Combined in-situ imaging of structural organization and elemental composition of substantia nigra neurons in the elderly

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Abstract

Human dopaminergic system in general, and substantia nigra (SN) neurons, in particular, are implicated in the pathologies underlying the human brain aging. The interplay between aberrations in the structural organization and elemental composition of SN neuron bodies has recently gained in importance as selected metals: Fe, Cu, Zn, Ca were found to trigger oxidative-stress-mediated aberration in their molecular assembly due to concomitant protein (alpha-synuclein, tau-protein) aggregation, gliosis and finally oxidative stress. In the present study, we demonstrate an integrated approach to the analysis of the structural organization, assembly, and metals' accumulation in two distinct areas of SN: in the neuromelanin neurons and neuropil. By using the highly brilliant source of PETRA III and the Kirkpatrick-Baez nano-focus, large area histological brain slices are scanned at the sub-neuronal resolution, taking advantage of continuous motor movement and reduced acquisition time. Elemental analysis with synchrotron radiation based X-ray Fluorescence (SRXRF) is combined with X-ray Phase Contrast Imaging (XPCI) to correct for inherent aberrations in the samples' density and thickness, often referred to as the mass thickness effect. Based on the raw SRXRF spectra, we observed the accumulation of P, S, Cl, K, Ca, Fe, Cu and Zn predominantly in the SN neurons. However, upon the mass thickness correction, the distributions of Cl became significantly more uniform. Simultaneously with the fluorescence signal, the Small Angle X-ray Scattering (SAXS) is recorded by a pixel detector positioned in the far-field, enabling fast online computation of the darkfield and differential phase contrast (DPC). The data has demonstrated the SN neurons and neuropil produces excellent contrast which is due to their different mass density and scattering strength, indicative of differences in local structure and assembly therein. In all, the results show that combined SRXRF-XPCI-SAXS experiments can robustly serve as a unique tool for understanding the interplay between the chemical composition and structural organization that may drive the biochemical age-related processes occurring in the human dopaminergic system.

Abbreviations

SN – Substantia Nigra;

SAXS – Small Angle X-ray Scattering;

XPCI – X-ray Phase Contrast Imaging;

CTF – Contrast Transfer Function;

KB – Kirkpatrick-Baez mirrors;

α -syn – alpha-synuclein;

DA – dopamine;

ROS - reactive oxygen species;

PD – Parkinson's disease;

OS – oxidative stress;

STXM - Transmission X-ray Microscopy;

NDP - nano-diffraction patterns;
DPC – differential phase contrast;
SR – synchrotron;
DFI – dark field intensity;
WG – waveguide;

Introduction

Human brain aging constitutes a mixture of neuropathological structural and chemical processes that cause the progressive neuronal loss [1,2]. Current evidence points to the aberration in the structure and assembly of neurons that is thought to be due to misfolding and aggregation of proteins: α -synuclein (α -syn) [3], tau [4] and A β [5], considered as major driving forces underlying both the physiological ageing and the most common neurodegenerative disorders: Parkinson's and Alzheimer's disease [6]. In the affected brains, the progression in the pathology is significantly faster, although the "clinical" boundaries between the normal aging and dementias are ambiguous and often contentious [7]. The transition bio-metals (Fe, Cu, Zn, Mn) have been shown to trigger the protein aggregation that poisons the neurons and promotes their excessive apoptosis or excitotoxicity, but the nature of this interaction remains unclear [8,9]. In particular, the age-associated neurotoxicity processes that take a toll on the neuromelanin-pigmented substantia nigra (SN) dopaminergic neurons generated considerable research interest due to their elevated propensities for accumulation of genetic mutations, improper handling of metabolic products of dopamine (DA) oxidation, and oxidative stress (OS) that result in production of cytotoxic reactive oxygen species (ROS), damage to cellular lipids/proteins, increased rate of neuronal loss, and dopamine depletion underpinning PD [10]. Notably, the nigral α -syn has recently emerged as a major causal risk factor to the pathology, since its aggregated form is particularly vulnerable to metals-driven ROS production [9]. In the basal conditions, the protein plays physiological functions, however, the presence of formidable amounts of Fe, together with DA and H₂O₂ causes the protein to fibrilize and become toxic [11]. Also note, in hyper-oxic conditions, Fe(II) was shown to be bound to the misfolded form of the nigral α -syn, oxidized to Fe(III), and released. As a matter of fact, H₂O₂ is a byproduct of this transition, which augments the OS-mediated neuronal burden [12]. Oppositely, Zn, Cu, Mn were found to participate in the anti-oxidative mechanisms that prevent the cells from the deleterious effects of OS, though some evidence argues they can also exert neurotoxic effects [9,13,14].

These and many other examples figure out the critical role of the interplay between metals and structural changes that underlie age-associated biochemical and structural burden of the neurons in the elderly [14]. However, to compromise sometimes contradictory evidence, and make the findings more integrated, a special attention must be paid to chemical/structural mapping (2D/3D) of the local extent to the neuronal burden. Currently, to probe *in-situ* the structural-chemical processes with the highest possible sub-cellular spatial resolution, highly brilliant synchrotron sources of coherent X-ray radiation have been offered for the non-invasive analyses of biological specimens in dynamic mapping and tomography modes [15]. Specifically, combined Small-Angle X-ray Scattering (SAXS) and Scanning Transmission X-ray Microscopy (STXM) allow for determination of both orientation and density of the nano-scale biological scaffolds throughout the analysis of nano-diffraction patterns (NDP) [16,17]. With the possibility for imaging of the differential phase contrast (DPC) and dark field intensity (DFI) signal, several studies demonstrated it is possible to extract the information on the scattering strength, electron density gradient, radius of gyration, and orientation of molecular assemblies (cytoskeleton, actomyosin fibers) from the NDPs of cells with excellent sub-micron resolution [18–20]. Unfortunately, to our knowledge, far too little attention has been paid to STXM studies of structural properties of SN tissue samples. Besides, there is an increasing body of enquiry on the 2D imaging of bio-metals in SN using Synchrotron Radiation-based X-ray Fluorescence spectroscopy (SRXRF) [14,21]. By using highly collimated 10-20 keV SR-beams of X-rays,

aberration in the levels of selected transition metals: Fe, Cu, Zn, Mn, Se was reported in thin (10-20 μm) freeze dried autopsy SN specimens taken from PD-affected [22–24] and normally-aged [25] human individuals as well as in the cell cultures of primary DA-ergic cells [13]. However, due to variation in the sample's thickness and density, often referred to as the mass thickness effect, the quantification of elemental mass fractions is a challenging task [26]. To get rid of this issue, SRXRF experiments must be combined with methods to determine the sample's electron density which is, in turn, proportional to the mass thickness of biological elements (in the absence of large stoichiometric variations in hydrogen). One of these methods is the X-Ray Phase Contrast Imaging (XPCI), which has been proven its usefulness in the fully quantitative SRXRF imaging of biological specimens [26]. In addition, unlike to the absorption X-ray imaging, the phase contrast radiography with highly collimated beams of X-rays has provided with significantly higher sensitivity, which was recently used to unravel the morphology of synuclein-positive Levy bodies found in the SN neurons of the PD-affected human individual [27]. Unfortunately, these and many other similar experiments were not combined with SAXS, SRXRF studies to provide with more cogent chemical–structural information on the SN tissue. Therefore, there still remains a need for co-localizing the metals' fractions to any possible alterations in structure and assembly of the human SN neurons.

Ramifications arising from exploring the interplay between chemical and structural age-associated changes within the human dopaminergic system may substantially boost our knowledge on the extent to the neuronal burden, necessary for understanding the processes that drive the human brain aging, Parkinson's disorder, and other dopamine-related alterations [13,14,21,22,24,36]. Recently, synchrotron X-ray based techniques: SRXRF [13,21,22,24–26,37], XPCI[26,27,38], SAXS [18–20] and many other have offered an *in-situ* analysis of biological specimens with the sub-cellular spatial resolution and ultra-low sub-ppm sensitivity for detection of chemical elements. However, despite huge technical advances in the state-of-the-art of instrumentation and methodologies, the integrated approach to tackling this critical problem by a single measurement is still pending [21]. Besides the instrumental issues, one has to stress the matrix-effects-related problems due to the inherent heterogeneity of biological specimens when the goal is the quantification of elements. So far, the problem has been addressed in our latest work, and the effect was found to significantly affect the elemental fractions in SN computed without correction [36]. In order to address current technical and analytical challenges in the combined structural/chemical analysis of thin (heterogeneous) biological specimens, the major objective of this study was to design, optimize and test a combined setup enabling quasi-simultaneous implementation of SRXRF, XPCI and scanning SAXS techniques. In addition, by combining the first two methods, our work extends the previous studies on the influence of the mass thickness effect and overcomes this artifact by a correction scheme.

In our present study, we demonstrate for the first time simultaneous high-resolution XPCI-SRXRF-STXM experiments using the highly brilliant source of PETRA III. Unlike to previous studies, this unique combination extends the chemical information obtainable from sole SRXRF imaging by structural information which can be derived from SAXS nano-diffraction patterns. We also show that by applying combined SRXRF-XPCI, a significant mass thickness effect can be avoided, yielding a corrected elemental ratio between bio-metals in the neurons with respect to the surrounding tissue. In addition, we also demonstrate the usefulness of the approach for possible structural and chemical analysis of sub-micron cellular deposits.

Sample preparation

The substantia nigra tissue specimens were taken during routine autopsy section from the aged individual not affected by any persistent neurological alterations. Following the autopsy, the tissue samples were rapidly frozen in -80°C . Just before the experiment, the SN samples were cryosectioned in -18° onto either 20 or 25 μm thick serial sections ($n=2$ sections used in this study), mounted onto 200 nm-thick silicon nitride membranes (Si_3N_4 , Silson Ltd., UK) and dried in a deep freezer at -80°C .

in darkness. Neither any paraffin embedding and chemical fixation were utilized to end up with a specimen in a pristine state. More details on the sample preparation protocol could be found elsewhere [24,25].

The beamline

All the experiments have been performed on the P10 coherence beamline at PETRA III (Hamburg, Germany) using the nano-focus GINIX setup, equipped with the pair of elliptically-shaped Kirkpatrick-Baez mirrors' [28,29]. In the focal plane, the X-ray beam spot size was 326 nm x 392 nm in horizontal and vertical dimensions, respectively [20]. The setup is sketched in Fig 1.

XPCI full-field measurements

The beamline arrangement for the full-field XPCI experiments is shown in Fig 1a. The 8 keV X-ray beam was primarily focused using the KB mirrors. In the primary focus, to provide the cone-like full-field coherent illumination, the waveguide optics was installed, and the sample tower was located downstream. The raw images were acquired using an sCMOS camera (2048 x 2048 pixels) with the directly coupled fiber-optics input (Photonic Science, UK) at the pixel size of 6.54 μm , while the sample-detector distance was 5.187 m. The effective pixel size was of 0.189 nm, which enabled the areas of 387 μm x 387 μm per a single exposition to be scanned. For the sake of the phase-retrieval procedure, the images were measured in either four or five different z_1 positions, as described below. The waveguide optics was aligned before and after the measurement of a whole sample area by using an automated script. The flat-field images were measured twice: following to the waveguide alignment (25 expositions, each of 1 s), and after completion of a sample's scanning. The SN sample areas were raster scanned in a manner that the resultant image was compromised of 6 x 6 (horizontal x vertical) adjacent shots. Every single SN area was measured with the dwell time set to 1 s. To control for the sample position, a motorized stage (with a nanometer precision) and an on-line microscopy were utilized.

Combined nano-SAXS and SRXRF experiments

For the scanning nano-SAXS and SRXRF microprobe experiments, the energy of the primary beam was switched to 9.68 keV, and the sample was put directly to the focus of the KB mirrors' system (cf. Fig 1b). The samples were analyzed by translating the sample in the XY focal plane (with fixed z coordinate). For the SRXRF experiment, an SDD single-point detector (KETEK, Munich, Germany) was installed at the angle of about 60° with respect to the oncoming SR beam. The intensity of the primary exciting beam was about $I_0 = 1.1 \times 10^{11}$ ph/s. To control for the localization of the regions of interest, an on-line microscope was utilized. A specimen was raster scanned with dwell time set to 5 s and with the 2- μm step in both directions. At the same time, the nano-SAXS experiments were performed using a distant Pilatus 300K (Dectris, Baden, Switzerland) detector placed 5.187 m upstream the sample position.

Phase retrieval

The transmission of an X-ray wave through a sample causes simultaneous attenuation of the wave amplitude and a shift in its phase as governed by the complex transmission function (for one dimension) presented below:

$$T(x) = e^{-B(x)/2} e^{i\varphi(x)} \quad (1)$$

Where: $B(x)$ denotes the absorption coefficient, and $\varphi(x)$ describes the phase shift by the sample.

The main idea behind all phase retrieval algorithms is to separate and extract the phase shifts from the X-ray intensity images. By assuming the slowly varying phase and weak absorption, the information on the phase retardation due to the propagation of X-rays across a specific specimen is encoded within the intensity images in a way it is filtered out by a periodic function of the second power of the selected spatial frequency as shown in Eq. 2, often referred to as the Contrast Transfer Function (CTF). For the slowly varying phase ($|\varphi(x) - \varphi(x + z\lambda f)| \ll 1$) and by neglecting the absorption ($B \ll \varphi$), one can devise a formula for computing the intensity in Fourier space:

$$\tilde{I}_z(f) = \delta(f) + 2\sin(\pi z\lambda|f|^2)\tilde{\varphi}(f) \quad (2)$$

Where: $\tilde{I}_z(f)$ - intensity in the reciprocal domain; $\tilde{\varphi}(f)$ - Fourier transform of the phase; z - the propagation distance; λ the wavelength of the X-ray beam; the $2\sin(\pi z\lambda|f|^2)$ term is often referred as the phase-contrast transfer function.

According to the propagation distance z , the contribution arising from some of the spatial frequencies is lost as the filtering sinus function reaches its zero-crossing points. To get rid of it, the lost frequencies must be included in a manner some other images are acquired in different positions to shift up the set of their zero-crossing points. The phase retrieval procedure used herein was based the CTF phase retrieval algorithm involving measurements of sample images at several positions downstream the X-ray beam [30]. In our experiments, the data was analyzed using the home-made code written in MATLAB. For each area scanned, a single shot was reconstructed separately, and the resultant set of pictures was finally stitched by means of the procedure implemented in Fiji [31]. Upon the phase retrieval, the phase-reconstructed images represent the phase shifts, which are inversely proportional to the electron density since the phase shift is described as [30]:

$$\varphi(x, y) = -\frac{2\pi}{\lambda} \int \delta(x, y, z) dz \quad (3)$$

$$\delta \approx \frac{2\pi r_e}{k^2} \rho_e(x, y, z) \quad (4)$$

Where: λ - wavelength, δ - the decrement, r_e - the classical radius of an electron, k - the wavenumber, ρ_e - the electron density.

The distributions of the phase in the sample plane reconstructed by the CTF algorithm were then used to correct the fluorescence maps, by simple division, justified by the proportionality of phase and projected mass following from Eq. 3 and 4, by assuming the real part of the refractive index is directly proportional to the sample density:

$$\delta(x, y, z) = \alpha \cdot \langle \rho(x, y, z) \rangle \quad (5)$$

Where: α - a constant, $\langle \rho(x, y, z) \rangle$ - the sample projected density.

By combining Eq. 4 to the Eq. 3, it is apparent the sample mass thickness (m_p) is directly proportional to the phase shift determined (cf. Eq. 5):

$$\varphi = C\langle \rho \rangle T = C m_p \rightarrow m_p \sim \varphi \quad (6)$$

Where: T - the projected sample thickness, $\langle \rho \rangle$ - the average thickness of a sample, C - a constant.

However, since the absolute phase does not have a meaning here, and only the relative phase shifts between the empty beam and matter are of importance, for the data processing, the images should be shifted so that the empty beam corresponds to zero. Furthermore, to avoid division by zero, a small offset (regularization) has to be added to the phase. In the present example, we ended up with a range

of 0.1-1.5 radian for the entire phase map used for normalization of the fluorescence map to avoid the mass thickness matrix effect. For this study, before the autopsy sections, the written consents from the individuals' family members had been obtained. The whole study was carried out in line with the Polish and European law, and approved by the Jagiellonian University Medical College.

Curve fitting of SRXRF spectra

The X-ray spectra were analyzed by means of a curve-fitting approach with the routine batch-fitting tool of PyMca [32]. The PyMca's STRIP algorithm was used to approximate the background. For the sake of curve-fitting, the least-squares Levenburg-Marquardt iterative algorithm involving the pseudo-Voigt fitting model was used. For the further analysis, the net-peak values of the background-subtracted K_α lines of P, S, Cl, K, Ca, Fe, Cu, Zn were taken to showcase the elemental distribution in a form of 2D-maps. The characteristic spectral lines arising from other biologically active elements could not be recognized.

Correction of the mass thickness effect

Since the net-peak area of a K_α line of an element is directly proportional to its areal mass, the quantity that is directly proportional to the elemental fraction may be obtained upon division by the phase shift (cf. Eq. 5) [33]:

$$C_i \sim \frac{A_i}{\varphi} \sim \frac{m_i}{m_p} \quad (6)$$

Where: A_i - the net peak area of the K_α line of the i -th element; φ - the phase shift; m_i - the areal mass of the i -th element; m_p - the projected areal mass of a sample.

To implement the normalization by the phase map, the SRXRF maps were first resized and bilinearly interpolated. Following this, the maps were then registered with the XPCI one used as a reference image using the DFT-based algorithm implemented in MATLAB [34]. Once completed, the registered elemental distributions were carefully divided by their size-matched XPCI images (cf. Fig 4).

Analysis of nano-SAXS patterns

The data was processed based on an automated script (MATLAB) with interactive feedback, containing the following steps. First, the direct beam on the pixel detector (beamstop and surrounding area with KB tails) was identified and removed by the rectangular mask. Next, the differential phase contrast, and the darkfield intensities were computed according to [35]. For the differential phase contrast, the first moment of the diffraction pattern, and its respective components, referred to as DPCx and DPCy, were computed and mapped for the entire scan (cf. Fig 5-6). In addition, the dark field was computed as the integrated intensity of the pattern integrated without counting the masked area. Next, the average diffraction patterns of the neurons and neuropil areas were determined separately, based on a one-step binarization of the DPCy image to single out the points belonging to distinct tissue areas of SN (cf. Fig 5).

Results and Discussion

Fig 2 shows the CTF-reconstructed overview image of a selected large SN tissue area. From this image, one can conclude that the contrast for SN neurons is sufficient to visualize them without using any chemical fixation, based on their significantly elevated electron density as demonstrated previously by [27]. This is in line with our previous work, suggesting an inhomogeneous distribution of the mass thickness in the SN tissue [36]. Also visible in the image are streak-like objects which can

be attributed to cholesterol crystals previously identified by Fourier Transform Micro-spectroscopy [39]. The bright area surrounding the crystal in the middle was possibly due to its elevated refraction index which could violate the assumption of slowly varying phase, which could lead to the reconstruction artifact. Based on the large XPCI images, the smaller areas containing SN neurons were identified and subjected to further combined high-resolution SRXRF-STXM scanning. Turning now into SRXRF analyses, Fig 3a shows the characteristic lines attributed to major biologically active elements: P, S, Cl, K, Ca, Fe, Cu and Zn could be identified which is in line with [22,24,25]. Following curve fitting, selected spatial distributions were presented for the two selected sample areas (see Figs 3b-c). All of the above elements show elevated elemental concentrations within the neural cells. Importantly, the foci are perfectly colocalized with the neuromelanin rich dark spots, as presented in its microscopic image in Fig 2b. In the right corner of the maps in Fig 2c, the object with a very high contribution of all the elements is apparent, which, as demonstrated in Fig 2c is likely to be due to its dramatically elevated density. Fig 4 shows that upon the mass thickness correction, the distribution becomes significantly less peaked than the uncorrected equivalent in Fig 3. This remark illustrates that the mass thickness effect indeed influences the results of the quantification, and could lead to overestimated numerical values of the chemical fractions for high-density tissue areas when using simply the external standard method. The most important observation emerging from this data is the distribution of Cl becomes significantly more homogenous, and the values of its K_{α} lines in the neuron bodies seem to be even reduced as compared with the surrounding tissue, which reflects the extraneuronal concentrations of Cl^{-} which contribute to the resting membrane potential [40]. This result is also in agreement with our previous studies utilizing the relative area of the incoherently scattered X-ray radiation used as a correction factor in the quantitative analysis of SN tissue [36]. The group of neurons in Fig 3b contains four separate cells. Interestingly, in the left corner of this picture, we could recognize one single object with the elevated abundance of both Zn and Fe.

Finally, to get deeper understanding towards the structural organization of the SN tissue areas, the nano-SAXS experiments have been performed and the data was analyzed as described above. Fortunately, building upon the differential phase contrast and dark field signal, the SN neurons were found to come up with sufficient contrast without any sort of staining, which reflects their physical properties (density, the organization of the material) are different than those of the surrounding neuropil tissue. Specifically, Fig 5c, which shows the dark field intensity, confirms that the neurons exhibit increased scattering intensity compared to the surrounding tissue areas. At the same time with the dark-field, Fig 5a-b shows that differential phase contrast components: DPCx and DPCy are also elevated in the neuronal bodies. Note that the different level of DPCx and DPCy results from the scanning direction. Since the differential phase is proportional to the gradient of the projected mass across an irradiated sample spot [18], this supports the idea of significantly increased variability in the distribution of the electron density in the SN neurons, reflecting their pronounced internal histological structure. At the same time, the extraneuronal spaces appear more homogenous in the DPCy and DPCx. To compute the average nano-diffraction patterns for the tissue areas of SN, the DPCy distribution was then subjected to the one-step binarization (cf. Fig 5b). As presented in Fig 5c, the average diffraction for the neuron-like areas was found of the significantly higher intensity of the dark field in the SN neurons implying their increased scattering length which reflects their internal structural heterogeneity and the increased concentration of the matter. The rectangular artifact next to the center of the patterns is caused by the far field of the KB mirrors' system. Fig 6 shows the same analysis performed for the second SN area analyzed. The values of the DPCx, DPCy and dark field intensities behave in the same way as for the specimen in Fig 5. What is also interesting, a small focalized object was identified at the top of Fig 6a, exhibiting the significant increase in the dark field. The object was identified with peculiar scattering patterns of two distinct types: a regular ring-like intensive scattering with the presence of multiple tiny scattering patterns (Fig 6a), and a streak-like profile with oriented aberration of the dark-field distribution [19]. This might be attributed to possible

fibrillar tissue deposits in the specimens that could be the glial cell. At the same time, the object was of the elevated fraction of Fe and Cu. Recently, the GFAP-positive glia in the aged brains of rodent brains in the subventricular zone were found to accumulate excessive amounts of redox active metals – Cu, Fe [41]. Therefore, given the size of the object (several microns), one may conclude the object found in our specimen may be a glial cell enriched in Fe and Zn, while the structure of the deposit seems to be ordered. Another possibility is the deposit may be an iron-rich micro-domain that could be present in the brains of human individuals as highlighted by *Bohic et al*, though, besides the chemical mapping, the authors did not show any structural analysis to assess for their structural origination and assembly [23]. Nevertheless, this still unresolved issue illustrates combined SRXRF-nano-SAXS measurements could possibly be capable of identifying tiny tissue deposits, and can extend the chemical information by showing some new means to describe physical properties of biological specimens throughout a carefully combined analysis of SRXRF spectra and NDPs.

Altogether, the present work demonstrates the SN neurons have radically increased electron density, which causes their mass thickness to be higher as compared with the surrounding neuropil areas. The analysis of NDPs led to the conclusion that the matter inside the neurons is of unordered structure, though the Fe-rich inclusions found in the neuropil seem to represent ordered fibrillar material, which, given the size, could be possibly either the glial cell or Fe-rich micro-domain. The correction of the K_{α} lines against the mass thickness produced distributions that are more blurred and less focalized as the raw ones, suggesting the matrix effect should be taken into consideration while analyzing heterogeneous biological samples using quantitative imaging by the SRXRF microprobe.

Conclusions

Our study introduces a combined XPCI-SRXRF-nano-SAXS analysis of heterogeneous biological specimens for the structural and aberration-free quantitative imaging of biological elements *in-situ*. Also, the methods allow for the dynamic raster scanning using coherent synchrotron X-ray beam with high spatial resolution. A careful analysis of diffraction patterns, along with the assessment of SRXRF spectra may in turn complement the information gathered from fluorescence spectra in such a manner that the chemical information may be combined with the local organization and assembly of analyzed material. In all, our work, though preliminary, paves the way towards multi-mode analysis of biological specimens combining purely chemical information with structural organization of the human SN tissue.

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Fig.1. The schemes of the beamline arrangements used in the experiments: (a) the XPCI setup (b) the setup for simultaneous nano-diffraction and SRXRF scans. Abbreviations: KB - the Kirkpatrick-Baez mirrors, F - the primary focus, WG – the waveguide optics, S – the sample tower, and D – the detector, XRFD - the XRF detector, BS – the beam stop. The WG-S (Z_1) distance was of 15 cm, while the S-D (Z_2) distance was of 5.187 m. It is assumed the SR beam propagates toward the Z-axis.

Fig 2. (a) The CTF-reconstructed overview image of a large part of the SN tissue specimen; (b) microscopic image of the labeled tissue area (c) the magnified area that was further the subject of high resolution SRXRF-SAXS experiments.

Fig 3. (a) The sum curve-fitted SRXRF spectrum with the K_{α} lines of the biologically active elements labeled; (b), (c) the 2D distribution of the raw net peak areas for some elements in two selected samples cut onto 25 (b) and 20 μm (c) (scalebars=25 μm).

Fig 4. The electron-density-corrected distributions of the K_{α} elements presented for selected elements for two different SN sample areas. In the first row of each line, there is a CTF-reconstructed image of a specific area, while the remaining ones represent the corrected maps (scale bars=25 μm).

Fig 5. The results of the nano-SAXS experiments, where: (a), (b), (c) show the 2D distribution of DPcx, DPcy and dark field intensities, respectively; (e) shows the binarized DPcy image; (d), (f) are the averaged nano-diffraction patterns for neuropil and neuronal areas, respectively; (scalebars=25 μm).

Fig 6. (a), (b), (c) the DPCy, DPCx and dark field images of the specimen, respectively; (d),(e) the scattering patterns taken from two adjacent points drawn from the tiny object could be found at the top of Fig 6a (scalebars=25 μm); (f) the raw SRXRF spectrum taken from the deposit.