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Sample preparation for energy dispersive X-ray imaging of biological tissues

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Abstract

Traditional electron microscopy (EM) can be complemented with analytical EM to increase objective sample information enabling feature identification. Energy dispersive X-ray (EDX) imaging provides semi-quantitative elemental composition of the sample with high spatial resolution (~ 10 nm) in ultrathin sections. However, EDX imaging of biological samples is still challenging as a routine method because many elements are at the detection limit for this technique. Moreover, samples undergo extensive preparation before analysis, which can introduce disruptive X-ray cross-talk or artifacts. EDX data can, for instance, be skewed by (i) osmium interference with endogenous phosphorus, (ii) chlorine present in EPON-embedded tissues, (iii) lead interference with endogenous sulfur, and (iv) potential spectral overlaps with grid material, contrast agents, and the in-microscope sample holder. Here, we highlight how to circumvent these potential pitfalls and outline how we approach sample preparation and analysis for detection of different elements of interest. Utilization of well-considered a priori sample preparation techniques will best ensure conclusive EDX experiments.

1 Introduction

(Scanning) transmission electron microscopy, or (S)TEM, is widely applied to provide high-resolution images of cellular ultrastructure. The contrast is caused by varied electron density within a sample and results in a singular, gray-scale read-out. Therefore, TEM/STEM image analysis is sometimes subjective and requires expert human interpretation. There are several ways to enhance the information using multimodal microscopy ([Ando et al., 2018](#)), including correlated light and electron microscopy (reviewed in [de Boer, Hoogenboom, & Giepmans, 2015](#) as well as elemental imaging (reviewed in [Pirozzi, Hoogenboom, & Giepmans, 2018](#)). Multimodal microscopy allows better identification of biological structures and its addition to (S)TEM is explored by many. The analytical method of energy dispersive X-ray (EDX) analysis provides elemental composition information that can be displayed as color overlays for “ColorEM” ([Pirozzi et al., 2018](#)). Here, we present our routine approach of EDX imaging of ultrathin epoxy resin-embedded biological samples with emphasis on the workflow from living sample to final analysis of ColorEM.

1.1 EDX

Energy dispersive X-ray (EDX) analysis is a method of elemental detection via the collection of characteristic X-rays induced by electron beam radiation (Fig. 1). The characteristic X-rays, generated by ionization events (Fig. 1B), are mapped (Fig. 1C) resulting in imaging of elemental concentrations (Fig. 1D and E) aiding in objective feature identification. Cellular components can be distinguished with EDX by analyzing the presence of carbon, oxygen, nitrogen, phosphorus, and sulfur (Pirozzi et al., 2018).

Although EDX is routinely applied in geology and material science, recent technological advances in silicon drift X-ray detectors (Newbury & Ritchie, 2015) make it feasible for the life sciences. EDX can be implemented with the addition of an X-ray detector to a SEM system or a TEM system with STEM mode. Here, we focus on EDX imaging in a SEM, and areas are selected after analysis of large-scale STEM imaging. ColorEM via EDX of biological samples has the potential to answer research questions with a palette of colors, almost as vast as the periodic table of elements. Proper foresight of sample preparation methods will best ensure the success of the EDX analysis.

1.2 Sample preparation

Before entering the EM, biological samples undergo extensive sample preparation steps: fixation, dehydration, resin-embedding, and heavy-metal contrasting. Biological samples are composed of light-weight elemental species, thus heavier elements, with higher electron density, are added for contrast, including neodymium, osmium, lead, and uranium. These methods have been optimized for ideal ultrastructural imaging. However, for proper EDX analysis sample preparation must be considered and tailored to specific elements of interest. In addition, the material of the section holder, as well as the in-microscope sample holder, can affect analysis. Here, we show the differences in EDX elemental mapping from varied sample preparation protocols to support biological EDX experimental design.

2 Routine chemical fixation of tissues

Primary aldehyde fixation crosslinks proteins to secure cellular structure however, it neither adds electron density nor adequately fixes lipids (Maunsbach & Afzelius, 1999). A secondary fixation with osmium tetroxide adds heavy-metal contrast and retains lipid membranes. The exact chemical reactions of the osmium ions are not completely known, but they have been shown to most readily bind to the carbon-carbon double bonds in unsaturated lipids. Further oxidation states of osmium ions react with some carbohydrates and finally with some amino acids, cross-linking the lipids to the protein scaffold created by the aldehyde fixation (Glauert & Lewis, 1998). The osmium contrast can be further enhanced if applied with an reducing

FIG. 1

EDX analysis to map atoms. (A) Nitrogen electron binding and $K\alpha$ X-ray energies. (B) The incident electron beam ionizes the atoms by ejecting valence electrons. When ionization occurs in the inner electron shells, an electron from a higher energy electron orbital fills the created vacancy. The electromagnetic radiation released is roughly equal to the surplus of the electron binding energies and is classified as an X-ray. (C) A detector collects X-rays in the vacuum chamber. (D) The X-ray spectrum provides a semi-quantitative elemental fingerprint. (E) The counts of each element are spatially mapped to reveal relative elemental composition at the cellular level.

Adapted and modified from Pirozzi, N. M., Hoogenboom, J. P., & Giepmans, B. N. G. (2018). ColorEM: Analytical electron microscopy for element-guided identification and imaging of the building blocks of life. *Histochemistry and Cell Biology*, 150(5), 509–520. doi: 10.1007/s00418-018-1707-4.

agent (White, Mazurkiewicz, & Barnett, 1979), like iron-containing cyanides (de Bruijn, 1968). Potassium ferrocyanide forms a stable complex with osmium tetroxide (Karnovsky, 1971) and more intensely stains membranes, microtubules, and filaments than with osmium tetroxide alone (Maunsbach & Afzelius, 1999).

Despite its advantages, osmium can obstruct characteristic X-rays from potential elements of interest. Most osmium characteristic X-rays are detected at the $M\alpha$ energy level at 1.910 keV. A disadvantage of osmium-containing post-fixation is the qualitative analysis of phosphorus, which has a $K\alpha$ energy level of 2.014 keV. Phosphorus is an essential element for life and important for cellular component elemental fingerprinting of DNA, RNA, and phospholipids (Pirozzi et al., 2018). For EDX questions regarding phosphorus localization, sample preparation with osmium-containing post-fixation may affect the results. An alternative, non-heavy metal post-fixation protocol utilizes tannic acid (Maunsbach & Afzelius, 1999) and *p*-phenylenediamine (Hayat, 1986) to stabilize lipid membranes and other cellular components, preventing them from extraction during dehydration. A related heavy metal compound, ruthenium tetroxide, reacts to similar functional groups: unsaturated hydrocarbons, alcohols, aldehydes, ethers, and amines (Sawyer, Grubb, & Meyers, 2008), without EDX interference with phosphorus. Here, we compare STEM images, elemental maps, and spectra of four post-fixation protocols: Non-heavy Metal, Osmium, Reduced Osmium, and Ruthenium.

2.1 Protocol

1. Rat pancreas tissue was chosen because it offers a variety of cell types. Fresh tissue was cut into 1-mm cubes, then fixed by immersion in 2% paraformaldehyde/2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2). Tissue was stored at 4 °C in fixative until further processing.
2. Tissue was cut into ~40 μ m sections with a vibratome (HM 650 V, MICORM International GmbH, Dreieich, Germany). Before sectioning, tissue was rinsed four times with phosphate buffered saline (PBS) and then embedded in 4% low melting point agarose, which was subsequently glued to the mounting stage with cyanoacrylate. The vibratome bath was filled with PBS. During sectioning, the 40 μ m sections were collected with a paint brush and placed into four separate 8 mL glass vials containing circa 3 mL PBS.
3. The tissue sections were processed by varied post-fixation protocols in the 8 mL glass vials at room temperature unless stated otherwise:
 - a. Non-heavy Metal: Tissue sections were placed in 1% tannic acid in 0.05 M maleic acid (pH 5.2) for 40 min on ice. Sections were rinsed twice with maleic acid, and serially dehydrated with 30% ethanol for 10 min, 50% ethanol for 10 min, 70% ethanol with *p*-phenylenediamine for 20 min, 100% ethanol thrice for 30 min.
 - b. Osmium: Tissue sections were placed in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer for 1 h at 4 °C. Sections were rinsed with water

three times and then serially dehydrated with 30%, 50%, 70%, 100%, 100%, and 100% ethanol for 10, 10, 20, 30, 30, and 30 min, respectively.

- c. **Reduced Osmium:** Tissue sections were placed in 1% osmium tetroxide and 1.5% potassium ferrocyanide in 0.1 M sodium cacodylate buffer for 1 h at 4 °C and then treated as described above.
 - d. **Ruthenium:** Tissue sections were placed in the contents of a freshly broken ampule of 0.5% ruthenium tetroxide in water for 1 h at 4 °C and then treated as described above.
4. After dehydration, the 100% ethanol was removed from the vials containing the tissue sections and replaced with a 1:1 mixture of 100% ethanol and EPON. The vials were placed on a laboratory roller shaker overnight. The next day, fresh EPON replaced the 1:1 mixture. The glass vials were covered with Parafilm under the screw-top to prevent any tissue sections from getting stuck in the cap. The vials were placed on rollers for at least 3 h. Then tissue sections were flat embedded between two Aclar embedding film sheets (Electron Microscopy Sciences, Hatfield, Pennsylvania), with a small piece of paper denoting the EM number for sample record keeping. After polymerization at 58 °C overnight, sections were visibly inspected for presence of islets of Langerhans with a bright field Leica DM E compound microscope (Leica Microsystems, Vienna, Austria). Sections containing islets (when applicable) were cut out and glued to blank resin stubs with non-polymerized EPON and cured overnight at 58 °C.
 5. Blocks were coarsely trimmed with a Leica EM RAPID (Leica Microsystems, Vienna, Austria) and finely trimmed by hand with a single edge razor blade. Ultra-thin sectioning at 100-nm thickness was performed on a Leica EM UC7 ultramicrotome (Leica Microsystems, Vienna, Austria). Sections were collected from the diamond knife water boat (Diatome Ltd, Nidau, Switzerland) with a carbon single hole grid and placed on formvar film prepared on a domino rack (Electron Microscopy Sciences, Hatfield, Pennsylvania). Sections were dried for at least 15 min at 58 °C. Note that 100 nm is thicker than the standard 60–80 nm typically used for EM, but provides a larger interaction volume leading to more EDX signal without relevant loss in resolution.
 6. To stabilize the section under the electron beam before large-scale EM acquisition, sections were pre-irradiated in CM100-bio TEM (FEI, Eindhoven, Netherlands) for at least 1 h at 80 keV acceleration voltage at low magnification (200–400×); longer pre-irradiation for non-contrasted samples is recommended, because less electron scattering delays stabilization.
 7. Large-scale nanotomography (Sokol et al., 2015) scans were acquired of the islet-containing region with a STEM detector in a Zeiss Supra 55 SEM (Zeiss, Jena, Germany). Imaging was performed with samples in the STEM 12-sample carousel (Fig. 2A) at 28 keV acceleration voltage, 352 pA current, 30 μm aperture, 3 μs dwell time, and 2.5 nm pixel size. Selected regions were further analyzed with EDX.

FIG. 2

EDX holders. Our standard STEM holder ((A) at the bench and (B) in the microscope) is an aluminum-based carousel with 12 grid positions fastened by a copper ring. The EDX holder (C, D) is a set of carbon cylinders fabricated to hold one grid each. In SEM, the holders are tilted to 25° toward the X-ray detector and a working distance of 7.5 mm is used to achieve the best X-ray counts per second for our system. Note that this excludes use of the STEM detector as it no longer fits beneath the sample. The carbon cylinders of the EDX holder are fastened to stubs with a double-sided carbon adhesive. The diameters of the two centered holes drilled are 1.5 and 3.05 mm with depths of 3.0 and 0.50 mm, respectively (E).

8. EDX imaging was performed with an X-Max 150 mm² silicon drift detector (Oxford Instruments, Abingdon, UK) in the Zeiss Supra 55, using a type II secondary electron (SE2) detector for sample navigation. The samples were placed in a homemade, dedicated EDX holder (Fig. 2C–E), composed of carbon cylinders attached to aluminum SEM stubs with double-sided carbon adhesives. Two centered holes were drilled into each carbon cylinder, 3 mm deep with a

1.5 mm diameter and 0.5 mm deep with a 3.05 mm diameter (Fig. 2E), fitting one sample. Acquisition settings were: 20 keV acceleration voltage, 6.1 nA current, 120 μ m aperture, 1 ms dwell time, 7.5 mm working distance, 25° tilt toward detector as shown in Fig. 2D, 1 frame with 1024 pixels width, 5 nm pixel size, and process time 5, resulting in an imaging time of approximately 20 min. The acceleration voltage of 20 keV was used in order to analyze the $L\alpha$ line of osmium (8.912 keV).

9. The pre-irradiation of the experiment in Fig. 3 was inadequate to stabilize the section resulting in nanotomy imaging artifacts of areas exposed more to the electron beam. The bleaching at the tile overlaps and focus spots was corrected by:
 - (a) Large regions void of tissue were manually selected and set to a gray value of 130.
 - (b) Tile overlap areas were automatically corrected using a home-built FIJI macro included on the website, which normalizes gray values line by line (3 pixels wide) horizontally and then vertically.
 - (c) Bleached focus spots were corrected in FIJI by subtracting the intensity by approximately 15 in the manually selected areas.
 - (d) Tiles were stitched using Photomerge/collage/blending option in Adobe Photoshop.
10. Elemental maps were constructed by Oxford AZtec software from signal from energy ranges noted in Table 1. The osmium maps depict the $M\alpha$ line signal and the “osmium high energy” map the $L\alpha$ line signal. The maps were exported as TIF files and the levels were adjusted linearly in Adobe Photoshop.
11. Spectra represent the map sum of all X-rays collected in the EDX imaged region. Both spectra were smoothed and normalized (low energy normalization at 1.125–1.175 keV and high energy normalization at 13.20–13.55 keV) by Oxford AZtec software. Characteristic X-ray lines were manually placed with Adobe Illustrator.

2.2 Results and discussion

Nanotomy of tissue overviews and exocrine cells showed the varied appearance due to post-fixation (Fig. 3A). Endocrine cells in the Non-heavy Metal protocol sample were indistinguishable due to lack of contrast. In the Ruthenium protocol sample, there was no islet to image for the nanotomy scan because ruthenium tetroxide readily reacted with the 40 μ m tissue sections, obscuring tissue organization and thereby preventing islet selection. Furthermore, the ruthenium tetroxide did not fully penetrate the tissue (Fig. 3A). EDX elemental maps (Fig. 3A) were affected by the close proximity of P $K\alpha$ and Os $M\alpha$ energy lines (Table 1; Fig. 3B). The phosphorus signal strength of the heterochromatin was compared to the endoplasmic reticulum (ER), the secretory granules, and the lipid membranes (Table 2). The phosphorus signal in the Non-heavy Metal protocol sample was highest in the heterochromatin

FIG. 3

Overlap of phosphorus and osmium. EDX elemental maps from different post-fixation methods reveal discrepancies of perceived phosphorus localization. (A) Large-scale STEM overview and exocrine cells show varied contrast per protocol as indicated. Heterochromatin of the nucleus (h), secretory granules (g), endoplasmic reticulum (er), and membranes (m) are indicated. The structures have different relative phosphorus signals depending on post-fixation protocol used. (B) Map sum spectra of the exocrine cells exhibit the phosphorus-osmium spectral overlap, Os $M\alpha$ at 1.910 keV and P $K\alpha$ at 2.014 keV. The iron (Fe $K\alpha$) is most prominent in the Reduced Osmium sample. Higher energy osmium signal (Os L lines) produces a lower quality elemental map (A, Osmium High Energy). Elements with potential overlap are indicated. Bars: top 10 μm ; all others 2 μm . See www.nanotome.org for full resolution maps.

Table 1 Energy ranges used to construct each elemental map.

Element	X-ray line	Energy (keV)	Map range (keV)
Nitrogen	K α	0.392	0.362–0.421
Phosphorus	K α	2.014	1.966–2.061
Sulfur	K α	2.308	2.254–2.361
Chlorine	K α	2.622	2.569–2.675
Ruthenium	L α	2.559	2.505–2.612
Neodymium	L α	5.230	5.157–5.304
Osmium	M α	1.910	1.863–1.958
	L α	8.912	8.821–9.003
Lead	M α	2.346	2.291–2.400

Table 2 Perceived phosphorus content of cellular components varies by post-fixation protocol.

Structure	Post-fixation			
	Non-heavy metal	Osmium	Reduced osmium	Ruthenium
Heterochromatin	++	++	++	++
Endoplasmic reticulum	+	+	++	+
Secretory granules	–	+	+	–
Membranes	–	+	++	–

The interpretation is based on the data shown in Fig. 3. The phosphorus signal is denoted as negligible (–), present (+), and strong (++). Non-heavy Metal and Ruthenium protocol samples have the same pattern of perceived phosphorus.

of the nucleus, with a weaker signal in the ER, very little signal in the secretory granules, and no distinct signal from the membranes (Fig. 3A). The similarity of the phosphorus maps of Non-heavy Metal and Ruthenium protocols suggests that they represent the validity of phosphorus content for aldehyde-fixed, EPON-embedded tissue. This pattern varied with the samples post-fixed with osmium-containing protocols. Measured phosphorus with the Osmium protocol increased in secretory granules and membranes compared to the Non-heavy Metal protocol (Table 2; Fig. 3A). Compared to the Osmium protocol, the Reduced Osmium protocol increased the osmium in the sample (Fig. 3) and had more signal in the membranes and ER as expected (Glauert & Lewis, 1998; Maunsbach & Afzelius, 1999; White et al., 1979). The features standing out in the osmium maps of Osmium and Reduced Osmium protocols (Fig. 3A) were consistent with those increased in phosphorus (Table 2). This was also found in Durcupan and Spurr's resins (results not shown). Correction of the phosphorus map by image subtraction is difficult because the osmium signal is much higher than the phosphorus signal. Analysis

of osmium at a higher energy characteristic X-ray, $L\alpha$ at 8.912 keV, was possible (Fig. 3B) but levels of detection did not have the signal to noise ratio to produce maps with the same spatial clarity (Fig. 3A). The iron is derived from potassium ferrocyanide. Iron's $L\alpha$ line potentially disrupts manganese and/or fluorine analysis (Table 3).

Table 3 Potential spectral overlaps according to sample processing step.

Post fixation				Embedding			
Osmium (Os) [76]		Iron (Fe) [26]		Chlorine (Cl) [17]		Calcium (Ca) [20]	
$M\alpha_1$	1.910	$L\alpha_1$	0.7050	$K\alpha_1$	2.622	$K\alpha_1$	3.691
Si $K\alpha$	1.740	Mn $L\alpha$	0.6374	S $K\alpha$	2.308	Sn $L\alpha$	3.444
W $M\alpha$	1.775	F $K\alpha$	0.6768	Pb $M\alpha$	2.346	Sb $L\alpha$	3.605
Sr $L\alpha$	1.807			Bi $M\alpha$	2.423	Te $L\alpha$	3.769
Re $M\alpha$	1.843	$K\alpha_1$	6.404	Tc $L\alpha$	2.424	I $L\alpha$	3.938
Y $L\alpha$	1.923	Eu $L\alpha$	5.846	Ru $L\alpha$	2.559		
Ir $M\alpha$	1.980	Mn $K\alpha$	5.899	Rh $L\alpha$	2.697		
P $K\alpha$	2.014	Gd $L\alpha$	6.057	Pd $L\alpha$	2.839		
Zr $L\alpha$	2.042	Tb $L\alpha$	6.273				
Pt $M\alpha$	2.051	Dy $L\alpha$	6.495				
		Ho $L\alpha$	6.720				
$L\alpha_1$	8.912	Co $K\alpha$	6.930				
Cu $K\alpha$	8.048	Er $L\alpha$	6.949				
Ta $L\alpha$	8.146						
W $L\alpha$	8.398						
Zn $K\alpha$	8.639						
Re $L\alpha$	8.653						
Ir $L\alpha$	9.175						
Ga $K\alpha$	9.252						
Pt $L\alpha$	9.442						
Au $L\alpha$	9.713						
Contrasting							
Neodymium (Nd) [60]		Lead (Pb) [82]		Uranium (U) [92]			
$M\alpha_1$	0.9780	$M\alpha_1$	2.346	$M\alpha_1$	3.171		
Ce $M\alpha$	0.8830	Au $M\alpha$	2.123	Pd $L\alpha$	2.839		
Pr $M\alpha$	0.9292	Nb $L\alpha$	2.163	Ag $L\alpha$	2.984		
Cu $L\alpha$	0.9297	Hg $M\alpha$	2.195	Th $M\alpha$	2.996		
Zn $L\alpha$	1.012	Tl $M\alpha$	2.271	Cd $L\alpha$	3.134		
Na $K\alpha$	1.041	Mo $L\alpha$	2.293	In $L\alpha$	3.287		
		S $K\alpha$	2.308	K $K\alpha$	3.314		

Continued

Table 3 Potential spectral overlaps according to sample processing step.—Cont'd

Contrasting							
Neodymium (Nd) [60]		Lead (Pb) [82]		Uranium (U) [92]			
Lα_1	5.230	Bi M α	2.423	Sn L α	3.444		
Ce L α	4.840	Tc L α	2.424				
V K α	4.952	Ru L α	2.559				
Pr L α	5.034						
Cr K α	5.415	Lα_1	10.552				
Pm L α	5.432	Au L α	9.713				
Sm L α	5.609	Ge K α	9.886				
		Hg L α	9.989				
		Tl L α	10.269				
		As K α	10.544				
		Bi L α	10.839				
		Se K α	11.222				
Grid				Microscope holder			
Nickel (Ni) [28]		Copper (Cu) [29]		Magnesium (Mg) [12]		Aluminum (Al) [13]	
Lα_1	0.8510	Lα_1	0.9297	Kα_1	1.253	Kα_1	1.487
Fe L α	0.7050	Ce M α	0.8830	Eu M α	1.131	Ho M α	1.348
Co L α	0.7762	Pr M α	0.9292	Ge L α	1.188	Se L α	1.379
La M α	0.8330	Nd M α	0.9780	Tb M α	1.240	Er M α	1.406
Ce M α	0.8830	Zn L α	1.012	As L α	1.282	Tm M α	1.462
Pr M α	0.9292	Na K α	1.041	Dy M α	1.293	Br L α	1.480
				Ho M α	1.348	Yb M α	1.521
				Se L α	1.379	Lu M α	1.581
Kα_1	7.478	Kα_1	8.048				
Co K α	6.930	Yb L α	7.416				
Er L α	6.949	Ni K α	7.478				
Tm L α	7.180	Lu L α	7.656				
Yb L α	7.416	Hf L α	7.899				
Lu L α	7.656	Ta L α	8.146				
Hf L α	7.899	W L α	8.397				
Cu K α	8.048	Zn K α	8.639				
Ta L α	8.146	Re L α	8.653				

Here, we focus on the α lines of the primary introduced/enhanced elements (Element (abbreviation) [atomic number]) and all the other α lines within 10% of the primary α line's energy, excluding noble gases. X-ray energies in keV.

Previously, a reduction in perceived phosphorus was found in pancreatic alpha cell granules when protocols similar to the Non-heavy Metal and Reduced Osmium were compared (Scotuzzi et al., 2017). It remained unclear whether the reduction in phosphorus signal was due to the absence of the interfering osmium signal or more extraction of phosphorus-containing biomolecules under osmium-free conditions. As a further control, ruthenium tetroxide was used because it reacts with many of the same molecules as osmium tetroxide, with the exception of acid and ester functional groups (Sawyer et al., 2008). The phosphorus map of the Ruthenium protocol matched neither phosphorus maps of Osmium nor Reduced Osmium protocol samples. This also suggests that the added phosphorus signal in osmium-treated samples was indeed from either the retention of phosphorus-rich molecules or spectral overlap with osmium. Thus, EDX imaging of phosphorus is impacted by osmium, an alternative using ruthenium made tissue very brittle and dark in color.

3 Resin

EPON became one of the epoxy resins of choice for its rapid penetration of tissues, sufficient contrast, widely tunable hardness, and adequate sectioning (Luft, 1961). Other epoxy resins have specific uses such as Durcupan and Spurr's (Luft, 1973). Although EPON is widely used in biomedical EM, an unexplained chlorine artifact has been detected when the samples were analyzed with EDX. Relatively high chlorine content in EPON-embedded tissue has been reported since the early days of X-ray microanalysis (Becker, 1979). Here we demonstrate the chlorine artifact observed in EPON-embedded tissue and how it can be mitigated by using an alternative epoxy resin.

3.1 Protocol

1. Tissue was processed as described in Step 1–2 of Section 2.1 using the Reduced Osmium protocol (Step 3.c of Section 2.1).
2. Tissue sections were flat embedded (Step 4 of Section 2.1), but in three resins:
 - (a) EPON
 - (b) Durcupan
 - (c) Spurr's
3. Vibratome sections were selected and thin sections were made (Step 5 of Section 2.1) and pre-irradiated for at least 2 h per sample and imaged as described in Step 6–7 of Section 2.1.
4. EDX analysis of cells selected from the nanotome scans was performed with the sample in the EDX holder at 15 keV acceleration voltage, 5.6 nA current, 120 μ m aperture, 100 μ s dwell time, 7.5 mm working distance, 25° tilt toward the detector (Fig. 2D), 10 frames with 1024 pixels width, 4 nm pixel size, and a process time of 4, resulting in an imaging time of approximately 20 min. Spectra of 100 nm thick sections of blank resin were acquired similarly but in 30 s

over a $1\text{ }\mu\text{m}^2$ area because spectra for these homogeneous areas remained consistent with longer analysis times.

5. Chlorine elemental maps (Table 1) were constructed by AZtec software, exported as TIF files, and the levels were adjusted linearly in Adobe Photoshop.
6. Spectra represent the map sum of all X-rays collected in the EDX imaged region. Spectra were smoothed and normalized at 3.1–3.5 keV by AZtec software. Characteristic X-ray lines were manually placed with Adobe Illustrator.

3.2 Results and discussion

The nanotomography scans showed minimal ultrastructural variation among tissue embedded in EPON, Durcupan, and Spurr's resins (Fig. 4A). Ultrathin sections of Spurr's resin were most challenging to acquire because the sections readily adhered to each other and all pre-irradiated sections contained folds (Fig. 4A). Chlorine signal was strongest in tissue embedded in EPON resin (Fig. 4B), while absent in blank EPON (Fig. 4B) and in cryo-sectioned cells without resin (data not shown). Similar to the unexplained chlorine in EPON-embedded tissue, calcium was detected in Durcupan-embedded tissue but not in blank Durcupan (Fig. 4B) and there was no specific localization in the calcium map. The EDX chlorine maps (Fig. 4A) represented cellular features more clearly in EPON-embedded tissue than tissue embedded in Durcupan or Spurr's resins. In early spectroscopy experiments, Becker (1979) described the chlorine signal being higher in tissue compared to EPON resin regions void of sample. The chlorine map displays a specific pattern in the nuclear envelope, secretory granules, and mitochondria. Chlorine signal could have a connection with post-fixation because less chlorine was detected when the tissue was post-fixed with the Non-heavy Metal protocol compared to Osmium and Reduced Osmium protocols (data not shown). Furthermore, Non-heavy Metal post-fixed tissue had more chlorine and calcium when embedded in Spurr's resin than when embedded in EPON and Durcupan, respectively (data not shown).

For EDX analysis and imaging, the presence of chlorine or calcium is mostly disruptive for EDX analysis of the elements themselves. Analysis of chlorine is a challenge because chloride ions will be lost during sample preparation. There are no endogenous elements with potential spectral overlap, however, exogenous elements with potential spectral overlap are shown in Fig. 4B. Although sulfur ($K\alpha$ at 2.308 keV) and lead ($M\alpha$ at 2.346 keV) are within 10% of Cl $K\alpha$, we have never experienced an analysis problem. This may hold true with the other possible conflicting elements.

4 Contrasting

Adding heavy metals to biological samples, or contrasting, is necessary to visualize ultrastructure. However, for EDX, use of certain contrast agents can interfere with the characteristic X-rays from the elements of interest. Routine contrasting is done

FIG. 4

EDX chlorine artifact from EPON embedding. Rat pancreas tissue post-fixed with Reduced Osmium Protocol and embedded as indicated. (A) Nanotomography (top), SE2, and the chlorine elemental map from the boxed areas are shown. Spurr's resin was readily adhesive to itself during sectioning resulting in folds. (B) Map sum spectra of exocrine cells in three epoxy resins are compared to spectra of sections of blank epoxy resin. Note that more chlorine is seen in EPON-embedded tissue. Durcupan-embedded tissue contributes to a calcium peak. The chlorine or calcium signals can potentially interfere with the elements shown below the x-axis. Bars: top 10 μm , all others 1 μm . See www.nanotomography.org for full resolution maps.

with uranium, lead, and -in our lab- most recently neodymium (Kuipers & Giepmans, 2020). Aqueous uranyl acetate binds to phosphate groups in nucleic acids and phospholipids, and the carboxyl groups in proteins (Dykstra, 2012). Contrasting with lead salts, such as Reynolds lead citrate (Reynolds, 1963), stains membranes and glycogen granules (Maunsbach & Afzelius, 1999), as well as amplifies what has already been stained by osmium tetroxide (Dykstra, 2012) or uranyl acetate (Hayat, 1986). Uranium is radioactive and toxic, an alternative is neodymium acetate (Kuipers & Giepmans, 2020). Lead citrate overlaps with (endogenous) sulfur (Tables 1 and 3). Through consideration of potential spectral overlap, contrast agents can be chosen or omitted for successful EDX experiments.

4.1 Protocol

1. Prepared tissue (Step 1, 2.b of Section 3.1) was sectioned (Step 5 of Section 2.1).
2. Contrasting with neodymium acetate or uranyl acetate:
 - (a) Neodymium acetate contrasting was performed on droplets on Parafilm. The section-side of the grid was placed on a droplet of aqueous 4% neodymium acetate for 20 min, followed by rinsing with water on subsequent droplets.
 - (b) Uranyl acetate contrasting was performed similarly with 5% uranyl acetate.
3. Sections were further contrasted with lead citrate on droplets on Parafilm in a glass petri dish containing pellets of sodium hydroxide to absorb excess carbon dioxide, which would cause precipitates on the sections. The section-side of the grid was placed on a droplet of Reynold's lead citrate for 2 min. Then, the grid was dipped 20 times in five sequential beakers of water to quickly remove lead citrate. The grid was air-dried on filter paper.
4. The regions chosen for EDX analysis were pre-irradiated, followed by SE2 and EDX imaging. The EDX imaging parameters were: 15 keV acceleration voltage, 6.1 nA current, 120 μ m aperture, 1 ms dwell time, 7.5 mm working distance, 25° tilt toward detector, 1 frame with 1024 pixels width, 8 nm pixel size, process time 5, resulting in an imaging time of approximately 20 min.
5. Elemental maps were constructed by Oxford AZtec software for sulfur, lead, and neodymium (Table 1).
6. The spectra shown in Fig. 5B were constructed from regions containing the cell depicted in the lower right corner of both views (Fig. 5A). The (insulin-producing beta) cell chosen has a higher sulfur content to better illustrate the sulfur-lead overlap. The spectra shown in Fig. 5C are map sums from neodymium/lead and uranium/lead contrasted sections to illustrate the different neodymium and uranium characteristic X-ray lines. Using Oxford AZtec software, spectra were smoothed and Fig. 5B were normalized at 2.8–3.0 keV, Fig. 5C “Nd M lines” was not normalized, and Fig. 5C “U M lines” “Nd L lines” were normalized at 4.5–5.0 keV. Characteristic X-ray lines were manually placed with Adobe Illustrator.

FIG. 5

Contrasting with lead obscures sulfur detection. (A) Serial sections of rat pancreas were imaged with SE2 and EDX; one without added contrast, and the other contrasted with neodymium acetate and lead citrate. The elemental maps of sulfur and lead for the contrasted section are nearly identical due to the close proximity of sulfur's K α and lead's M α lines. (B) Spectra of the sections show how the sulfur signal is overpowered by the presence of lead. (C) Contrasting with neodymium acetate or uranyl acetate adds numerous potentially troublesome peaks to the EDX spectrum. Bars: 5 μ m.

4.2 Results and discussion

While the contrast agents added electron density to certain components, improving contrast in the electron EM images (Fig. 5A, SE2 images), the addition disrupted the EDX spectrum (Fig. 5B and C). The biggest conflict was lead's interference with endogenous sulfur. The lines' close proximity caused the elemental maps of lead and sulfur to be identical (Fig. 5A). If sulfur is interesting for analysis, we therefore do not contrast with lead.

Heavier contrast agents have more characteristic X-ray lines which simplify fingerprinting an individual element but cause more spectral overlaps with other elements. In Fig. 5C, the major and minor lines are shown for neodymium and uranium. Note that even lines with low relative energy can be troublesome when analyzing conflicting elements of interest in low concentration.

5 Grids and sample holder

The materials of the single hole section holder, or grid, as well as that of the sample holder should be tailored to the element(s) of interest of a given experiment.

5.1 Protocol

1. Rat pancreas tissue was prepared (Step 1–2, Step 3.c of Section 2.1, Step 2.b of Section 3.1).
 - (a) For the grid comparison, sequential sections were cut (Step 5 of Section 2.1) but on 1 mm hole grids of carbon, nickel, and copper. The sections were contrasted (Step 2.b–3 of Section 4.1), regions were pre-irradiated in the SEM, and EDX imaging was performed with the EDX holder.
 - (b) For the in-microscope sample holder comparison, sections were cut (Step 5 of Section 2.1) and contrasted (Step 2.a, Step 3 of Section 4.1). Two regions were both analyzed in the EDX holder and STEM holder (Fig. 2B and D) with brief pre-irradiation in the SEM for stabilization.
2. EDX imaging parameters were: 15 keV acceleration voltage, 5.6 nA current, 120 μ m aperture, 1 ms dwell time, 7.5 mm working distance, 1 frame with 1024 pixels width.
3. Elemental maps were constructed by AZtec software for nitrogen, phosphorus, osmium ($M\alpha$), and lead (Table 1).
4. Spectra represent the map sum of all X-rays collected in the EDX imaged region. Spectra were smoothed by AZtec software. Characteristic X-ray lines were manually placed with Adobe Illustrator.
 - (a) For the grid comparison, the lower energy spectra were normalized at 1.2–1.4 keV and the higher energy spectra were normalized at 3.05–3.20 keV.
 - (b) For the in-microscope sample holder comparison, the spectra were similar for both regions; spectra from region A are shown. The lower energy spectra were not normalized and the higher energy spectra were normalized at 5.4–5.5 keV.

5.2 Results and discussion

There was no notable difference observed with SE2 and EDX imaging of sections on grids of carbon, nickel, and copper (Fig. 6A). Elemental maps of light and heavy elements showed little variation. The nickel and copper signals were clear in the map sum spectra (Fig. 6B). Care should be taken if the element of interest is in close proximity to the α lines, shown in Fig. 6B, but also the lower intensity β lines noted in Fig. 6C. For example, a β line of copper (Cu $K\beta_1$ at 8.905) has an overlap with an α line of osmium (Os $L\alpha$ at 8.912). Such spectral overlaps could be problematic for interfering elements of low concentration. Use of carbon grids ensures no addition of hindering metals, however, the geometry of the grid calls for altered handling. Pyrolytic carbon grids are not completely flat and have a thickness of approximately 200 μm compared to flat TEM copper or nickel grids with a 25 μm thickness. Due to the uneven base surfaces of carbon grids, the grid often shoots away when handled with tweezer contact on the bases and thus should be gripped on the 200 μm thick lateral sides. Other considerations to keep in mind with carbon grids are possible air bubbles when placed on a liquid droplet and X-ray shielding during EDX imaging, depending on grid orientation. The sample section is supported by a formvar film on one base side, creating a shallow 200 μm valley which traps air when placed on a droplet. For any on-grid protocols, such as section contrasting or post-embedding immunolabeling, copper or nickel grids are recommended. All EDX analysis is performed with the film-side closest to the X-ray detector to prevent any X-ray shielding caused from the thickness of the carbon grid.

The in-microscope sample holder used for EDX imaging had minimal effect on the quality of the elemental maps, but the EDX spectrum was affected by introduction of elemental peaks from the material of the holder (Fig. 7). The STEM holder is aluminum-based with a copper grid holder and contributed to the EDX spectrum with signal from oxygen, aluminum, magnesium, calcium, and copper (Fig. 7B). The maps of these added elements resembled the pattern of electron density, like the SE2 image, due to extra scattering at those regions, causing more ionization of the sample holder. The EDX holder produced elevated background signal (Fig. 7B, right) but was effective in not adding any other elements to the EDX spectrum. The homemade EDX holder is conductive, reusable, and the hole keeps the grid stable during analysis at a 25° tilt. To remove the grid, minimizing possible damage to the sample, the SEM stub was loosened from the multi-holder for pin stubs, lifted with pin stub SEM mount grippers, and inverted over clean filter paper. The only disadvantage of using the EDX holder after STEM imaging is time of holder transfer, including SEM chamber vacuum venting and pumping.

6 Other considerations

6.1 Section thickness

Section thickness can be used to maximize signal to noise in favor of the element of interest. The interaction volume is the region from which ionization events occur within the sample, and the origin of the element of interest determines if a larger

FIG. 6

See figure legend on opposite page.

or smaller interaction volume eases analysis. A thicker section is better suited for EDX analysis of sulfur-rich insulin, for example. The granules are approximately 300 nm in diameter, thus resin sections of 100 nm result in complete cross-sections of many granules, providing a concentrated volume rich of the targeted structure containing the element of interest. Thicker sections (up to 150 nm) would be beneficial for spatially clear maps of sulfur in the insulin granules compared to thinner sections. On the contrary, if small nanoparticles are sought to be detected, atop a section from post-embedding immunolabeling, for example, a thinner section would be advantageous. Decreasing the interaction volume would increase the signal to noise ratio thus, a thinner section is best.

6.2 Current

With higher current the ionization events in a sample increase, resulting in higher sensitivity. Lighter elements and low concentrations of higher weight elements make up the bulk of biomedical EDX imaging, and thus require high current for adequate signal. For all the EDX imaging shown here, the largest aperture (120 μm) and High Current Mode were used on the Zeiss Supra 55 SEM. These settings increased current at the cost of resolution compared to the settings of nanotomography STEM imaging (30 μm aperture).

6.3 Acceleration voltage

The ionizing energy of the incident electron must be greater than or equal to the binding energy of the electron. The surplus energy (overvoltage) generally accepted is a factor of 1.5–3 to increase the signal to noise ratio of EDX spectroscopy (Friel *et al.*, 2017). It is unclear if this is always sufficient for EDX imaging. For example, an overvoltage of >2 produced a clear osmium $L\alpha$ peak in the map sum spectra (Fig. 3B) but, it was not sufficient for a clear EDX image (Fig. 3A).

FIG. 6

Grid material must be considered if the element of interest has an overlap with the grid material. Serial sections of rat pancreas were placed on single hole grids of similar geometry (1 mm hole) but different material as indicated at the top (carbon, nickel, and copper). (A) SE2 and EDX analysis show little variation between elemental maps for light elements (nitrogen, phosphorus) or heavy elements (osmium, lead). (B) Grid choice needs to be taken into account if the characteristic X-rays of the element(s) of interest have a potential overlap with the grid material. Note the alteration of background signal in the second view. There appears to be a drop in background signal at energies higher than the $K\beta$ emission lines of the grid material. (C) Use of a nickel or copper grid introduces more characteristic X-ray lines than use of a carbon grid. Bars: 2.5 μm .

FIG. 7

In-microscope sample holder adds to EDX spectrum. (A) The sample was imaged first in the EDX holder, followed by placement in the STEM holder for region (A), and vice versa for region (B). No striking difference is observed in the elemental maps of light elements (nitrogen, phosphorus) nor heavy elements (osmium, lead). (B) The map sum spectra reveal compositional differences of the holders. There is an expected increase in the carbon (C K α) signal when the sample is imaged in the EDX holder. The STEM holder increases the oxygen signal (O K α) and introduces magnesium (Mg K α), aluminum (Al K α), calcium (Ca K α), and copper (Cu K α) signals. Bars: 2.5 μ m.

6.4 Sample tilt

Because X-rays emit from the sample in all directions, the sides of the in-microscope sample holder can block some X-rays from reaching the detector. The shielding is alleviated by tilting the sample. In addition, tilting the thin section changes the angle at which the incident electron beam meets the sample, elongating the path through the sample and increasing the interaction volume. For all the EDX imaging shown here, the in-microscope sample holder was tilted 25° toward the X-ray detector in the SEM.

7 Conclusion

Biological EDX imaging is still in its infancy with much potential for improvement. It is difficult to predict whether an elemental peak overlap will hinder analysis, as there is no rule of thumb for the energy proximity. There are system artifacts that vary per EDX set-up and sample preparation protocol thus, it is very important to keep all conditions similar throughout an experiment. Slight alterations in sample preparation can reap dramatically different results in regard to post-fixation, resin embedding, and contrasting. We hope the methods explained here provide a foundation for the design of an EDX experiment tailored to the elements of interest.

8 Materials

8.1 Equipment

1. Vibratome, HM 650 V, MICORM International GmbH, Dreieich, Germany
2. Refrigerator (4°C) for fixative, buffer, and sample storage
3. Refrigerator (4°C) for osmium storage and post-fixation
4. Laboratory roller mixer
5. Oven at 58°C
6. Leica DM E compound microscope, Leica Microsystems, Vienna, Austria
7. Stereo microscope, Zeiss, Jena, Germany
8. Leica EM RAPID, Leica Microsystems, Vienna, Austria
9. Ultramicrotome: Leica EM UC7, Leica Microsystems, Vienna, Austria
10. CM100-bio TEM, FEI, Eindhoven, Netherlands
11. Supra 55 SEM and STEM detector, Zeiss, Oberkochen, Germany
12. STEM 12-sample carousel, Zeiss, Oberkochen, Germany
13. X-Max 150 mm² silicon drift detector, Oxford Instruments, Abingdon, UK
14. Aztec software, Oxford Instruments, Abingdon, UK

8.2 Materials

15. Fixative: 2% paraformaldehyde/2% glutaraldehyde in 0.1 M sodium cacodylate buffer
16. 0.2 M Sodium cacodylate buffer pH 7.3

17. Phosphate buffered saline
18. Low melting point agarose, VWR Chemicals
19. Cyanoacrylate glue 10 V, Ruplo Lijmtechniek, Ten Boer, Netherlands
20. Paintbrush
21. 8 mL glass vials
22. 2% osmium tetroxide in water
23. 3% potassium ferrocyanide in 0.2 sodium cacodylate buffer
24. 0.5% ruthenium tetroxide in water
25. EPON: Glycid ether 100; 2-Dodecenylsuccinic acid anhydride; Methyl nadic anhydride, SERVA Electrophoresis GmbH, Heidelberg, Germany
26. Durcupan ACM, MFCD00130983, Sigma
27. Spurr's resin: Nonenylsuccinic anhydride; D.E.R. 736; Dimethylaminoethanol; ERL 4221, Sigma-Aldrich Inc.
28. Parafilm
29. ACLAR 33C Embedding Film, Cat. # 50425-10, Electron Microscopy Sciences, Hatfield, Pennsylvania
30. Single edge razor blades, Cat. # 71974, Electron Microscopy Sciences, Hatfield, Pennsylvania
31. Wooden sticks
32. Tweezers, no. 5 Dumont, Montignez, Switzerland
33. Ultra 45° diamond knife, Diatome Ltd, Nidau, Switzerland
34. Glass slides
35. 1% Toluidine blue in 1% sodium borate
36. Hot plate
37. Domino rack, Cat. # 70620, Electron Microscopy Sciences, Hatfield, Pennsylvania
38. Formvar, Article F6146, Sigma
39. Film-making apparatus
40. Copper 1 mm hole grids, Cat. # 1000-CU, Electron Microscopy Sciences, Hatfield, Pennsylvania
41. Nickel 1 mm hole grids, Cat. # 1000-NI, Electron Microscopy Sciences, Hatfield, Pennsylvania
42. PELCO Pyrolytic carbon grids 1 mm hole, Ted Pella, Inc., Redding, California
43. EDX holder: 10 mm JEOL carbon stub, G323 Agar Scientific; 12 mm diameter Carbon Tabs, G3347N, Agar Scientific; 0.5" SEM Pin Stubs 6 mm length, G301F, Agar Scientific
44. Multi Holder for 8 Pin Stubs, Zeiss, Jena, Germany
45. Pin Stub SEM Mount Grippers, Balzers Union Ltd, Liechtenstein

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Author contributions

N.M.P. designed and performed experiments, processed the data, and wrote the manuscript. J.K. designed the EDX holders and provided feedback on figures and discussion. B.N.G.G. conceived of the study and edited the manuscript.

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