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X-ray nano-diffraction on cytoskeletal networks

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Abstract. The nano-scale structure of cytoskeletal biopolymers as well as sophisticated superstructures determine the versatile cellular shapes and specific mechanical properties. One example is keratin intermediate filaments in epithelial cells, which form thick bundles that can further organize in a cross-linked network. To study the native structure of keratin bundles in whole cells, high-resolution techniques are required, which do at the same time achieve high penetration depths. We employ scanning x-ray diffraction using a nano-focused x-ray beam to study the structure of keratin in freeze-dried eukaryotic cells. By scanning the sample through the beam we obtain x-ray dark-field images with a resolution of the order of the beam size, which clearly show the keratin network. Each individual diffraction pattern is further analyzed to yield insight into the local sample structure, which allows us to determine the local structure orientation. Due to the small beam size we access the structure in a small sample volume without performing the ensemble average over one complete cell.

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1. Introduction

Cytoskeletal protein networks play an important role in, e.g. the determination of cellular shape, mechanical properties and consequently specific cellular functions [1]. These networks are mainly built of three different classes of proteins: actin filaments, microtubules and intermediate filaments (IFs). IF proteins are encoded by a large group of different genes and their expression is highly linked to specific cell-types [2, 3]. The most diverse group of IFs are keratins. Keratins can further be subdivided into cyto-keratins, which are expressed in various epithelial cells, and so-called ‘hard’ keratins, which are used to build, e.g. hair or nails [4]. A major function of cytokeratins is to provide epithelial cells with the necessary mechanical properties to withstand external stress [5]. Mutations in genes encoding for keratin proteins can result in skin diseases such as Epidermolysis bullosa simplex showing an intolerance of the skin against external forces and the formation of blisters as a consequence of light touches [6–8]. All cyto-keratin IFs show a similar hierarchical architecture. In a complex assembly process, keratins first form heterodimers of two different keratins (a basic and an acidic keratin), which then assemble into filaments with a diameter of about 10 nm. These filaments further form bundles with diameters ranging from a few tens to a few hundreds of nanometers, which organize in a superstructure. A variety of studies accesses the assembly mechanisms into single filaments [4, 9], and also the structure, morphology and dynamics of intracellular keratin networks as a whole [10–13]. However, knowledge about the detailed filament arrangement in keratin bundles in cells is still lacking. The small structure sizes require high-resolution imaging techniques, which are compatible with soft matter samples and non-invasive sample preparations. Further, structural variations of keratin bundles between different tissues and, at least in terms of the diameter, also within the same cell, make it difficult to probe and determine a general bundle structure.

An ideal probe for studying structures at the nano-scale are x-rays. The small wavelength in principle allows for structure determination with atomic precision and due to the high penetration depth of hard x-rays, comparatively thick samples can be investigated non-invasively. Today’s x-ray optics further make it possible to achieve focal spot sizes below 100 nm [14–16], which allows for probing the local sample structure in small volumes of the order of the beam

size. By scanning the small beam across the sample and employing different contrast mechanisms such as absorption, dark-field or differential phase contrast, real space images of the sample with a spatial resolution of the order of the beam size can be obtained [17–19]. At each scan point, a diffraction pattern is recorded and can further be analyzed to yield structural information averaged over the probed sample volume. For micrometer-sized beams, this method has been applied to a variety of different samples including biological materials such as bone, tooth, muscle or wood [20–26]. The step toward the use of a sub-micron beam reduces the probed sample volumes to dimensions, which allow to investigate structural properties of nano-scale objects without averaging over a large ensemble [27]. This is particularly important for samples with a structural heterogeneity where the local structure differs from the ensemble average. Scanning diffraction experiments with a nano-focused x-ray beam have been applied to study samples in material science [27], but for biological samples this method was so far not employed.

In this paper, we present scanning diffraction experiments using a nano-focused x-ray beam with a focal spot size of about $150 \times 150 \text{nm}^2$ on freeze-dried cells, which are transfected with genes encoding for keratins K8 and K18. In these cells, keratin filaments form bundles and organize in a cross-linked network. X-ray dark-field contrast is employed to generate real space images of the sample which show the keratin network. Diffraction patterns in reciprocal space are analyzed with respect to signal orientation and anisotropy, which allows us to determine the local structure orientation in the sample.

2. Materials and methods

2.1. Cell culture

Human adrenal cortex carcinoma-derived SW-13 cells (ATCC CCL-105) [28], stably transfected with DNA encoding for fluorescent human keratin hybrids (HK8-CFP, HK18-YPF) [29–31], are generously provided by Rudolf Leube (RWTH Aachen, Germany). These so-called SK8/18-2 cells are grown in culture dishes in high glucose (4.5 g l$^{-1}$) Dulbecco’s modified eagle medium (E15-810, PAA Laboratories GmbH, Pasching, Austria) with 10% fetal calf serum (Invitrogen GmbH, Darmstadt, Germany), 100 units ml$^{-1}$ penicillin and 0.1 mg ml$^{-1}$ streptomycin (Sigma-Aldrich, Munich, Germany) at 37°C in a water-saturated atmosphere with 5% CO$_2$.

2.2. Sample preparation

Silicon nitride ($\text{Si}_3\text{N}_4$) membranes (frame size: $5 \times 5 \text{mm}^2$, frame thickness: 200 µm, membrane size: $1.5 \times 1.5 \text{mm}^2$, membrane thickness: 200–1000 nm, from Silson Ltd, Blisworth, England) are placed with the flat side facing up in a culture dish and covered with cell culture medium. Cells are detached from a confluent cultured dish with 0.25% (v/v) trypsin (Sigma-Aldrich) and 0.02% (w/v) EDTA (Roth, Karlsruhe, Germany) in phosphate buffered saline (PBS), suspended in medium and spread over the $\text{Si}_3\text{N}_4$ membranes [32]. After 1–2 days of incubation at 37°C in a water-saturated atmosphere with 5% CO$_2$, the samples are briefly washed with PBS, fixed by adding 4% paraformaldehyde solution (Sigma-Aldrich) for 15 min at room temperature and washed three times with PBS. At this stage, phase contrast and epifluorescence microscopy images of all samples are taken using an inverted microscope (IX 71, Olympus, Hamburg, Germany) equipped with 10× and 20× objectives. Fixation of the
cells with paraformaldehyde solution prevents changes in cellular morphology during further sample preparation and therefore allows for the comparison of microscopy images of single cells taken at different steps of the sample preparation. The samples are washed in ultrapure water to avoid salt precipitation on the membrane and plunge-frozen by injection into liquid ethane (Leica EM GP, Leica, Vienna, Austria) to prevent crystallization of water in the sample [33]. All samples are stored for at least 24 h in liquid nitrogen and subsequently lyophilized in a home-built freeze-drier. Afterwards, the samples are warmed up to room temperature and imaged again by phase contrast microscopy. The keratin network could not be imaged with fluorescence microscopy in the dry state, because the fluorophores are destroyed during sample preparation. Samples are stored over silica gel in a desiccator.

2.3. X-ray diffraction experiments

X-ray diffraction experiments are performed at the experimental hutch III of the beamline ID13 at the European Synchrotron Radiation Facility (ESRF, Grenoble, France). The beam is pre-focused by refractive beryllium lenses and monochromatized by a channel-cut Si(111) monochromator to a photon energy of 15.25 keV. Figure 1 shows a sketch of the setup downstream of the monochromator. The beam is focused on the sample (S) by nano-focusing parabolic refractive x-ray lenses (L) [34, 35] and cleaned by an electron microscopy aperture (A) and a pin hole (P), yielding a spot sizes of about $140 \times 110$ and $200 \times 125$ nm$^2$ (horizontal $\times$ vertical) at two different beamtimes and a primary beam intensity of about $3 \times 10^9$ cps. The samples are mounted on a scanning stage comprising a hexapod for coarse sample positioning and a piezo stage for fine translations during scans. Behind the sample, the primary beam is blocked by a beamstop (BS) and the scattered intensity is recorded.
Table 1. Overview of step sizes $\Delta_x$ and $\Delta_y$ in lateral directions, number of scan points $N_x \times N_y$, exposure time $T$, beam size $F$ in horizontal and vertical directions, and approximated radiation dose $D$ during diffraction scans. For a description of the estimation of the radiation dose during the scans see section 3.3.

<table>
<thead>
<tr>
<th>Scan</th>
<th>$\Delta_x$ ($\mu m$)</th>
<th>$\Delta_y$ ($\mu m$)</th>
<th>$N_x \times N_y$</th>
<th>$T$ (s)</th>
<th>$F$ (nm$^2$)</th>
<th>$D$ (Gy = J kg$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2</td>
<td>2</td>
<td>51 $\times$ 51</td>
<td>0.1</td>
<td>140 $\times$ 110</td>
<td>$2.7 \times 10^4$</td>
</tr>
<tr>
<td>2</td>
<td>0.25</td>
<td>0.25</td>
<td>37 $\times$ 37</td>
<td>10</td>
<td>140 $\times$ 110</td>
<td>$1.8 \times 10^8$</td>
</tr>
<tr>
<td>3</td>
<td>0.1</td>
<td>0.1</td>
<td>101 $\times$ 101</td>
<td>1</td>
<td>200 $\times$ 125</td>
<td>$1.1 \times 10^8$</td>
</tr>
</tbody>
</table>

using a Maxipix detector (ESRF) with a resolution of 516 $\times$ 516 pixels and a pixel size of 55 $\times$ 55 $\mu m^2$ at a sample-to-detector distance of about 0.9 m. For sample alignment prior to the x-ray measurements, a visible light microscope (M) is moved into the beam path. The focus of the microscope is adjusted to coincide with the x-ray focus, which allows for finding the focal plane of the x-ray beam for all samples easily. After alignment with the visible light microscope, coarse mesh scans with a step size of 1–2 $\mu m$ and 0.1 s exposure time are performed on a large sample area to assure precise sample positioning. X-ray dark-field images in real space are calculated by integrating the scattered intensity outside a chosen dark-field radius around the primary beam for each diffraction pattern in one scan. These values are then arranged in a two-dimensional array with dimensions corresponding to the number of scan points in the lateral directions. Therefore, each pixel in an x-ray dark-field image corresponds to the total scattered intensity at the corresponding scan position. The dark-field radius is chosen to just cover the primary beam on the detector. Figure 2(b) shows an x-ray dark-field image of a coarse mesh scan on the sample. Scans with smaller step sizes and longer exposure times are performed on selected regions of interest, as shown in figures 2(c) and (d). Here, we present results from three scans for which the experimental parameters are listed in table 1.

3. Results and discussion

3.1. Structure orientation in the sample

Based on the fluorescence microscopy images of the keratin network, as shown in figure 2(a), we particularly select cells with a distinct network morphology allowing for a correlation of results from x-ray diffraction experiments with structural information obtained from visible light microscopy. One interesting example is the cell in the center of figure 2(a) exhibiting a long extension (red arrow), which contains several thick keratin bundles. The corresponding x-ray dark-field image in figure 2(b), reconstructed from a coarse mesh scan with a step size of 2 $\mu m$ and an exposure time of 0.1 s, shows a high contrast between cellular material and substrate, even though biological samples are generally weak scatterers. In particular, the nuclei of the imaged cells show high scattered intensity, but also the periphery and the cellular extension pointed out in figure 2(a) can be clearly identified. Note that not all cells express keratin and therefore some cells, which are visible in x-ray dark-field images (and also in visible-light phase contrast images, not shown here), are not visible in the corresponding fluorescence microscopy images.
Figure 2. X-ray diffraction from highly aligned keratin bundles in freeze-dried cells. (a) Inverted fluorescence microscopy image of the keratin network in SK8/18-2 cells. The image is taken on paraformaldehyde-fixed cells, before plunge-freezing and freeze-drying. (b) X-ray dark-field image from a coarse scan on a large sample area with a step size of 2 µm and an exposure time of 0.1 s. (c) X-ray dark-field image from a region of interest scan recorded on the keratin-rich extension of one cell with a step size of 250 nm and an exposure time of 10 s. (d) Composite image of the diffraction patterns corresponding to the marked region in (c) showing a strong degree of orientation in the scattering signal.

On the keratin-rich extension, a region of interest scan with a smaller step size of 250 nm and a longer exposure time of 10 s is performed. Again, the dark-field image in figure 2(c) shows a high contrast between the cellular extension and the substrate. Further, the integral intensity is not homogeneous over the whole extension, but varies from scan point to scan point indicating a substructure in the sample. When looking at individual diffraction patterns, shown as composite image for a small region of this scan in figure 2(d), highly anisotropic scattering signals are visible, which indicate an oriented substructure in the sample. We can therefore not only distinguish between cellular material and empty regions, as shown by the dark-field images, but we can also use x-ray scattering patterns in reciprocal space to obtain information about the local sample structure.

Individual diffraction patterns from the sample exhibit highly anisotropic scattering signals, as can be seen from the raw data diffraction pattern in figure 3(a). The anisotropy of the
scattering signal, and accordingly the anisotropy in the sample structure, provides additional information to the x-ray dark-field contrast images. To capture the signal anisotropy we determine the overall degree and direction of signal orientation in each diffraction pattern by approximating the thresholded intensity distribution by an ellipse. For this analysis, we first process the raw data by masking dead or hot pixels and pseudo pixels without sensitivity, filtering the image with a $3 \times 3$ median filter to reduce the noise and thresholding the image.

*Figure 3.* Analysis of the orientation of structures in the sample. (a) Diffraction pattern obtained on the keratin-rich extension shows highly oriented scattering. (b) Processed and thresholded diffraction pattern together with a fitted ellipse (magenta), which has the same second moments as the intensity distribution. The major and minor axes of the ellipse are shown in cyan. (c) The orientation of the minor axis and the eccentricity of the ellipse are plotted as a vector field (arrowheads are omitted, due to no physical relevance in this case) in overlay with the corresponding dark-field image.
with a threshold value of 0.1 cps. To be able to treat diffraction patterns from empty regions, which show nearly no intensity in the thresholded diffraction patterns, with the same method, we add a circular intensity distribution in the central region of the diffraction pattern where the intensity is blocked by the beamstop. This assures that diffraction patterns from empty regions yield an approximation by a circle, which corresponds to no anisotropy in the scattering signal. The thresholded intensity distribution is then approximated by an ellipse, which has the same second moments as the intensity distribution. Figure 3(b) shows a thresholded diffraction pattern along with the approximated ellipse and the principle axes. From the ellipses we determine the direction of the minor axis in reciprocal space, which indicates the average direction of oriented structures in the sample in real space at the measured position, and the eccentricity, which gives a measure for the average degree of orientation. Together, the degree and direction of orientation in the sample can be visualized as a vector field, where the arrow length and direction are determined by the eccentricity and the direction of the minor axis of the ellipse, respectively. An overlay of the vector field, where the arrowheads of the vectors are omitted due to no physical relevance in this analysis, along with the corresponding dark-field image for the scan on the cellular extension is shown in figure 3(c). The vector field indicates structures in the sample that are oriented in parallel with the direction of the cellular extension, which is in-line with the fluorescence microscopy images of the keratin network in figure 2(a).

The same analysis is applied to a mesh scan with a step size of 100 nm covering an area of $10 \times 10 \mu\text{m}^2$ on a different cell of the same type. An overlay of the corresponding dark-field image and the orientation map is presented in figure 4 and a fluorescence microscopy image of the keratin network, where the scanned region in the periphery of the cell is marked by a red box, is shown as an inset. The dark-field image shows a network-like intensity distribution with apparent feature thicknesses of about 200–400 nm. This is at the resolution limit due to the used beam size. The local orientation, determined from scattering signal anisotropy, agrees very well with the direction of the elongated structures in the dark-field image. The feature thickness in the x-ray dark-field image along with the structure orientation indicate that the observed structures correspond to the keratin network in the sample. Differences between the x-ray dark-field image in combination with the orientation map, and the fluorescence microscopy image might be attributed to different factors. The fluorescence images have comparatively low resolution and therefore thin bundles are invisible. Further, the fluorescence microscopy images are taken on hydrated samples in buffer, whereas the diffraction experiments are performed on freeze-dried samples and artifacts due to plunge-freezing and freeze-drying cannot be excluded.

A typical method to determine the anisotropy in the scattering signal is by radial integration of the scattered intensity in a ring in reciprocal space and fitting the obtained azimuthal intensity curve. Here, the amplitude yields a measure for the degree of sample orientation. Further, specific structures with characteristic length scales in the sample can be visualized by selecting a certain region of momentum transfer. Recently, this method has been also applied to small angle scattering from biological samples, i.e. a mouse soleus muscle [26] or the dentinal collagen network in human teeth [25, 36, 37]. In contrast to these samples with well-defined and well-known real space structures, the structure of keratin bundles in our samples is probably not perfectly homogeneous and would therefore lead to scattering in different $q$-regions. Hence, in our case, it is important that the analysis allows for the determination of the anisotropy of the complete diffraction pattern and not solely of a selected region of momentum transfer in reciprocal space corresponding to a specific range of structure sizes in real space.
Figure 4. Orientation map and dark-field image of the keratin network in a freeze-dried eukaryotic cell reconstructed from a mesh scan with a step size of 100 nm and 1 s exposure time. The inset shows a fluorescence microscopy image of the keratin network recorded before freeze-drying and the scanned region is marked by a red box.

3.2. Radial intensity of averaged and single diffraction patterns

At each scan point a scattering pattern in reciprocal space is recorded. The scattering signal is analyzed by azimuthal integration of single and averaged diffraction patterns. To account for anisotropy in the scattering signal, the reciprocal space is divided into eight angular segments and the first and fifth segments are centered symmetrically around the major axis of the ellipse approximating the scattering signal. Average diffraction patterns recorded on the cellular extension and on the empty region are shown in figures 5(a) and (b) and the angular segments are indicated by dashed, white lines. For azimuthal integration of the average diffraction pattern from the empty region, the same positions of the angular segments as for the cell region are used. The insets indicate from which regions of the scan the diffraction patterns are used to obtain an average diffraction pattern from the cellular extension and the empty region. Only
Figure 5. Azimuthal integration of averaged diffraction patterns in eight angular segments. (a) Averaged diffraction pattern from the keratin-rich extension of the cell shown in figure 2. The angular segments 1–8, indicated by dashed white lines, are chosen symmetrically around the principal axes (cyan) of the ellipse (magenta) describing the orientation of the scattering signal. (b) Averaged diffraction pattern from the empty region. The positions of the angular segments are chosen identically to (a). The insets in (a) and (b) show from which regions of the scan (black pixels) the diffraction patterns are used to obtain an average diffraction pattern from the cellular extension and the empty region. The side length of the insets is 9.25 μm. (c) Radial intensity profiles from cell and background regions and (d) segment-wise background subtracted radial intensity profiles integrated in eight angular segments.

diffraction patterns recorded at the black positions are used for averaging. Azimuthal integration of the average diffraction pattern from cell and background regions yield the radial intensities shown in figure 5(c) and segment-wise background subtraction results in the radial intensity
profiles shown in figure 5(d). As expected intuitively, the radial intensity profiles in figure 5(d) can be classified into three groups: the intensity profiles in the direction of the major and minor axes of the ellipse show highest and lowest scattering intensities, respectively, and the intensity profiles in between the principle axes show intermediate scattering intensity. All radial intensity profiles can be well described by a power-law decay of the form $f(q) = aq^b + c$, as shown in figure 6 exemplarily for one segment. The power-law exponents obtained from the fits are in the range from $-4$ to $-4.25$. Here, leaving out data points in the lowest $q$-region for fitting slightly changes the power-law exponents, which stay, however, all below $-4$. Similar diffraction experiments on freeze-dried bacterial cells yielded exponents $-3 \leq b \leq -4$ for the decay of the radial intensity [38]. Corresponding to the well-known Porod law [39], compact two-phase systems with a sharp interface exhibit a power-law decay with $b = -4$ and further, power-law exponents smaller than $-4$ have been attributed to structures with diffuse boundaries [40, 41]. In our case, the power-law exponents are still close to the classical Porod exponent of $-4$, but consistently deviate to smaller numbers.

The radial intensities obtained from single diffraction patterns do not show a smooth decay, but a structured signal with weak scattering maxima and minima. Figure 7(a) shows a diffraction pattern recorded on the edge of the cellular extension along with dashed white lines indicating the segmentation for azimuthal integration. In figure 7(c), the corresponding radial intensities after segment-wise background subtraction are shown. For background subtraction the radial intensities of the averaged empty diffraction pattern (see figure 5(b)) are calculated with the segmentation of the single diffraction pattern and subtracted for each segment separately. Particularly, the radial intensities in the first and fifth segment, which are perpendicular to the local structure direction, show scattering maxima as indicated by arrows in figure 7(c). This intensity distribution might be explained as the form factor of a cylinder with a diameter of about 42 nm, which could correspond to a keratin bundle or sub-bundles. Deviations of the radial intensity from the form factor of a cylinder (fit data are shown in the supplementary information, available from stacks.iop.org/NJP/14/085013/mmedia) might be attributed to the
Figure 7. Azimuthal integration of a single diffraction pattern and an average of \(2 \times 2\) diffraction patterns in eight angular segments. (a) Single diffraction pattern from the edge of the keratin-rich extension and (b) average of \(2 \times 2\) diffraction patterns including the diffraction pattern shown in (a). The angular segments 1–8, indicated by dashed white lines, are chosen symmetrically around the principal axes (cyan) of the ellipses (magenta) describing the orientation of the scattering signal in each image. The insets in (a) and (b) show the positions (black pixels) of the displayed diffraction patterns in the scan. The side length of the insets is \(9.25\,\mu\text{m}\). (c) Radial intensity profiles from a single diffraction pattern and (d) from an average of \(2 \times 2\) diffraction patterns after background subtraction.

rough bundle surface due to protein side chains and the substructure of the bundle, which is made of several filaments with a diameter of about 10 nm.

In contrast to the above observation, the same analysis applied to an average of \(2 \times 2\) adjacent diffraction patterns, including the diffraction pattern shown in figure 7(a), reveals a smoother decay of the radial intensity profiles without pronounced maxima or minima in the scattering signal (see figures 7(b)–(d)). This finding indicates small changes in the peak
positions, which result in a smooth intensity decay after averaging. Therefore, the detailed intensity distribution in the scattering patterns in reciprocal space and correspondingly also the local sample structure in real space, sensitively depend on the position on the sample. Taking into account the beam size and step size during the scan, the average of $2 \times 2$ diffraction patterns covers an area of about $400 \times 400 \text{nm}^2$ on the sample and thus the maximum beam size for probing the local structure in this type of samples needs to be smaller than about $400 \times 400 \text{nm}^2$.

3.3. Dose estimation and radiation damage

Due to the small beam size and the high x-ray flux in our experiments, damage of the samples caused by the radiation cannot be avoided. Following the approach by Howells et al [42], we estimate the radiation dose based on the assumption that all cellular material consists of protein with the empirical average formula $\text{H}_{50}\text{C}_{30}\text{N}_{9}\text{O}_{10}\text{S}$ and mass density $\rho = 1.35 \text{g cm}^{-3}$. The average radiation dose during the scan is calculated using

$$D = \frac{I_0 T h \nu}{d \rho \Delta x \Delta y},$$

with $I_0 = 3 \times 10^9 \text{s}^{-1}$ being the primary beam intensity, $T$ the exposure time, $h \nu = 15.25 \text{keV}$ the photon energy, $d = 4.964 \text{mm}$ the attenuation length in the sample as obtained for the assumed average cellular material [42] (http://www.cxro.lbl.gov/), $\rho$ the mass density, and $\Delta x$ and $\Delta y$ the step sizes in the lateral directions. The estimates of the radiation dose in gray ($\text{Gy} = \text{J kg}^{-1}$) for the three scans as well as the scan parameters are listed in table 1. For fine scans with long exposure times and small step sizes the radiation dose is of the order of $10^8 \text{Gy}$. After these scans, sample thinning and shrinkage at the edges was observed, indicating visible damage of the sample due to the radiation. The visible effects of radiation damage on the sample might be reduced by cooling the sample with a cryo jet and by using frozen-hydrated instead of freeze-dried sample. However, this imposes further geometrical limitation to the set-up and especially the temporal stability might decrease due to thermal drifts.

4. Conclusion

We performed x-ray nano-diffraction experiments on freeze-dried eukaryotic SK8/18-2 cells to gain insight into the structure of cellular keratin bundles and networks. X-ray dark-field contrast was used to generate real space images of the sample. Here, structure sizes in the range of 200–400 nm could be observed. The signal anisotropy in individual scattering patterns was used to determine the local bundle orientation in the sample. Overlay images of orientation maps and x-ray dark-field images show a network structure that is in-line with fluorescence microscopy images of the keratin network in the sample.

The analysis of single diffraction patterns using azimuthal integration in different angular segments reveals maxima and minima in the scattering signal in the radial intensity profiles perpendicular to the local structure direction in the sample. The intensity distribution can be approximated by the form factor of a single bundle, which is assumed to be a cylinder with a diameter of about 42 nm. An average of $2 \times 2$ diffraction patterns shows no scattering maxima and minima, but a smooth decay of the scattered intensity. This indicates that the cellular samples show a heterogeneity in structural properties. Fits of a power-law decay to radial intensity profiles obtained from an average diffraction pattern of a complete scan yields...
exponents ranging from $-4$ to $-4.25$. The deviation from the well-known Porod law might be attributed to a rough sample surface or rough internal surfaces, which might be possibly caused by small molecules or peptides or substructure of the sample.

We have demonstrated that small-angle x-ray scattering as a well-established biophysical method for in vitro structure analysis of biomolecular assembly can be generalized in a way that is compatible with cellular structure analysis. To this end, a controlled nanometer-sized beam has been scanned over the cell, recording diffraction patterns at each scan position. In this proof of concept study, we have concentrated on the protein network of keratin IFs in epithelial cells. The examples of data analyses illustrate the different options of combining real space and reciprocal space information. Further progress can be anticipated if the dynamic range and the $q$-window of the diffraction data are increased, i.e. by stitching patterns recorded with and without a beamstop. In this case, a more complete description of the bundle structure will be possible. In summary, the combination of a high resolution in real space as well as in reciprocal space allows to probe different structural length scales simultaneously in the same samples. This method is applicable to a variety of different cellular and subcellular samples. Further, the high penetration depth of hard x-rays in biological material and water opens the possibility to study, e.g. hydrated cells in buffer or comparatively thick tissue samples.

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References

[34] Schroer C G et al 2003 Appl. Phys. Lett. 82 1485–7