l yeast extract, 5 g/l glucose, 5 g/l NaCl, 15 g/l agar). Prior to preparation the actively growing cells were washed off the culturing medium with ca. 1.5 ml buffer solution (2 g/l KH₂PO₄, 0.36 g/l Na₂HPO₄·2H₂O, pH 7.2). After placing a droplet of cell suspension onto the substrate, a polyimide foil of 12.5 µm thickness (diffraction experiment) and Si₃N₄ membranes of 0.5 µm thickness (PCDI experiment), the cells were allowed to adhere for 60 seconds. The remaining buffer was blotted, the foil mounted on top of a solid stainless steel rod cryo-plunged into liquid ethane to prevent crystallization [28] and afterwards lyophilized in a home-built freeze-drier.

2.2. Beamline and instrumentation

The experiment has been carried out at the coherent Small Angle X-ray Scattering beamline (cSAXS) of the Swiss Light Source (SLS) at the Paul Scherrer Institut in Villigen, Switzerland. Starting from the undulator source consisting of Nₑ = 96 magnet periods with undulator period 19 mm, the beam was defined by the following optical elements, as indicated schematically on Fig. 1: (i) horizontal slit S₀ at 12.1 m distance from source, (ii) horizontal and vertical slits S₁ at 26 m, (iii) a Si(111) double crystal monochromator at 28.6 m (2nd crystal). The first crystal was LN₂-cooled. The monochromator was set to a photon energy of 6.2 keV. Following (iv) further slit systems S₂, S₃, and S₄ as well as the fast shutter system (FS) for detector acquisition, the beam impinges on a Fresnel zone plate (FZP) with preceding central stop (CS). We used a a highly efficient FZP fabricated of Au on a Si₃N₄ membrane using 100 keV e-beam lithography and electroplating [29,30] with a diameter of φ = 200 µm, zone height of 1 µm and outermost zone width dr = 100 nm. The FZP was located at ∼34 m from the source and at a distance of ∼0.1 m to the sample. An order sorting aperture (OSA) with a diameter of φ = 20 µm was placed between FZP and sample. The sample was positioned by a high precision piezo stage (Physik Instrumente, Germany) mounted on an air bearing rotation stage (Micos UPR160F) on top of a hexapod (PI M-850).
The first part of the experiment was optimised for ptychography: The coherent slit setting of S0 (gap width 10 µm) was chosen. In addition, a long distance between sample and detection plane is favoured for sufficient sampling of the recorded diffraction pattern (fulfilment of the oversampling condition). Therefore, the zero read-out noise photon counting detector PILATUS 2M (SLS detector group, [31]) was placed at the long working distance of \( z_{12} \approx 7.22 \text{m} \) (ptychographic imaging of resolution chart) behind the sample and a helium-filled flight tube of length 7 m was used. Ptychographic imaging results of \textit{D. radiodurans} have been obtained during a second experiment at cSAXS using the same experimental setup. However, the detector was placed at \( z_{12} \approx 7.65 \text{m} \) (see [32] for distance callibration) with an air path of \( \approx 0.4 \text{m} \) between the flight tube and the PILATUS. A cryojet, beamstops and filters were not used for ptychographic imaging.

The second part of the experiment was optimised for diffraction: a cryojet was used to cool the sample and the detector was placed \( z_{12} \approx 2.28 \text{m} \) behind the sample (according to a calibration using silver behenate), with 1475 \times 1679 pixels of size 172 µm \times 172 µm. A flight tube of length 2.063 m was used between sample and detector to reduce air-scattering of the diffracted X-ray beam. Beam damage of the detector was suppressed by using multiple beamstops between sample and detection plane and/or by using silicon filters of appropriate thickness. The coherence properties of the beam were altered by changing the gap of the horizontal slits S0. Importantly, increasing the gap yields a beam with lower spatial coherence, but more flux.

Fig. 1. Schematic of the experimental setup used for X-ray cellular nano-diffraction and PCDI accentuating the important parameters: 1. gap of the source slit S0, which controls the flux and coherence properties of the beam, 2. the distance \( Z_0 \) between the zone plate optics (FZP) and the sample, which defines the size of the beam at the cellular specimen, and 3. the detector distance \( Z_{12} \) with respect to the sample, which determines sampling of the recorded diffraction pattern and attainable diffraction angle. For different choices of these three parameters the biological specimen can then be translated in the \( XY \) – directions and additionally be rotated with respect to \( \phi \). In detail, the beamline setup consists of the X-ray undulator (period 19 mm) source (S), horizontal and vertical slits (S1-S4), Si(111) monochromator (LN2-cooled), fast shutter (FS), filter (Fi), central stop (CS), order sorting aperture (OSA) and focus (F) with bacterial specimen. Flight tube and beamstops are not shown.

2.3. Ptychography

Before going into the experimental details of the ptychographic data, we will give a very brief formulation of the underlying theory (see also [33, 34]). Assuming that the exit surface wave...
\(\Psi_j(x)\) (ESW) directly behind the sample can be formulated as a product of the illuminating wave field \(P(\vec{x} - \vec{x}_j)\) and the complex transmission of the specimen \(S(\vec{x})\), a Fourier transform relation holds between the ESW and the intensity \(I_j\) at a Fraunhofer or farfield detection plane

\[
I_j = |\mathcal{F}\{\Psi_j(x)\}|^2 = |\mathcal{F}\{P(\vec{x} - \vec{x}_j)S(\vec{x})\}|^2,
\]

where \(\vec{x}, \vec{x}_j \in \mathbb{R}^2\) are vectors in direct space of the plane of the sample. The index \(j\) indicates a lateral shift of the probe function \(P\). In the process of measuring the diffraction signal, only the intensity of the diffracted beam can be recorded, thus preventing a direct inversion of Eq. (1) under general conditions. However, the lost phases can be recovered through iterative reconstruction if the problem fulfills the oversampling condition [35] and is constrained by the experimental conditions. In this experiment, a set of diffraction patterns was collected in an iterative reconstruction scheme for Eq. (1) to hold: These are experimental uncertainties such as positioning errors due to the limited experimental accuracy, drifts and vibrations, noise in the recorded data, unusable areas of the detector, a limited stationarity of the probe and partial spatial coherence. In particular, phasing of weakly scattering specimens such as bacterial cells is a challenging task [1, 36].

We made use of two different ptychographic algorithms as described in [33] (and references therein) and [34], respectively. We refer to the former algorithm as ‘DM’ (difference-map) and to the latter as ‘ePIE’ throughout the paper.

Following the strategy of [36], we used a Siemens star resolution test chart (model ATN/XRESO-50HC, NTT-AT, Japan) in order to get a good estimate of the probe, which could be used as an initial guess for the phasing of the bacterial specimen. The sample was placed into a defocus position of \(\sim 1\) mm. 323 diffraction patterns have been measured using a scan pattern with scan points on concentric circles (cf. Fig. 2(a)), a dwell time of 0.2 s or equivalently, an exposure time of \(\sim 1\) min in total. The distance between adjacent concentric circles was 0.5 \(\mu m\) in comparison to \(\sim 0.75\mu m \times 1.5\mu m\) FWHM values of probe intensity, \(h \times v\) respectively. A subset of 800 \(\times\) 800 pixels of the recorded whole diffraction patterns (cf. Fig. 1) was used for phasing (\(\sim 10\) nm real space pixel size). Dead or hot pixels of the detector were masked and their intensity values were defined by the current iterate in the reconstruction scheme. Moreover, the ptychographic reconstruction algorithm (‘ePIE’ algorithm) was initiated using the complex field of a 1 mm forward propagated, circular aperture with uniform amplitude and constant phase as probe function and uniform amplitude with constant phase as object guess. Additionally, the amplitude of the object was clipped onto the interval \([0.7, 1]\) during each iteration. Note, that the interval has been chosen close to the theoretical expectation. The final reconstruction (cf. Fig. 2(a)) was obtained after 320 iterations by averaging over the last 200 iterations with an average interval of 2. Here, we found superior results using the ‘ePIE’ algorithm with parameters \(\alpha = \beta = 1/2\) (cf. Eq. (6), (7) in [34]).

For the interpretation of the results we made use of the ‘phase retrieval transfer function’ (PRTF), which indicates the goodness of the reconstruction in comparison to the measured intensities (cf. [1, 17, 38], Fig. 3). Best results were obtained through the averaging procedure over many iterations and thereby cancelling randomly fluctuating phase contributions. There-
Therefore, the closest estimate for the measured intensities will be that corresponding to the exit surface waves \( \langle \Psi_j(\vec{x}) \rangle_{\text{it}} \) of the average over iterates. An overall estimate of the full ptychographic reconstruction can be achieved by averaging over all probe positions (corresponding to measured diffraction patterns \( j \)). In addition, we performed an azimuthal average. We thus use the PRTF as defined by:

\[
PRTF(|\vec{q}|) = \frac{\langle \langle \sqrt{f_{PCDI}} \rangle_j \rangle_{\varphi}}{\langle \langle \sqrt{f_{\text{meas}}} \rangle_j \rangle_{\varphi}} = \frac{\langle \langle |\mathcal{F}\{\langle \Psi_j(\vec{x}) \rangle_{\text{it}}\}| \rangle_j \rangle_{\varphi}}{\langle \langle |\mathcal{F}\{\langle \Psi_j(\vec{x}) \rangle_{\text{it}}\}| \rangle_j \rangle_{\varphi}}.
\]

The phase reconstructions of \( D. \ radiodurans \) (cf. Fig. 4) were obtained using the ‘DM’ algorithm for the simple reason that it can be parallelized. Here, both algorithms yield reconstructions of comparable quality, as judged from single projections. Two different regions with bacteria have been studied, Fig. 4(a) and Figs. 4(b)–4(d) respectively. The samples were measured in the focal region (probe size: \( \sim 0.5 \mu m \times 0.4 \mu m \) full width at half maximum (FWHM) values of probe intensity, \( h \times v \) respectively; see Table 1 for scan details).

We recorded 77 ptychographic datasets for a tomographic reconstruction on the cellular sample seen in Fig. 4(b). The angular increment between successive projections is \( \Delta \Phi = 2^\circ \), leaving a missing-wedge of \( \pm 13^\circ \). In each projection a dwell time of 0.2 s was used. The grid spacing between adjacent concentric circles was 0.35 \( \mu m \). Three projections could not be reconstructed and have been omitted for the tomographic reconstruction. To quantify the errors resulting from the missing data, we have simulated the reconstruction based on a test phantom with parameters comparable to our experimental situation. An upper limit of 10% error in the reconstructed density values was found. These errors were dominated by the missing-wedge, while the three missing projections were found to have a negligible effect.

A subset of 192 \( \times \) 192 pixels of each PILATUS image corresponding to a real space pixel size of 46 nm has been chosen for all reconstructions. Importantly, the ptychographic reconstructions were initiated with the reconstructed illuminating function from the Siemens star as a probe guess and a uniform amplitude, constant phase object guess. In addition, we introduced a further constraint onto the data. We defined a rectangular support \( \chi \) of the visible cell cluster and during each iteration the phase outside the support was set close to zero using the following phase constraint:

\[
\mathcal{P}(O(\vec{x})) := \begin{cases} 
|O(\vec{x})| \cdot \exp(i \min(\text{Arg}(O(\vec{x})), 0)) & \forall \vec{x} \in \chi \\
O(\vec{x}) \cdot \exp(i \gamma \cdot \text{Arg}(O(\vec{x}))) & \forall \vec{x} \notin \chi
\end{cases},
\]

where \( \gamma \in \mathbb{R} \) was chosen close to zero. It should be noted that \( \mathcal{P}(O(\vec{x})) \) enforces a negative phase shift on the object transmission. However, this represents no restriction since one usually is interested in the relative phase shift between different materials. Moreover, the object transmission can be easily adapted to cover the full phase range by multiplying \( \mathcal{P}(O(\vec{x})) \) by \( \exp(i \pi) \). The final results were obtained by averaging over 500 iterations during the so-called steady state of the ‘DM’ algorithm [33].

### 2.4. Tomography

At first, the ptychographically reconstructed projections (PCDI parameters for 2D reconstructions as above) have been aligned with respect to the axis of rotation using a method described in [39]. Afterwards, the 3D tomographic reconstruction was done by applying the filtered back-projection algorithm using the standard ‘Ram-Lak’ or ramp filter [40]. Visualisation was done with ‘Avizo’-Software.
the cell material is protein of the empirical formula H50C30N9O10S1 of density 

Afterwards, the scan

In a first step diffraction on

2.6. Cellular diffraction

We used the reconstructed Probe \( P \) from the ptychographic reconstruction to investigate the applied surface dose \( D \). In a first step we calculated the 2D-photon density \( \rho_P(\vec{x}) \) by scaling the normalized intensity of the probe at each scan position \( j \) to the actual photon number \( N_j \):

\[
\rho_P(\vec{x}) = \sum_j N_j |P(\vec{x} - \vec{x}_j)|^2 / \int_{R^2} dS |P(\vec{x})|^2.
\]

Given the characteristic function \( \chi(\vec{x}) \) of a certain part of the cell area of the scan, the photon energy \( E \), the mass density of the cell material \( \rho_c \) and the absorption coefficient \( \mu \), one obtains the applied surface dose \( D \) [41] for this region:

\[
D = \frac{\mu E}{\rho_c} \cdot \frac{\int_{R^2} dS \cdot \chi(\vec{x}) \cdot \rho_P(\vec{x})}{\int_{R^2} dS \cdot \chi(\vec{x})},
\]  

(4)

All dose calculations in this work are based on the assumption that the main consistency of the cell material is protein of the empirical formula H50C30N9O10S1 of density \( \rho_c = 1.35 \, \text{g/cm}^3 \) [41].

2.6. Cellular diffraction

In a first step diffraction on \( D. \text{radiodurans} \) data was taken by translating the object in the XY-plane of the focal region using a coherent slit setting of S0 on the sample region A of Fig. 5 (see Table 2 for a summary on scan details). The performed mesh scans differ in dwell time \( \Delta T \), step size \( \Delta x \), between adjacent scan points and field of view (FOV). The data set A1 was recorded without a beamstop but with a Si filter of 50 \( \mu \text{m} \) thickness. A2, A3, A4 were carried out with a beamstop and no filters. The two diffraction patterns of A4 were taken on a cell region and on an empty region of the polyimide foil, respectively.

Secondly, more diffraction data was taken with the same settings on the sample region B (cf. Fig. 5, Table 2). However, a rather incoherent beam was used by opening the slits S0 to a width of \( \Delta = 0.42 \, \text{mm} \). Two beamstops were used during recording of the datasets B1 and B3. Afterwards, the scan B2 was performed in the coherent setting without any beamstop but with a Si filter of 50 \( \mu \text{m} \) thickness.

For each mesh scan four contrast values can be calculated from every single diffraction pattern: the integral signal (transmission), the ratio of the integral signals from a region defining the central beam and from its counter set (darkfield) and the relative centre of mass movement in X- and Y-direction (differential phase contrast, DPC-x and DPC-y respectively). The darkfield contrast is a measure for the scattering strength of the sample. In the case that attenuation effects in the sample are negligible and all photons behind the sample can be detected, it reflects the ratio of scattered photons to incident photons. As explained in [42–44], in addition to transmission and darkfield the differential phase contrast DPC-x and DPC-y reflects the gradient in averaged phase shift over the illuminated sample spot. Note that by blocking a part of the primary beam using a beamstop, none of these contrasts reflect quantitative measurements anymore. However, the tails of the primary beam can still be used for the analysis to obtain

<table>
<thead>
<tr>
<th>Description</th>
<th>( \Delta T ) [s]</th>
<th>( \Delta x ) [( \mu \text{m} )]</th>
<th>( F ) [photons/( \mu \text{m}^2 )]</th>
<th>( D ) [Gy]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fig. 4(a)</td>
<td>0.2</td>
<td>0.28</td>
<td>( 2.3 \cdot 10^9 )</td>
<td>( 4.9 \cdot 10^8 )</td>
</tr>
<tr>
<td>Fig. 4(b)</td>
<td>0.4</td>
<td>0.28</td>
<td>( 4.7 \cdot 10^9 )</td>
<td>( 9.9 \cdot 10^8 )</td>
</tr>
<tr>
<td>Fig. 4(c)</td>
<td>0.4</td>
<td>0.25</td>
<td>( 5.9 \cdot 10^9 )</td>
<td>( 1.3 \cdot 10^9 )</td>
</tr>
<tr>
<td>Fig. 4(d)</td>
<td>0.2</td>
<td>0.35</td>
<td>( 1.5 \cdot 10^9 )</td>
<td>( 2.4 \cdot 10^9 )</td>
</tr>
</tbody>
</table>
sufficient contrast. Here, only a subset of 194 × 194 pixels around the centre of the beam of each diffraction pattern has been used to obtain the different contrasts.

Next, we segmented the corresponding diffraction patterns according to the contrast (darkfield or DPC) into ‘cell and sample holder’ Σ₁ and ‘sample holder’ Σ₂ (cf. e.g. subgraphs in Fig. 6, 7). This was done either by thresholding the darkfield image or by thresholding a contrast image followed by further image processing. Diffraction curves $I(\vec{q})$ have been calculated for both sets of diffraction patterns by averaging over the set $\langle \cdot \rangle_{\Sigma_{i} \in 1,2}$ and azimuthally averaging $\langle \cdot \rangle_\phi$ with respect to constant radii. The difference signal

$$\Delta I(\vec{q}) = \langle (I_j(\vec{q}))_{\phi} \rangle_{\phi \in \Sigma_1} - \langle (I_j(\vec{q}))_{\phi} \rangle_{\phi \in \Sigma_2} \tag{5}$$

is the basis for our analysis. Here, the momentum transfer $|\vec{q}| = k \sin \alpha/2$ is used with $\alpha$ being the angle between optical axis and scattering vector, and $k$ denoting the wavenumber. Note, that this diffraction data significantly exceeds the $q$-range of the ptychographic analysis. Due to spurious OSA scattering, some sectors of the diffraction patterns had to be excluded. Dead and hot pixels have been masked. The absence of correlations between the scattering signal from the sample holder and the scattering of the cellular specimen justifies background subtraction similar to conventional (incoherent) diffraction. The fact that we have used both coherent and incoherent illumination settings with consistent results can be regarded as a validation of this assessment. We stress that background subtraction based on a pixel by pixel analysis of the sample (sample pixels versus background pixels) was found to be significantly more robust than background subtraction based on a second empty sample.

One may be concerned about the effect which a highly convergent beam has on small-angle X-ray scattering, since SAXS measurements usually require collimated beams. By placing a sample in the planar wave-front of the focal plane, the far-field pattern of the sample structure is measured. More precisely, the measured far-field pattern corresponds to the Fourier transform of the sample convolved with the Fourier transform of the probe, which is a plane wave with for example a Gaussian envelope in the focal plane. Since for cellular nano-diffraction we are by definition interested in diffraction from structures, which are smaller than the beam size, one realizes that the convolution of the far-field pattern with the Fourier transform of the probe has only a negligible effect, if the structure size is much smaller than the beam size, which is the case in the present work. Furthermore, one may even argue that it will be easier to control the wave-front flatness of a coherent nano-beam by ptychography than the collimated (stochastic) wave-fronts of macroscopic SAXS measurements.

<table>
<thead>
<tr>
<th>region A</th>
<th>$\Delta T$[s]</th>
<th>$\Delta_{xy}$[(\mu m)]</th>
<th>$N_x \times N_y$</th>
<th>Si[(\mu m)]</th>
<th>S0[(\mu m)]</th>
<th>$\nu$</th>
<th>$D$ [Gy]</th>
<th>$q_t$ [1/(\mu m)]</th>
</tr>
</thead>
<tbody>
<tr>
<td>$A_1$</td>
<td>0.1</td>
<td>0.1</td>
<td>161 × 191</td>
<td>50</td>
<td>10</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>$A_2$</td>
<td>1</td>
<td>0.2</td>
<td>41 × 41</td>
<td>BS</td>
<td>10</td>
<td>-3.2</td>
<td>3.6 · 10^7</td>
<td>0.75</td>
</tr>
<tr>
<td>$A_3$</td>
<td>10</td>
<td>0.8</td>
<td>11 × 11</td>
<td>BS</td>
<td>10</td>
<td>-3.7</td>
<td>2.2 · 10^7</td>
<td>0.95</td>
</tr>
<tr>
<td>$A_4$</td>
<td>100</td>
<td>200</td>
<td>2 × 1</td>
<td>BS</td>
<td>10</td>
<td>-3.6</td>
<td>1.3 · 10^8</td>
<td>1.88</td>
</tr>
<tr>
<td>region B</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$B_1$</td>
<td>0.1</td>
<td>0.2</td>
<td>61 × 61</td>
<td>BS</td>
<td>420</td>
<td>-3.5</td>
<td>5.5 · 10^7</td>
<td>2.30</td>
</tr>
<tr>
<td>$B_2$</td>
<td>0.1</td>
<td>0.2</td>
<td>61 × 61</td>
<td>50</td>
<td>10</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>$B_3$</td>
<td>100</td>
<td>200</td>
<td>2 × 1</td>
<td>BS</td>
<td>420</td>
<td>-3.8</td>
<td>2 · 10^8</td>
<td>1.89</td>
</tr>
</tbody>
</table>

Table 2. Overview of diffraction data sets according to dwell time $\Delta T$, step size $\Delta_{xy}$ and number of scan points $N_x \times N_y$ within the rectangular mesh. In addition, Si filter thickness (BS denotes usage of beamstops instead of Si filter) and nominal gap of slits S0 are shown as well as the results from diffraction analysis for the exponent of the diffraction curves $\nu$, highest scattering vector $q_t$ and dose estimations $D$. 

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3. Results

3.1. Ptychographic imaging of the resolution chart and probe retrieval

The ptychographic reconstruction of the 500 nm thick layer of tantalum suitably matches the corresponding theoretical phase shift of \( \Delta \Phi(E = 6.2 \text{keV}) = -0.34 \pi \) and transmission of \( I/I_0 = 0.77 \) (not shown). Its innermost part consisting of 50 nm structures is clearly resolved (cf. Fig. 2). The ‘overall’-PRTF (cf. Eq. (2)) in Fig. 3(a) (green curve) shows signal at higher frequencies beyond the black dash-dotted line of \( v = 34 \mu \text{m}^{-1} \) or equivalently of \( dx = 15 \text{nm} \) half-period length. In addition, we calculated the PRTF according to an average over a single scan point (red curve). Note that the contribution of the probe to the diffraction patterns is confined to a small circular area up to \( v_{\text{max}} \approx 5 \mu \text{m}^{-1} \). The disc-like image of the FZP and OSA can be clearly distinguished from the contribution of the Siemens star at high frequencies. We thus attribute the resolution to the sample and not the probe. Let us briefly consider the imaging regime of the diffraction patterns. Using a Gaussian beam model, the radius of curvature of the probe \( R(z) = z(1 + (z/z_0)^2) \), \( z_0 \) being the Rayleigh range, can be approximated by \( R(z) \approx z \) at a distance of ca. 1 mm to the focus. Estimating the probe diameter to be 1 – 3 \( \mu \text{m} \) one thus obtains Fresnel numbers \( F \) in the range of \( 4 \leq F \leq 45 \). Note that in this regime one may rather speak of ptychographic Fresnel coherent diffractive imaging (cf. [10, 45]). The relationship between the resolution and the highest angle at which signal is recorded may thus not be applicable due to the phase curvature of the illuminating probe (cf. Fig. 2(b)). As an alternative, we make use of the power spectral density (PSD) and line scans at the sharp edges to determine the resolution (see appendix for details of calculations of the PSD). A fit of the vertical and horizontal edges to an error function yields line-spread functions with FWHM-values of 16 nm \( (v) \) and 17 nm \( (h) \), respectively. In addition, the PSD of the reconstructed phase (cf. Fig. 3(b)) supports the conclusion that features in the range corresponding to \( 15 - 10 \text{nm} \) half-period length are resolved.

The reconstruction of the probe can be used to analyse the complex field of the focus [46–48]. Consider the following operation on the complex field of the probe \( P \) (‘sharpness’, cf. [49])

\[
\Upsilon(\Delta z) := \int_{\mathbb{R}^2} dS |\mathfrak{F}\{P(\vec{x}, \Delta z)\}|^4, \tag{6}
\]

where \( \mathfrak{F}\{\cdot, \Delta z\} \) denotes the free space back propagation by \( \Delta z \). We have determined the points of highest focusing by locating two maxima of \( \Upsilon(\Delta z) \). The numerical back propagation of the complex wave field thus reveals an astigmatism in the focal region (cf. Media 1). The difference of propagation distances yields a distance of \( \Delta F_{\text{bw}} \approx 0.7 \text{mm} \) between the two orthogonal directions of highest focusing. The field along the optical axis is displayed as planes of constant x and y through the centre (cf. Fig. 2(b)). A Gaussian fit to the regions of highest focusing yields FWHM-values of 89 nm in the vertical direction and 93 nm in the horizontal direction.

3.2. Ptychographic imaging of Deinococcus radiodurans

The results of the ptychographic reconstructions of freeze-dried \( D. \text{radiodurans} \) are presented in Fig. 4. In Fig. 4(a) the typical tetrad form of \( D. \text{radiodurans} \) can be seen. Extending previous results [1], we can clearly identify disc-like regions exhibiting a large phase shift up to 0.4 rad. Given their size and shape we attribute them to the bacterial nucleoid and DNA.

There are features in the reconstructions visible within sizes of one to two pixels indicating a half period length resolution in the range of the pixel dimension of \( dx = 46 \text{nm} \). The PRTF reflects a good reconstruction up to the highest frequencies. In comparison to the PRTF of the Siemens star (cf. Fig. 3(a)), we observe a lower trend in the high frequency region of the reconstruction of Fig. 4(b). The PSD (blue curve, Fig. 3(b)) of the reconstruction in Fig. 4(b)
Fig. 2. (a) Phase from ‘ePIE’ reconstruction of the tantalum test structure. Scan points are indicated as magenta points. A fit of the error function to one of the edges of the line scans yields a line-spread function with FWHM-values of 16 nm in the vertical direction and 17 nm in the horizontal direction. The inner part of (a) is shown in (c). (b) Complex representation of reconstructed probe at the plane of the sample (top) and horizontal and vertical line scans through the centre of the back-propagated probe (bottom) (cf. Media 1). The white dashed lines highlight the position of the different focal positions. (d) (top) Gaussian fits (solid red lines) to the peaks of the line scans of the probe intensity are depicted below together with the data from the line scans (black dots). We obtain FWHM-values of 87 nm in the vertical direction, and 93 nm in the horizontal direction. (d) A typical diffraction pattern of the PCDI dataset (log-scale) is shown in the lower part.
Fig. 3. The PRTFs of the ptychographic reconstruction of the resolution test chart and of a single projection of cells (Fig. 4(b)) are depicted in (a), green curve and blue curve respectively. In addition, the PRTF of the test sample according to an average over a single scan point is shown as red curve (cf. Eq. (2)). The high frequency ranges of the azimuthally averaged power spectral densities (PSD) of the reconstructed phase distributions are displayed in (b). Real space half-periods of 50nm, 25nm and 15nm are indicated as dashed, solid and dashed-dotted black lines, respectively. Legend of (b) is legend in (a).

clearly shows structures up to $dx = 50$nm (half period length). The cutoff is very small and can be better seen in the 2D PSD (see appendix) indicating a resolution in the range of $dx = 50 - 55$nm.

The tomographic reconstruction Fig. 4(d) reveals the 3D structure of bacterial cells seen in (Fig. 4(b)). A video is provided in the supplementary material (cf. Media 2). We can identify the 3D distribution of the high-density regions (red). Assuming the ratio of the mass number and the atomic number on average over a voxel to be nearly constant, the 3D phase distribution $\phi(\vec{r})$ can be used to estimate the effective mass density of the cell [1]

$$\rho_c(\vec{r}) \approx - \frac{2u}{\lambda r_0} \phi(\vec{r}),$$

where $u$ denotes atomic mass unit, $r_0$ the classical electron radius and $\lambda$ the wavelength. For cell material of $\text{H}_50\text{C}_{30}\text{N}_9\text{O}_{10}\text{S}_1$ the error is less than 10% [1]. We used the tomographic reconstruction to estimate the mass density of the high-density regions. With the help of software tools for analysing tomographic data, we defined a sub-volume which can be seen as a magenta label in the lower part of the tomogram (front-view, Fig. 4(d)). The average over the sub-volume yields $<\rho_c> \approx 1.6\text{g/cm}^3$.

The results show that ptychographic tomography is a suitable approach not only for strongly scattering samples (e.g. mineralized tissue [14] and stained bacteria [15]) but also for weakly scattering specimens such as freeze-dried cells. Note that ptychography from unmineralized tissues and in particular cells is limited by the signal to noise ratio. Thus weakly scattering samples are much more difficult to reconstruct than for example mineralized tissues such as bone or more generally solid state samples.

In comparison to the reconstruction of the single projection in Fig. 4(b) which has been taken before the tomographic dataset, the quality of the tomographic reconstruction is affected by the overall consistence of all ptychographic reconstructions. The inner structure slightly deviates from the first phase map indicating a moderate radiation induced change of the inner structure. The applied dose for the tomographic dataset was estimated to be $\sim 2.4 \cdot 10^8\text{Gy}$. Another tomographic dataset has been taken after the first one in order to achieve an angular increment of $\Delta \Phi = 1^\circ$. However, the combined tomogram (not shown) shows less contrast in the high-density regions and was thus discarded. The image in Fig. 4(c) has been recorded after an integral dose of $>5 \cdot 10^8\text{Gy}$ and clearly shows radiation induced structure changes in
comparison to its initial state in Fig. 4(b). Note, that the structural changes may also have an effect on $<\rho_c>$. Our dose estimations are summarized in Table 1 (see also Fig. 8). We thus conclude that in contrast to single projections, medium and high resolution tomography will necessitate cryogenic sample conditions.

3.3. Diffraction data of Deinococcus radiodurans

The darkfield and differential phase contrast of the scanned regions A and B clearly reveal the outline and some internal structure of the cells in the scanned FOV (see Fig. 5). The images of the sample regions A and B show typical shapes of $D.\ radiodurans$ aggregates, as known from light and electron microscopy [2, 3, 50] (and our previous ptychographic study [1]). Importantly, scanning diffraction with the four observables transmission, phase shift DPC-x, DPC-y and darkfield allow for fast, robust visualisation of biological cells without staining and with the additional benefit of a large FOV that can be easily adapted. The images have been recorded with samples kept in the cryojet, demonstrating that cryo samples can be made compatible with the vibration requirement needed for nano-beam scanning diffraction. In case of the coherent datasets ptychography yields an accurate picture of the actual illuminating wave field. To this end, we have propagated the reconstructed wave field $P(\vec{x})$ (Fig. 2(b)) along the optical axis back to the position of this sample. We stress the advantage of combining scanning diffraction images with ptychographic phase retrieval, since the probe and hence the resolution can be exactly quantified, presenting a significant advantage over conventional knife edge measurements (see appendix for detailed discussion). This part is illustrated by the overlay in Fig. 5(d). The size of the beam at the sample matches the visual impression of the resolution. In addition, the reconstructed probe can be used to find the position of the focus in the beam. Moreover, the knowledge of the probe can be used to simulate experimental images and to design an experiment (defocus, step sizes, convolution effects and dose expectations) as shown in part C of Fig. 5. Here, the effect of a double peak in the illumination function on the differential phase contrast can be seen, which is not seen in simulated knife-edge scans (cf. appendix).

Next we turn to the diffraction data in q-space, which can give complementary information to the ptychographic reconstruction, in particular since a larger q-range can be exploited. At the same time, the ptychographic reconstruction helps to ‘locate’ the source of the diffraction signal. Since each point of the images (differential phase contrast and darkfield) corresponds to a single diffraction pattern we can segment the image into pixels with cells and without cells, representing the empty sample holder (see insets in Fig. 6, 7). By making use of the whole sets of collected diffraction data we are able to discriminate diffraction signal of cellular material from diffraction signal of the polyimide foil, representing the background signal. The two diffraction intensities (averaged over the number of corresponding pixels) are displayed as a function of $q$ after azimuthal averaging, together with the difference signal, which can be interpreted as the average background subtracted diffraction signal of the bacterial cells. We find that these diffraction curves can be well described by a power-law behaviour $y = a \cdot q^\nu + c$ in the high q-region (Fig. 6, 7). All exponents $\nu$ lie between values of $-3$ and $-4$. Importantly, this is also true for the diffraction curves that have been obtained at singular positions ($A_4, B_3$, Table 2). One might expect the diffraction signal to be quite different at a single position of the cell in comparison to the case where the diffraction signal is averaged over a whole cell or a bunch of cells. However, for the case of a bacterial cell, without the pronounced organelles typical for eukaryotic cells, our results show that this is not really the case. In other words, the structure leading to the observed power law decay originates at length scales much smaller than the beam size used here ($\sim 508\text{nm} \times 538\text{nm}$ FWHM values of probe intensity $h \times v$). Thus a single shot represents a spatial average very similar to that of the entire cells. The fact that the functional form of the diffraction signal follows a power law with an exponent $-4 \leq \nu \leq
Fig. 4. (a)-(c) Phase reconstructions of *D. radiodurans* (‘DM’ - algorithm) showing dark areas, which we attribute to DNA rich regions. (d) 3D visualisation of the tomographic reconstruction of the set of projection images such as seen in (b) (cf. Media 2). The tomographic reconstruction is shown at three different angles. A magenta label in the tomogram (front-view) depicts voxel-regions which have been used to estimate the mass density $\rho_c$ in the high-density regions of the bacterial specimen (red). We obtained $\langle \rho_c \rangle \approx 1.6\,\text{g/cm}^3$. (a), (b) same colorbar as in (c).
−3 has been observed for the power spectral density of a ptychographic reconstruction of *D. radiodurans* [1] and is indicative of a self-affine distribution of scattering length density. Note that structures composed of compact regions with sharp interfaces would exhibit a power law with ν = −4, corresponding to the well known Porod law. We used the least-square fits shown in Fig. 6 to determine the cutoff \( q^\dagger \) where the diffraction signal reaches a constant white noise level. This cutoff can be defined as the resolution of the diffraction experiment. In the following it can be used to compare the influence of (a) counting time and (b) slit settings on \( q^\dagger \). In explicit terms \( q^\dagger \) is the intersection of the power-law decay \( a \cdot q^\nu \) and the constant background \( c \)

\[
q^\dagger := \left( \frac{c}{a} \right)^{1/\nu}. \tag{8}
\]

The upper graph in Fig. 6 shows the background corrected cellular diffraction signal as measured for \( \Delta T = 1 \) s and \( \Delta T = 10 \) s, leading to \( q^\dagger = 0.75 \text{ nm}^{-1} \) and \( q^\dagger = 0.95 \text{ nm}^{-1} \), respectively. This is in agreement to experimental expectation. The \( \Delta T = 100 \) s data set recorded at a single spot, displayed in the lower graph of Fig. 6 shows an even stronger signal, extending to \( q^\dagger = 1.88 \text{ nm}^{-1} \). This demonstrates that cellular structure can be analysed by diffraction at resolutions (in rec. space) which are currently not accessible by X-ray microscopy, either in the conventional form or the more recent Coherent Diffractive Imaging (CDI). The data shown in Fig. 6 also illustrates the difficulties associated with spurious scattering by the OSA, which is difficult to suppress due to thermal effects induced by the cryojet.

Finally we note, that at small \( q \) the \( A_4 \) data set shows a transition of the power law decay to a plateau (not fully reached due to the BS). This behaviour was captured by adapting the fitting function to \( I = a \cdot q^\nu(1 - \exp((-d \cdot q/2\pi)^2)) + c \), with \( d \) denoting the crossover. The resulting value of \( d \approx 60 \text{ nm} \) gives a typical length scale of the largest structure.

Next, we would like to know which effect opening the slit \( S_0 \) and the associated relaxation of coherence has on the resolution. Note that the high flux does not necessarily have to result in higher \( q^\dagger \) due to a possible decrease in signal to noise. However, the results shown in Fig. 7 of the \( B_1 \) data set, show \( q^\dagger = 2.3 \text{ nm}^{-1} \) which is indeed the highest value obtained. Furthermore, it seems that dose fractionation over the entire aggregate of cells presents an advantage over a single point long measurement where we see a moderate increase of resolution (in \( q \)-space), at least for detectors which are free of read-out noise.

We present the results of the dose estimations in Fig. 8, as described in the method section (cf. Eq. (4)). Note that our dose estimations are spatially more precise than a simpler estimation based on an average fluence, which can be best seen in Fig. 8(b). After all, a comparison of the attainable resolution in diffraction settings always should be accompanied by a corresponding comparison in dose, in view of the important issue of radiation damage in biological specimens. In case of the sample region \( A \) (coherent setting) the average photon flux was measured with the PILATUS to be \( \sim 7 \cdot 10^8 \text{ photons/s} \). Assuming the same photon flux at each scan position we obtain applied doses of \( 3.6 \cdot 10^7 \text{ Gy} \), \( 2.2 \cdot 10^7 \text{ Gy} \) and \( 1.3 \cdot 10^8 \text{ Gy} \) for \( A_2, A_3 \) and \( A_4 \), respectively. In case of the incoherent setting of sample region \( B \) we measured the increase in flux to be in the range of a factor of \( 10 - 17 \) due to the increased gap of the slit \( S_0 \). Therefore, we assumed an average photon flux of \( \sim 10^{10} \text{ photons/s} \) yielding applied doses of \( 5.5 \cdot 10^7 \text{ Gy} \) and \( 2 \cdot 10^8 \text{ Gy} \) in case of the incoherent datasets \( B_1 \) and \( B_3 \), respectively. The results are summarized in Table 2.

### 4. Conclusion, discussion, and outlook

In summary, we have carried out an X-ray structural analysis on bacterial cells of *D. radiodurans*, combining coherent imaging and cellular nano-diffraction. For this study, the bacterial cells were vitrified by plunge freezing followed by freeze-drying. However, in order to minimize radiation damage, samples were at least for experiments with highest flux (incoherent...
Fig. 5. **Sample region A - coherent setting**: differential phase contrast (a), (c) and darkfield (b), (d) of cells are shown for the mesh scans $A_1$ (top) and $A_2$ (bottom) - (cf. Table 2). The colorbar in (c) denotes the movement of the first moment whereas in (d) the grey scale denotes the ratio of scattered to unscattered photons, $n_s$ and $n_0$ respectively. The intensity of the beam from the ptychographic reconstruction of the Siemens star (cf. Fig. 2) after back propagation of 1 mm into its focus is visualized on top of the darkfield (d). Note, that the propagation of 1 mm corresponds to the lateral displacement of the sample between the two different measurements ($\pm 100$ µm due to an uncertainty of the position on the sample holder with respect to the curvature of the polyimide foil). The centre of the reconstructed beam is chosen to be at the cell-position of $A_4$ (cf. Fig. 6). **Sample region B - incoherent setting**: a section of the scan $B_1$ is shown as darkfield (f) and differential phase contrast (e). The position of the cell diffraction of $B_3$ is indicated on top of the incoherent darkfield as red circle (f). **Sample region B - coherent setting**: darkfield (h) and phase contrast (g) of a corresponding coherent mesh scan $B_2$ are shown. **Numerical simulations C**: synthetic cell sample (top) and calculated differential phase contrast images (below) according to Eq. (1) using the back propagated probe (cf. Fig. 2) at different positions on the optical axis. The distance of propagation is displayed and the corresponding intensity of the probe is visualised as an overlay using the same colorbar as in (d).
Fig. 6. **Sample region A - coherent setting:** (top) Diffraction data of $A_2$ and $A_3$ (cf. Table 2). The sets of diffraction patterns were segmented into areas with $\Sigma_1$ and without cell signal $\Sigma_2$ using the differential phase contrast image of $A_2$ (see inset). The difference between the two classes of diffraction sets averaged according to their set size is depicted next to the legend in the top right corner for $A_2$. Diffraction signals were obtained by azimuthally averaging each diffraction pattern of a scan and thereafter averaging according to their segmentation. The segmented diffraction curves of $A_2$ are shown and the difference signals are presented for both scans in combination with their power-law fits. Exponents of $\nu = -3.2$ and $\nu = -3.7$ were obtained for $A_2$ and $A_3$, respectively. (Bottom) presents the results of $A_4$ (cf. Fig. 5(d)). The diffraction signals obtained after azimuthally averaging are shown in the graph. The difference between the diffraction data of the cell material and of the sample holder is depicted in the lower left corner. A slightly modified power-law yields an exponent of $\nu = -3.6$. In both graphs the points $q = q^\dagger \equiv (c/a)^{1/\nu}$ are marked.
Fig. 7. **Sample region B - incoherent setting**: incoherent diffraction curves were obtained from data $B_1$ and $B_3$ (cf. Table 2). The image segmentation of $B_1$ according to areas with cell signal $\Sigma_1$ and without cell signal $\Sigma_2$ is shown as an inset. Power-law fits yield exponents of $\nu = -3.5$ and $\nu = -3.8$ for $B_1$ and $B_3$, respectively. The points $q = q_1 \equiv (c/a)^{1/\nu}$ are marked for both curves.

Fig. 8. Photon surface densities $\rho_\gamma$ of diffraction datasets $A_2$ (a), $A_3$ (b), $B_1$ (c) and ptychographic dataset (Fig. 4(a)) (d). The location of bacterial cells is indicated by black lines. (a)-(c) same colorbar as in (d).
setting) kept at cryogenic temperatures by use of a cryogenic \( N_2 \) jet. This shows that the experimental scheme is compatible with future extension to frozen hydrated samples with near optimum structure preservation.

At first, let us summarize the ptychographic results. The reconstructions yielded the projected phase (2D images) at a resolution in the range of 50nm for \( D. \text{ radiodurans} \) and 10nm for the tantalum test pattern. In the future, reducing the distance between focus and sample, as well as switching to Fresnel zone plates with higher resolution will maximize the fluence on the sample and improve the sampling in the detection plane. A first step towards ptychographic tomography of unstained biological cells was also presented. Imposing an additional phase constraint into the reconstruction scheme was found to be helpful. No additional phase unwrapping was necessary, due to the low phase shift of unstained cellular specimens. However, the tomogram shows that globular dense regions of ca. \( 1.6 \text{ g/cm}^3 \) in the bacteria exist with approx. isotropic shape. We attribute this pronounced feature observed in density contrast to DNA rich regions. In contrast to the 2D case, the resolution of the ptychographic tomogram was limited due to radiation induced damage. The next step must thus be ptychographic imaging of frozen-hydrated samples both in 2D and 3D.

Secondly, we have shown how the cellular diffraction signal can be extracted and analysed. Furthermore, we have compared the cellular diffraction curves as a function of momentum transfer after azimuthal averaging, for different parameters of the illuminating beam, notably flux and coherence. The observed curves could all be described by a power law decay with an exponent \( \nu \) in the range \( -4 \leq \nu \leq -3 \). Importantly, the cellular diffraction experiments open the analysis to maximum scattering vectors, up to a momentum transfer of \( q_1 \approx 2 \text{ nm}^{-1} \), which is to date unachievable by CDI. No indications for ordered structures associated with DNA compactification were observed in this range. Note, however, that the absence of peaks or modulations in the diffraction curves does not preclude ordered structures with signals below the dominating power-law decay.

In addition, we have shown how the ptychographically reconstructed probe can be directly used for enhanced control of the nano-diffraction experiment. Most importantly, the illuminated region of the sample can be well characterised. In particular, the size and shape of the beam does not have to be measured by knife-edge scans with the associated inaccuracies. Moreover, the exact complex-valued wave front is accessible, from which all beam parameters desired to better control the nano-diffraction experiment can be derived, such as wavefront curvature for example. Furthermore, the scanning X-ray microscopy images in phase contrast and darkfield contrast can be analysed with the full probe information at hand. Finally, we have shown how dose estimations become more precise by taking into account the exact beam profile, as well as the overlap between serial exposures during scanning.

However, we are also aware of the limitations in the presented approach. While the resolution can be extended by analysis in reciprocal space, this resolution applies to the averaged structure. The real space resolution of this averaging process as given by the focal spot is clearly lower in cellular nano-diffraction than in ptychographic imaging or more generally in CDI. At the same time, the overhead for detector read-out during scanning of three degrees of freedom needed for tomography limits the reasonable field of view, and hence in most cases the size of the object. Thus, for multicellular organisms, adaptable focal sizes and scanning steps would be desirable.

5. Appendix

5.1. Calculation of the PSD

The usage of a window function for the calculation of the PSD is common in digital signal processing. A very useful window is the so-called Kaiser-window \( w(x) \) (cf. [51]):
\[ w(x) = \begin{cases} I_0 \left( \beta \sqrt{1 - \left(\frac{2x}{L}\right)^2} \right) / I_0(\beta), & -L/2 \leq x \leq L/2 \\ 0, & |x| > L/2 \end{cases} \] (9)

where \( I_0 \) is the modified Bessel-function of the first kind, \( \beta \) is a free parameter and \( L \) is the window length. Note that one recovers the rectangular window for \( \beta = 0 \) which is intrinsically used for the calculation of the discrete Fourier transform of any discretised signal of finite length. The Kaiser-window with \( \beta > 0 \) helps to reveal the high frequency content of a signal that would be otherwise suppressed by noise or a huge amount of low frequencies in the data. We used the Kaiser-window for the calculation of the PSDs (Fig. 9).

![Fig. 9. (a) 2D PSD of ‘ePIE’ reconstruction of Siemens star test pattern. Note that the Siemens star structure can be clearly seen in the radial direction. (b) 2D PSD of ptychographic (‘DM’) reconstruction of \( D. \) radiodurans in Fig. 4(b). Scale bars correspond to spatial frequencies.](image)

5.2. Discussion knife-edge scanning

The complex-valued illumination field (probe) at the sample position is useful to design, analyse and to control nano-diffraction experiments. As in many other optical applications, beam properties (focus position, size, field of depth, aberrations) need to be known in order to perform controlled diffraction experiments, or more specifically nano-beam small-angle scattering experiments. Ptychographic reconstruction carried out in any plane gives access to the entire beam by means of propagation. Since beam properties can also be derived from measurements of caustics or multiple knife-edge scans, a brief comparison between the ptychographic approach and the conventional knife-edge approach shall be addressed here. To this end, we will assume that the pixel size of the probe intensities and the step size of knife-edge scans is equal to the pixel size of the ptychographically reconstructed probe of \( dx \approx 10nm \), in other words we do not question the practical implementation of knife edge scans at the 10nm range. In mathematical terms a knife-edge scan along \( x \) in a plane perpendicular to the optical axis spaced \( \Delta z \) from a reference plane (e.g. focus plane) is defined as

\[ K(x, \Delta z) = \int_{-\infty}^{x} dx' \int_{-\infty}^{\infty} dy \, |\mathbf{g}\{P\}(x', y, \Delta z)|^2, \] (10)
where \( \tilde{F}(P)(x, y, \Delta z) \) denotes the free-space propagation of the probe \( P \) by \( \Delta z \). Beforehand, it is important to note that a reconstruction of the full probe intensity \( |\tilde{F}(P)(x', y, \Delta z)|^2 \) or any function \( f(x, y) \) from \( K(x) \) is an analogous to 2D tomography. The derivative of \( K(x) \) with respect to \( x \) yields the line integral \( \Psi_{\alpha=0}(x) \) for a constant angle (here \( \alpha = 0 \)) which is known as Radon transform (see also [52])

\[
\frac{\partial}{\partial x} K(x) = \int_{-\infty}^{\infty} |\tilde{F}(P)(x, y, \Delta z)|^2 dy \equiv \Psi_{\alpha=0}(x).
\]

\( |\tilde{F}(P)(x, y, \Delta z)|^2 \) or any function \( f(x, y) \in \mathbb{R} \) can thus be reconstructed if the knife-edge scan is carried out for a sufficient number of orientations of the knife-edge. Since this is obviously not possible for practical reasons, conventional knife-edge scans in two orthogonal directions is based on the assumption that the probe intensity is separable in the \( x- \) and \( y- \) direction. With the popular assumption of a normal intensity distribution, the knife-edge integral is expressed in form of the error function

\[
|\tilde{F}(P)(x', y, \Delta z)|^2 = \exp\left(-\frac{(x-\mu)^2}{2\sigma^2}\right)/\left(\sigma\sqrt{2\pi}\right) \cdot f(y)
\]

\[
\Rightarrow K(x, \Delta z) = \frac{C_0}{2 \pi} \left(1 + \text{erf}\left(\frac{x-\mu}{2\sigma}\right)\right),
\]

where \( C_0 \) denotes a constant that arises from the integration along the \( y- \) direction. In this case a fit of \( K(x, \Delta z) \) to the measured data yields the width of the beam, namely FWHM\(_x = 2\sigma \sqrt{2\log 2} \).

At first, let us consider a simulated 'dumbbell-shaped' probe intensity (cf. Fig. 10(a)), which violates the assumption of factorization. Certainly, this model-probe may have pronounced and somewhat exaggerated side-lobes. However, the reader may note that the probe reconstructed experimentally in this work also exhibits side-lobes at least in the plane shown in Fig. 10(d). For this choice of probe, the fit of the error function to the calculated knife-edge scan along the horizontal direction (cf. Fig. 10(b)) yields FWHM = 0.635 \( \mu \)m in comparison to the theoretical value of FWHM = 0.1 \( \mu \)m at the centre (cf. Fig. 10(c)).

Next, let us consider the experimental case of the reconstructed probe field intensity (ptychographic reconstruction of the Siemens star resolution chart, cf. Fig. 2). After a calculation of the probe at several planes behind the sample, we performed 'numerical' knife-edge scans along the horizontal and vertical direction in a few hundred planes to obtain the beam-width along the optical axis. The horizontal and vertical beam-widths as a function of the distance to the sample plane are shown in Fig. 10(f) as red and blue curves, respectively. Note that the minimal beam-widths do not exactly coincide with the maxima of \( \Upsilon(\Delta z) \) (Eq. (6)), indicating that the horizontal and vertical axes do not coincide with the minor and major axes of the beam. As is known from knife-edge scanning of astigmatic Gaussian beams [53], it is not generally sufficient to perform knife-edge scans along the horizontal and vertical axes. Contrarily, one needs to scan at several angles to determine the major and minor axes in one plane. Furthermore, it becomes clear that features such as the double-peak and donut-shape (at certain positions of the optical axis, cf. Fig. 5(C)), will be extremely difficult to infer from a set of knife-edge scans along two orthogonal axes.

Finally, let us consider the intensity of the probe at \( \Delta z = -0.686 \) mm (cf. Fig. 10(d)) behind the plane of the sample. In order to get a FWHM estimate that becomes close to the theoretical value of FWHM = 0.093 \( \mu \)m (cf. Fig. 2) the fit of the error function to the knife-edge scan needs to be restricted to values close to the centre yielding a FWHM = 0.104 \( \mu \)m (cf. Fig. 10(e)). The outcome of the FWHM value of the fit could also be improved by manually weighting the data points near the centre. However, without a two-dimensional representation of the probe intensity, justification for both means would not be granted. We conclude that even if the information...
on the phase front of the beam is not needed, reconstruction of the intensity distribution from selected knife-edge scans is problematic and does not stand up to the full probe reconstruction by ptychography. Therefore, we recommend that ptychography should replace knife-edge scans whenever possible, i.e. if the beam is sufficiently coherent.

Fig. 10. (a) Intensity of a model-probe exhibiting a dumbbell-shape. The calculated knife-edge scan along the horizontal direction is shown in (b). The white, dashed line in (a) indicates the region of highest focusing which is shown in (c) (solid, black line). The resulting Gaussian shape as estimated from the knife-edge scan is depicted as solid, red curve. (d) shows the intensity of the reconstructed probe at $\Delta z = -0.686 \text{mm}$ behind the plane of the sample. A white, dashed line indicates the part which has been used for estimating the beam width. The corresponding result of the calculated knife-edge scan is shown in (e). The FWHM values from calculated knife-edge scans are shown as function of distance to the sample plane in (f). The red and the blue curve denote horizontal and vertical beam-width, respectively. In addition, $\Upsilon(\Delta z)$ is shown as solid, black curve which has been scaled arbitrarily for reasons of visualisation.

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