X-Rays Reveal the Internal Structure of Keratin

Bundles in Whole Cells

Clément Y. J. Hémonnot,[†] Juliane Reinhardt,[‡] Oliva Saldanha,[†] Jens Patommel,[¶]
Rita Graceffa,[†] Britta Weinhausen,[§] Manfred Burghammer,^{§,||}
Christian G. Schroer,^{‡,⊥} and Sarah Köster^{*,†}

Institute for X-Ray Physics, University of Göttingen, Friedrich-Hund-Platz 1, 37077 Göttingen, Germany, Deutsches Elektronen-Synchrotron, Notkestrasse 85, 22607 Hamburg, Germany, Institute of Structural Physics, Technische Universität Dresden, Zellescher Weg 16 01069 Dresden, Germany, European Synchrotron Radiation Facility, 71, Avenue des Martyrs, 38043 Grenoble, France, Department of Analytical Chemistry, Ghent University, Krijgslaan 281, 9000 Ghent, Belgium, and Institute for Nanostructure and Solid State Physics, Department of Physics, University of Hamburg, Luruper Chaussee 149, 22761 Hamburg, Germany

E-mail: sarah.koester@phys.uni-goettingen.de

Abstract

In recent years, X-ray imaging of biological cells has emerged as a complementary alternative to fluorescence and electron microscopy. Different techniques were established and

*To whom correspondence should be addressed

[†]Institute for X-Ray Physics, University of Göttingen, Friedrich-Hund-Platz 1, 37077 Göttingen, Germany

[‡]Deutsches Elektronen-Synchrotron, Notkestrasse 85, 22607 Hamburg, Germany

[¶]Institute of Structural Physics, Technische Universität Dresden, Zellescher Weg 16 01069 Dresden, Germany

[§]European Synchrotron Radiation Facility, 71, Avenue des Martyrs, 38043 Grenoble, France

^{||}Department of Analytical Chemistry, Ghent University, Krijgslaan 281, 9000 Ghent, Belgium

[⊥]Institute for Nanostructure and Solid State Physics, Department of Physics, University of Hamburg, Luruper Chaussee 149, 22761 Hamburg, Germany

successfully applied to macromolecular assemblies and structures in cells. However, while the resolution is reaching the nanometer scale, the dose is increasing. It is essential to develop strategies to overcome or reduce radiation damage. Here we approach this intrinsic problem by combining two different X-ray techniques, namely ptychography and nano-diffraction in one experiment and on the same sample. We acquire low dose ptychography overview images of whole cells at a resolution of 65 nm. We subsequently record high-resolution nano-diffraction data from regions of interest. By comparing images from the two modalities, we can exclude strong effects of radiation damage on the specimen. From the diffraction data we retrieve quantitative structural information from intracellular bundles of keratin intermediate filaments such as a filament radius of 5 nm, hexagonal geometric arrangement with an inter-filament distance of 14 nm and bundle diameters on the order of 70 nm. Thus, we present an appealing combined approach to answer a broad range of questions in soft-matter physics, biophysics and biology.

keywords: cytoskeleton; intermediate filaments; keratin; X-ray nano-diffraction; ptychography

Biological cells contain a wealth of nanometer-sized objects and structures. Much of what is known about cellular function has been learned from direct imaging. Mostly two techniques have been established for cellular imaging in the past: fluorescence microscopy and electron microscopy (EM). Fluorescence microscopy is used for living or fixed cells and relies on chemical labels specifically attached to certain molecules in the cell or fluorescent proteins expressed in the cell. The advent of super-resolution techniques has recently turned fluorescence microscopy into a nanoscale imaging technique.¹ With EM, even higher resolution can be achieved and structural details of cellular components can be visualized.^{2,3} Both techniques require fairly extensive sample preparation, like labeling, fixation or staining, and are characterized by a small penetration depth.

X-ray imaging of cells has recently evolved into a third, complementary class of techniques, which

overcomes some of the challenges not met by fluorescence microscopy or EM. The penetration depth of hard X-rays is much larger than for electrons or visible light and thick samples can be investigated. X-rays of 10 keV have an attenuation length of about 2 mm in liquid water, while electrons of the same energy have an average path length of $2.5 \mu\text{m}$,^{4,5} hence using X-rays it is possible to study samples in aqueous environment.⁶ The resolution reached in the hard X-ray regime is on the nanometer scale, depending on the specific setup used *i.e.* down to about ten nanometers reciprocal space information and down to 50 nm real space resolution by solving the phase problem. Furthermore, the electron density can be retrieved from the information contained in the phase. Labeling is not needed since the X-rays interact directly with the electron density of the sample. Here, biological samples are particularly challenging since the most abundant elements in biological matter - hydrogen, carbon, nitrogen and oxygen - all scatter very weakly.⁷ Nevertheless, in recent years several X-ray techniques have been successfully applied to and adapted for imaging of biological cells, such as ptychography,^{8–11} holography,^{12–15} tomography,^{16–18} phase contrast^{19,20} and scanning nano-diffraction.^{6,21–24} The techniques themselves are complementary with respect to the physical properties they are able to reveal. In diffraction techniques, the amplitude of the exit wave field is measured. From the reciprocal space information, the shape, internal structure, orientation and build-up of sub-cellular constituents can be retrieved.^{25–27} In coherent diffractive imaging techniques, such as ptychography, the electron and mass densities in real space can be accessed by solving the phase problem.^{23,28} In order to estimate the number of photons needed to image cells in diffractive techniques, we can assume the empirical formula $\text{H}_{50}\text{C}_{30}\text{N}_9\text{O}_{10}\text{S}$ for the chemical composition of the sample as suggested by Howells *et al.*²⁹ They have shown that the fluence needs to be at least 10^{11} photons/ μm^2 to obtain a resolution of 10 nm voxels. Within our configuration we use about 2×10^{11} photons/ μm^2 . In phase-contrast X-ray imaging, a map of the real part of the refractive index is obtained; this technique allows the investigation of large samples up to a few millimeters.^{30,31} In tomography, absorption is measured, thus density volumes of unstained or unsliced tissues and cells can be visualized. Nowadays tomography offers the possibility to probe large samples, such as bone.^{32–34} Tomography has been

combined with staining to localize vital and non-vital cells in clusters of thousands of cells.¹⁶

The idea to combine several X-ray techniques, thus obtaining complementary information, is highly attractive. In the past, we and others have combined nano-diffraction and ptychography on samples of the same kind. We have characterized intact hair cell stereocilia from the inner ear by analyzing ptychography and nano-diffraction data sets acquired on different samples of the same preparation.²³ We were able to measure diameters and local orientation of these cellular structures. The combination of these two methods was also used to study *Deinococcus radiodurans*, a bacterium famous for its resistance to high doses of radiation,¹¹ but again on different samples. However, by contrast to well-defined hard matter samples or some biological samples like proteins or viruses, cells are characterized by individual differences in their internal structure. Thus, averaging techniques such as conventional small angle X-ray scattering, using a large beam, obscure some or most of the nanoscale structures we are interested in. Furthermore, since each cell is different, when applying complementary methods, it is indispensable to perform the measurements on the identical sample.

Thus, we take this idea further and probe the exact same cell with both methods. We choose samples from epithelial SK8/K18 cells, which contain highly ordered networks of keratin bundles.^{35,36} We first record real space images of $30\ \mu\text{m} \times 30\ \mu\text{m}$ of whole cells at a resolution of 65 nm using ptychography while applying a comparatively low dose of 10^3 Gy, thus reducing radiation damage to the biological samples. Based on these high-resolution images, we select a region of interest (ROI) of $6\ \mu\text{m} \times 6\ \mu\text{m}$, where scanning X-ray nano-diffraction is performed on the identical sample to retrieve local, internal structural information. For these measurements the dose is five orders of magnitude larger. Keratin is an intermediate filament protein found in cells of the simple epithelium in organs such as lung and intestine and is believed to be a major contributor to cell mechanics and stability.³⁷ The 10-nm-diameter filaments organize into densely packed bundles of about 100 nm thickness.³⁸ A precise knowledge of the internal bundle structure is necessary in order to understand the mechanical properties of the keratin bundles. Thus, we present high spatial resolution X-ray imaging in real and reciprocal space and successfully resolve the internal structure

of the bundles, including single filament radius and inter-filament distance by further development of a filament bundle model from the literature.³⁹

Results and discussion

X-ray imaging in real and reciprocal space

Figure 1: Correlation between ptychography and X-ray scanning nano-diffraction. a) Ptychography image of a whole cell, scale bar $5\ \mu\text{m}$, and b) detail from the ROI shown in a) by the black box. c) Dark-field image from the same region as in b), calculated from nano-diffraction data with a step size of 100 nm. In b) and c) the field of view corresponds to $6\ \mu\text{m} \times 6\ \mu\text{m}$

Due to the high sensitivity of biological cells to radiation, and the fact that the dose is increasing as the resolution approaches smaller values,²⁹ it is essential to reduce energy input, when using multimodal techniques, especially, when employing scanning methods.^{6,26,40} Here we study freeze-dried cells, thus largely avoiding the impact of free radicals induced by the radiolysis of water.^{41,42} These radicals represent 80% of the energy deposition in cells when irradiated.^{41,43} For example, hydroxyl radicals ($\cdot\text{OH}$) are associated with changes in structural and functional molecules.⁴¹ Alternative techniques using cryo-streams of N_2 at 100 K reduce the diffusion of free radicals very efficiently as well.^{39,44,45} We combine and correlate two X-ray imaging techniques, ptychography and nano-diffraction.^{11,23} Ptychography images of the whole cell, as shown in Fig. 1a, serve to localize the sample with respect to the X-ray beam. For better comparison with

the dark field image, a detail of this image is shown in Fig. 1b.

In ptychography, the sample is scanned through the coherent beam, recording far-field diffraction patterns at each scan point with sufficient (≈ 60 to 70%) overlap. The spatial resolution of the ptychographic reconstruction (Fig. 1) is determined by Fourier Ring Correlation (FRC, see Materials and Methods for details) and we achieve a spatial resolution of about 65 nm. Previous studies of biological specimens using ptychography at similar energies reported a resolution of about 50 nm in freeze-dried conditions (at 6.2 keV).¹¹ A resolution of 30 nm in frozen-hydrated samples was recently reached at an energy of 5.2 keV.⁴⁶ Thus the resolution we reach in Fig. 1 is on the same order as literature values found for the hard X-ray regime.

Thus, we obtain high-resolution real space images at comparatively low dose (*i.e.* 3.4×10^3 Gy, see Materials and Methods section) and therefore reduced radiation damage. For comparison, cells exposed to a 17 keV X-ray beam showed shrinkage and mass loss after exposure.⁴⁷ We did not observe a shrinkage effect on the cells during or after our measurements. Moreover, a time-resolved study showed the structural changes of hair-keratin induced by synchrotron radiation.⁴⁸ Three levels of structural changes were identified while increasing the exposure: i) after 4 s the coiled-coil architecture of the keratin dimers is disrupted, ii) after 30 s the long-range organization along the filament is lost and iii) after 50 s the lateral organization of the filament packing is disturbed. Considering the different characteristics of the setups used (energy, intensity, exposure time and beam dimension) we estimated to be below the appearance of structural changes to keratin dimers. However, it has to be noted that hair keratins and cytoplasmic keratins are considerably different, so the comparison has to be made with care.

The phase shift ϕ (Fig. 1) depends on the refractive index n of the sample and thickness d :

$$\phi = \frac{2\pi}{\lambda} n d \sin \theta, \quad (1)$$

where λ is the wavelength and 2θ the scattering angle. The reconstructed phase retrieved by ptychography is directly proportional to the electron density of the given material, see Materials and

Methods section. Hence, the smallest phase shift values in the ptychographic reconstruction (Fig. 1a, b), represented by darker regions, depict higher electron density in the projection and highlight the correlation between the expected intracellular network structure and the appearance of the X-ray images.

The example in Fig. 1 demonstrates that it is possible to correlate nano-diffraction data and ptychography data recorded on the identical sample. When imaging the ROI from Fig. 1b by nano-diffraction, as shown in Fig. 1c, the computed dark field image reproduces the network structure, which resembles the keratin network that we had imaged previously.²⁶ Dark field images are obtained by integrating the photons on the detector plane, while excluding the primary beam or non-scattered intensity. In those previous studies we could identify the keratin network thanks to visible light fluorescence micrographs that had been recorded beforehand. Whereas that technique proved to be very useful at the time to characterize the new X-ray technique, we here refrain from employing this intermediate step. This approach will make the presented technique more versatile as it renders it applicable to non-labeled samples. The pseudo-real space resolution of the dark field images is limited by the beam diameter and employed step size (more precisely the larger of the two) and thus, in our case, 100 nm to 150 nm. This resolution could be improved by reducing the beam size, at the expense of the primary beam intensity and increased total scanning time. The reciprocal-space resolution of each nano-diffraction pattern does not depend on the beam-size, but only on the q -range used.

Reciprocal space data from individual SAXS patterns

Even though the network structure shown in Fig. 1c strongly resembles keratin structures we have imaged in the past,²⁶ additional information is needed to demonstrate that this network is keratin. Nano-diffraction provides this additional information, which we can exploit to characterize the sample: Each “pixel” of the dark field image represents the integrated result of a full diffraction pattern. A typical example of such a diffraction pattern is shown in Fig. 2a. We divide the pattern

Figure 2: a) typical diffraction patterns showing a clear anisotropy of the signal and modulations within the signal. b) Azimuthally integrated, radial intensity within the segments indicated in a). The local maxima are represented by blue and red diamonds. c) Radial intensity from segment 1 as in b, with the corresponding smoothed curve and threshold for maxima detection (dashed line). d) Histogram of the peak positions for three cells compared to a reference cell from literature²⁶ that showed a dense keratin network. e) Mean and standard deviation of the Gaussian distribution from d and Tab. 1. The regression and prediction bounds from the reference cell are also indicated.

into eight segments, as shown by the black lines, and integrate the segments, which are aligned with the main axis of the pattern azimuthally, *i.e.* segments 1 and 5. The resulting radial intensity $I(\mathbf{q})$ is then plotted against the magnitude $|\mathbf{q}| = \frac{4\pi \sin \theta}{\lambda}$ of the scattering vector $\mathbf{q}$ (Fig. 2b). For this analysis, we manually select the diffraction patterns that show anisotropy. Identification of local maxima in $I(\mathbf{q})$ is performed for 184 diffraction patterns originating from three distinct cells and a reference cell from previously recorded data.²⁶ The radial intensity is smoothed by a five-point moving average to remove noise (Fig. 2c). A local maximum is defined when the n^{th} data point is larger than the $(n-1)^{th}$ and the $(n+1)^{th}$ data point. The search for peaks is performed on the smoothed intensity with a minimal intensity threshold of 0.2, such that peaks with a lower intensity than the threshold value are disregarded to avoid detection of noise at large q -values. Furthermore, in order to account for possible small variations of the smoothed intensity curve, the

minimal distance between two peaks is set to 15 data points of the q -axis. For this experiment and setup, the distance between two consecutive data points Δq is equal to $4.5 \times 10^{-3} \text{ nm}^{-1}$. The curves and local maxima are then visually checked and in case of missing or false maxima, manual addition or deletion is performed.

The peaks are histogrammed, as shown in Fig. 2d. Here, cell 1, cell 2 and cell 3 are present data from this particular measurement campaign, whereas the reference cell is analyzed and plotted for comparison. Those data had been taken previously²⁶ and at the time we could assign the network structure to keratin by comparing to fluorescence micrographs. The histograms show good agreement in the peak positions found for these cells and in particular, the newly acquired data agree with the reference cell. This is a very strong indication that the network structure observed in the ptychograms and dark field images is indeed the keratin network in the cells. In total, we analyzed more than 1000 peaks in over 180 diffraction patterns. Some of the 1D radial intensity curves only display one single peak, whereas others show up to seven orders of oscillation. For each cell separately, the histograms are fitted by Gaussian distributions in order to extract the mean peak position and the standard deviation. Tab. 1 summarizes the positions of the first five peaks for each cell. The values for the mean agree very well and the standard deviations are small, when considering intrinsic structural variations. To further visualize this result, the means and standard deviations of the four cells are plotted against the peak number in Fig. 2e. The regression and prediction bounds for the reference cell are also shown. We use a 0.99 confidence level for the prediction bounds and we observe that cells 1, 2 and 3 agree with the prediction from the reference cell.

Structural information from diffraction patterns

When probing nanostructures with a nm-diameter beam, the ensemble averaging, which is typical of solution SAXS, along with its established interpretation of the peak positions becomes invalid. We have discussed this aspect previously^{23,26} and recently Priebe *et al.*³⁹ have presented a simple yet sufficient model to qualitatively describe the scattering pattern caused by bundles of filaments.

Table 1: Means and standard deviations of the positions of the maxima of the radial SAXS intensities as plotted in Fig. 2b for three different cells. The number of analyzed patterns for each cell is given in parentheses. The reference cell is taken from published data²⁶ for comparison and analyzed with the same procedure.

maximum	cell 1 (71)	cell 2 (77)	cell 3 (17)	ref. cell (19)
1 st	0.14 ± 0.05	0.14 ± 0.05	0.15 ± 0.04	-
2 nd	0.26 ± 0.05	0.26 ± 0.08	0.27 ± 0.07	0.24 ± 0.05
3 rd	0.34 ± 0.05	0.36 ± 0.09	0.40 ± 0.12	0.35 ± 0.06
4 th	0.46 ± 0.06	0.48 ± 0.07	0.49 ± 0.02	0.45 ± 0.05
5 th	0.57 ± 0.07	0.59 ± 0.03	-	0.56 ± 0.06

Here, we use this model, incorporate it into a combined 2D and 3D fitting routine and thus quantitatively determine the relevant structural parameter of the bundle like filament diameter and distance. The geometry of the setup and the model are sketched in Figure 3a-c and further described in the supporting information. The bundle consists of a predefined number of rod-like particles (seven in the example in Fig. 3b) with a length L and a radius r ; the diameter of the whole bundle is given by $\odot = 2(a + r)$ and $\odot = 2(2a + r)$ for the 7 and 19 filaments model, respectively. The filaments are modeled as cylinders with a Gaussian density distribution in longitudinal direction to account for the convolution with the Gaussian profile of the nano-beam, where we assume, for simplicity, a circular beam. The tilt of the filament with respect to the beam direction, the x -axis, defines the angle ϑ . We expect it to be close to 90° as the cells have a fairly small height⁴⁹ and thus the bundles lie mostly flat on the substrate. We thus fix this angle to 90° . The angle Ψ denotes the rotation of the bundle about its long axis and can be anything between 0 and 360° , however, depending on the chosen geometry and thus symmetry, only certain values have to be considered. We use a hexagonal lattice, thus by symmetry we can restrict the angle Ψ to an interval of 0° to 60° . Finally, χ determines the rotation of the filament in the $y - z$ plane when projected into the detector plane and can be directly estimated from the main axis of the anisotropic pattern (see Fig. 2a) which is perpendicular to the structure in real space.

The radial intensity $I(\mathbf{q})$ of the scattering signal from the bundle is calculated from the product of

Figure 3: Sketch of the setup; the X-ray beam points along the x -axis and the sample is scanned through the beam in y - and z -direction. b) Sketch of the model used, reproduced with permission from Priebe *et al.*³⁹ c) Cross section of the model composed of 19 filaments as well as the model with 7 filaments represented by dashed lines. d) Example of TEM image of the cross section of a keratin bundle showing hexagonal lattice, scale bar 50 nm.

the form factor F and the structure factor S ^{50,51}

$$I(\mathbf{Q}_z, \mathbf{Q}_p) = |F(\mathbf{Q}_z, \mathbf{Q}_p)|^2 S(\mathbf{Q}_p), \quad (2)$$

where $\mathbf{Q}_{x,y,z}$ are the scattering vectors in the coordinate system associated with the bundle and $\mathbf{Q}_p = (\mathbf{Q}_x^2 + \mathbf{Q}_y^2)^{0.5}$. The coordinate system is further described in supporting information. $F(\mathbf{Q}_z, \mathbf{Q}_p)$ is given by the Fourier transform of the particle shape:^{39,52–55}

$$F(\mathbf{Q}_z, \mathbf{Q}_p) = \frac{e^{-\mathbf{Q}_z^2 L^2 / 2}}{\sqrt{2\pi}/L} \frac{2J_1(r(\mathbf{Q}_p))}{r(\mathbf{Q}_p)}, \quad (3)$$

with the first order Bessel function of the first kind J_1 , the radius of one filament r , and the filament length L .

Figure 4: a) Example of fit of 1D experimental data (blue circles, see Fig. 2b) using two variants of the model, 7 filaments (orange line) and 19 filaments (gray line) arranged in a hexagonal lattice. b) Corresponding 2D simulated diffraction pattern of the fit with 19 filaments.

$S(\mathbf{Q}_p)$ depends on the geometric arrangement of the individual filaments in the bundle:^{39,55,56}

$$S(\mathbf{Q}_p) = \sum_{j,k} \exp(-i\mathbf{Q}_p(\mathbf{R}_j - \mathbf{R}_k)), \quad (4)$$

with the filament positions $\mathbf{R}_{j,k}$, that are given by the angle Ψ and the filament-to-filament distance a .

In vitro experiments employing electron microscopy (EM)⁵⁷ or solution SAXS⁵⁸ have shown that keratin IFs are extended rod-shaped particles that form densely hexagonally packed bundles in cells.³⁸ Thus, we follow the model from Ref.,³⁹ which also considers hexagonal packing. We extend the model by determining structural parameters of the bundles by a non-linear least-square optimization. The detailed fitting procedure is explained in the supporting information. We compare two distinct scenarios: first, a bundle consisting of seven individual filaments, thus three primitive “unit cells”, and second a bundle with an additional “shell” of filaments, resulting in nineteen filaments in total, thus twelve primitive unit cells.

The model contains six free parameters: the radius r of a single filament, which determines the peak heights and positions in the $I(q)$ curves, the length L of a filament that determines the lateral extension of the features in the 2D SAXS patterns, the distance a between the centers of mass of two filaments, which influences the position and height of the peaks, the angle Ψ , which again influences the positions and width of the peaks, the angle ϑ , which changes the shape of the fea-

tures in the 2D SAXS patterns, and finally the angle χ , which is given by the orientation of the anisotropic pattern in the detector plane. From the 1D azimuthally integrated data, we can determine r , a and Ψ , whereas L , ϑ and χ have to be derived from the 2D patterns.

Fig. 4a shows a typical example of a fit to the 1D $I(\mathbf{q})$ data (blue circles, integrated data from the diffraction pattern in Fig. 2a) for both, seven (orange line) and nineteen (gray line) individual filaments. Fig. 4b shows the corresponding 2D calculated data with nineteen filaments. The fits agree very nicely with the data and small deviations can be explained by simplifications we have made for the model. First, in the real system, the space between the filaments is not empty as it is in the model; second, we have considered all filaments to have identical parameters, which is certainly an oversimplification. Despite these approximations, the positions of the maxima are well reproduced by both fits and the norm of residuals is smaller for the nineteen-filament fit than for seven filaments. This finding is in line with our expectation from EM data³⁸ that the keratin bundles are approximately 100 nm in diameter.

Table 2: Comparison of the results for fitting the data shown in Fig. 4a with a 7 *versus* 19 filaments model, including the error estimate of each parameter. Additionally the expected values from EM experiments are given.^{38,59} The EM values of the bundle diameters are given as mean and standard deviation.³⁸ The norm of residuals is defined as the sum of the square of the difference between the estimated value and the data: $\text{resnorm} = \sum (I_{\text{estimated}} - I_{\text{data}})^2$.

parameter	7 filaments	19 filaments	EM
r (nm)	6.8 ± 0.5	5.1 ± 0.6	4-5
a (nm)	23.5 ± 0.5	14.3 ± 0.4	11-14
$\varnothing$ (nm)	60.6 ± 2.1	67.4 ± 2.9	81 ± 22
ψ (°)	139.8 ± 0.6	155.7 ± 0.6	-
resnorm	3.6	2.9	-

In Tab. 2 we summarize the results and compare them to values expected from EM images. Our observation is that the model including nineteen filaments describes the data better. When including only seven filaments, the fit results for the radius is $r = 6.8$ nm and for the distance between the filaments is $a = 23.5$ nm. These values are on the right order of magnitude. However, with nineteen filaments included, the numerical values $r = 5.1$ nm and $a = 14.3$ nm agree much better

with the literature.^{57,59} Certainly, the bundles are heterogeneous, both concerning the individual filament diameters^{60–63} and in the build-up and total thickness of the bundles. However, also the bundle diameter is well reproduced in our model with 60.6 nm and 67.4 nm for seven and nineteen filaments, respectively.³⁸

Additional 2D patterns and corresponding fits with the two models can be found in supporting information. When analyzing and averaging the values for eight individual patterns, we find the following structural parameters: i) For the model with 7 filaments $r_7 = 6.5 \pm 0.5$ nm, $a_7 = 25.8 \pm 2.7$ nm and $\phi_7 = 64.5 \pm 4.9$ nm and ii) for the model with 19 filaments we find $r_{19} = 5.5 \pm 0.7$ nm, $a_{19} = 15.2 \pm 1.4$ nm and $\phi_{19} = 72.0 \pm 5.5$ nm. Thus, the standard deviations are small. This result is surprising, since it shows that the bundles are more homogeneous concerning filament and bundle diameter and filament distance than originally expected.³⁸ When analyzing the angles χ in more detail, we find very similar values for adjacent scan positions, but values spread over all possible angles between 0° and 180° when comparing results from 161 different scan positions (see supporting information). These positions are spread over the full sample area and have been selected according to distinct modulations in the diffraction pattern. This result is expected since adjacent positions correspond to the same bundle structure and thus similar parameters, whereas distant positions correspond to independent structures.

Conclusion

By combining ptychography and scanning X-ray nano-diffraction we are able to localize protein networks in intact cells with nanometer precision and subsequently probe the local, internal structure of these hierarchical networks. Importantly, we obtain the ptychography overview images with relatively low dose imposed on the sample and thereby minimize the total radiation damage. Radiation damage is the main challenge in multimodal X-ray imaging techniques. Higher dose is introduced only to small ROIs *via* nano-diffraction. By comparing ptychography and nano-diffraction

images of the very same sample, we could show that visible radiation damage did not occur. This combined imaging technique offers an alternative to fluorescence or electron microscopy for *in situ* probing of protein structures on the nanoscale. The keratin bundles investigated here are a prime example of a well-ordered natural structure. By extending a qualitative model of such filament bundles to a quantitative fitting routine, we are able to measure the filament and bundle diameters as well as filament arrangement and interfilament distance at nanometer precision. However, the presented approach will also be applicable to many other biological and soft-matter systems which show a certain degree of order without being truly crystalline. Just to name a few examples, the method could be applied to other cytoskeletal proteins, such as actin network bundles and stress fibers, the microtubule spindle, or to chromatin structures in the nucleus.

Materials and Methods

Cell culture and sample preparation

Keratin-rich SK8/18 cells^{35,36} were grown, cultured and prepared on silicon nitride windows as described previously.^{6,26} Samples were chemically fixed, plunge-frozen and freeze-dried and inspected by bright-field and phase contrast microscopy at different stages of the sample preparation to identify possible artifacts from the preparation.

Ptychography

The ptychography experiments^{23,28,44} were performed using the nano-focus extension (experimental hutch III) at beamline ID13 at the European Synchrotron Radiation Facility (ESRF, Grenoble, France).⁶⁴ At a photon energy of 14.9 keV the beam was focused by nano-focusing refractive X-ray lenses.⁶⁵ In order to record the far-field diffraction patterns, a Maxipix detector (ESRF, 516×516 pixels, pixel size 55 μm) was placed 2.2 m away from the sample. For the ptychography measurements no beamstop was used. By closing the vertical and horizontal slits the primary beam

intensity was reduced to a flux of about 2×10^7 photons/s. By this procedure we could considerably increase the coherence of the beam.

The sample was scanned through the beam, recording diffraction patterns at each scan point with an overlap of about 60 to 70 %. In order to determine the spatial resolution of a ptychographic image, Fourier Ring Correlation (FRC) was applied. This technique measures the correlation of two images at different spatial frequencies in the Fourier space.⁶⁶ One dataset containing 100×100 diffraction pattern was split up into two datasets by taking all even rows of scan points for one and all odd rows of scan points for another half-dataset. The overlap of the scan points in each half-dataset was still sufficient for the ptychographic reconstruction. Both half-datasets were reconstructed separately and the phase image was transformed to Fourier space. Afterwards, the Fourier transforms of each final phase reconstruction were cross-correlated. The resulting cross-correlation function decreases as a function of spatial frequency. Different criteria can be applied to define which degree of correlation is required to offer a certain level of information. As discussed in van Heel and Schatz,⁶⁷ we chose the sigma criterion to indicate the resolution level at which sufficient information was collected significantly above the noise level. Thus, we achieve an upper bound for the spatial resolution of about 65 nm in the ptychographic reconstruction shown in Fig. 1.

The sample was positioned 1 mm out of focus in order to increase the beam size, thus reducing the number of required overlapping scan points. The ptychography measurements were performed with a step size of 300 nm and an exposure time per scan point of 40 ms. The corresponding dose is evaluated^{6,23,26} to about 3.4×10^3 Gy, and thus considerably lower than for nano-diffraction.

The refractive index $n(\mathbf{r})$ of a material is directly related to the electron density distribution $\rho_e(\mathbf{r})$ as:

$$n(\mathbf{r}) = 1 - \delta(\mathbf{r}) + i\beta(\mathbf{r}), \quad (5)$$

where β and δ are the imaginary part (absorption) and deviation from 1 of the real part of n , which are defined as:

$$\beta(\mathbf{r}) = \frac{\lambda}{4\pi} \mu(\mathbf{r}) \quad \text{and} \quad \delta(\mathbf{r}) = \frac{r_0 \lambda^2}{2\pi} \rho_e(\mathbf{r}) \quad (6)$$

with the X-ray attenuation coefficient μ and the classical electron radius r_0 . The ptychographic reconstruction was performed using an ePIE⁶⁸ based algorithm written in GPU accelerated C++ code. For the reconstruction, the diffraction patterns were cropped to 256×256 pixels resulting in a pixel size of 12.76 nm for the reconstructed images shown below. The illumination was initialized from a previous reconstruction of a resolution test pattern (NTT-AT, San Jose, CA, USA), made of a 500 nm thick tantalum layer, containing structures of 5 micrometers in the outer regions down to 50 nm lines and spaces in the innermost part. At the sample position, the FWHM of the illuminating amplitude is about $980 \text{ nm} \times 920 \text{ nm}$.

Scanning X-ray nano-diffraction

X-ray diffraction experiments^{22,26,69} were performed at the same beamline and, importantly, on the same sample as the ptychography experiments described above. At the focal position with a beam size of $100 \text{ nm} \times 150 \text{ nm}$ (horizontal $\times$ vertical, full width at half maximum), the primary beam intensity was about 3×10^9 photons/s. Behind the sample, a 6 cm long flight tube filled with helium was installed to reduce air scattering. A beam stop was attached to the exit window of the flight tube to block the primary beam. The scattered intensity was recorded with the same Maxipix detector as for ptychography, but placed at a sample-to-detector distance of about 0.9 m as a compromise to keep absorption of the scattering signal by air at a minimum and to ensure sufficient speckle sampling. The sample was aligned with the beam by performing a coarse scan with a step size of 500 nm and an exposure time of 30 ms. In order to obtain a real space visualization of the cell, X-ray dark-field images were computed by summing up the total number of scattered photons in each 2D diffraction pattern and plotting these values on a color scale. Finer scans were performed with smaller step sizes of 100 nm and longer exposure times of 1 s, corresponding to an estimated dose^{6,23,26} of about 10^8 Gy.

Unless otherwise stated, diffraction patterns were analyzed by self-written MatLab scripts (The MathWorks, Inc., Natick, MA, USA).

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Supporting Information Available

Additional images of ptychogram reconstruction and corresponding dark field images; fitting procedure of the 2D patterns and examples of fitting results. This material is available free of charge via the Internet at <http://pubs.acs.org/>.

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