UCLouvain
Institute of Mechanics, Materials and Civil Engineering (IMMC)

iMMC

LACAMI platform

in collaboration with the Louvain centre for Toxicology and Applied Pharmacology (LTAP) LTAP

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Scanning electron microscopy (SEM)-energy dispersive X-ray (EDX) studies of ovary tissue obtained from rats treated with a form of ionic silver (silver acetate)

High concentrations of Ag were measured (ICP-MS) in ovary tissue of rats exposed to silver acetate for 4 weeks in a toxicokinetic study (LabCorp study no. CC71MP). Following this finding, ovary tissue of rats exposed to silver acetate (160 mg/kg bw/d) for 8 weeks in a preliminary reproductive performance study (Labcorp study no. 8436495) were further investigated in the present study. The objective of the present study was to approach the speciation of Ag deposited in these ovary tissues. For this, we used scanning electron microscopy (SEM) coupled to energy dispersive X-ray (EDX) spectrometry to screen the elemental composition of Ag deposits. The SEM-EDX analysis was guided by an autometallographic (AMG) staining to reveals the presence of Ag in ovary tissues (Stoltenberg et al., 1994)[1].

Ovary samples from Labcorp study 8436495, preserved in PFA, were processed at LTAP for the present analysis. This report summarizes the results obtained with the ovaries from the high dose group (160 mg AgAc/kg bw/d) :

- rat 4F-137
- rat 4F-138
- rat 4F-139

The samples were embedded in paraffin, and cut serially for microscopic analyses :

- 5 µm thick sections were used for Hematoxylin-eosin (HE) staining, and AMG analysis
- thick sections (20 µm) were used for SEM-EDX analyses in order to increase the sensitivity of the analysis.

As paraffin is not transparent to electrons, the slides were first dewaxed in toluene. On usual glass support, the edges of the sections detached and curled in places, and these areas cannot be analyzed. Therefore, some sections were prepared with a "Plus Gold" support, and the adhesion of the section was improved. Next, the slides were covered with a thin layer of C-conductor.

The slides were inspected by scanning electron microscopy (SEM), using the signal of secondary electrons to obtain information on the topography of the biological section, and the signal of backscattered electrons (BSE) to look for the presence of heavy elements such as Ag.

In areas where the presence of Ag was suspected, elemental composition was investigated by EDX spectrometry, fixing the electron beam on a specific feature to analyze the very local composition, or collecting an average signal by scanning the electron beam on a delimited zone. Elemental maps were also collected on chosen areas to confirm the correlation between the location of Ag and other elements such as selenium and sulfur.

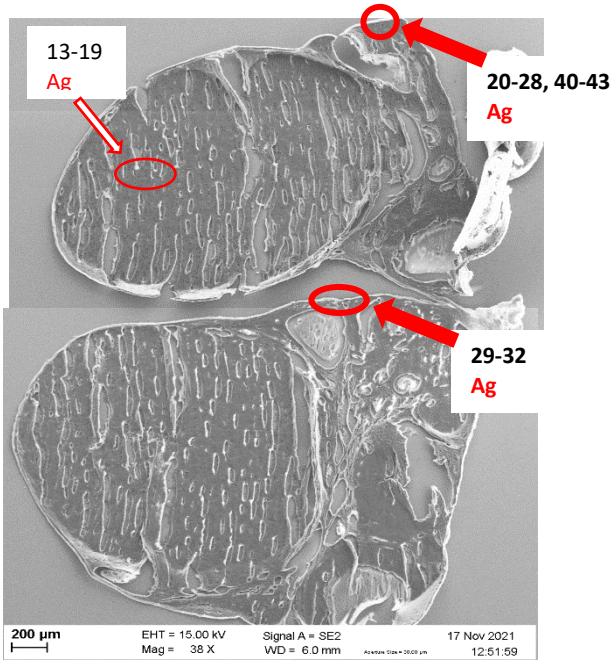
[1] Stoltenberg, M., et al. (1994). "Autometallographic detection of silver in hypothalamic neurons of rats exposed to silver nitrate." J Appl Toxicol **14**(4): 275-280.

The microscope was a Zeiss Ultra-55 FEG-SEM, operated at 15kV. EDX spectra were collected with a Bruker Quantax system equipped with an SDD-type detector. The acquisition time varied as a function of the signal intensity for the peaks of interest with regard to the background and to the noise: typically, 30 seconds for an individual spectrum, and 20 minutes for the acquisition of a map.

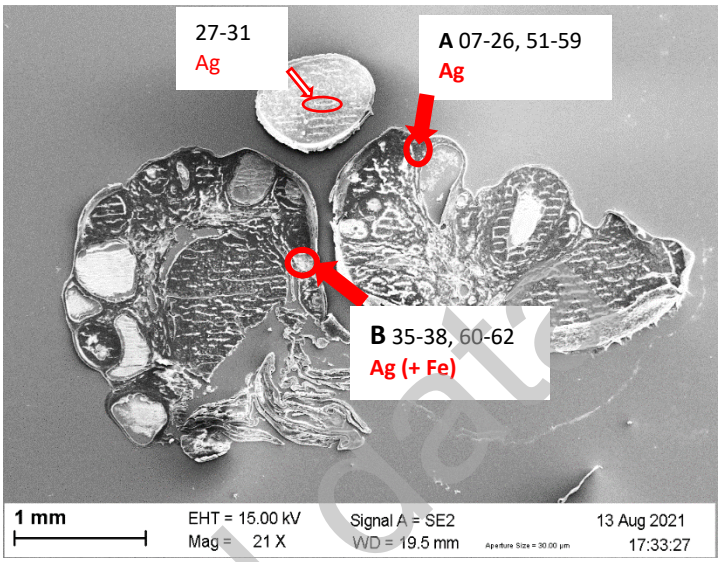
1. Ag detection

The detection of Ag-containing areas was guided by the AMG stained sequential slide, when available. If not, the signal of backscattered electrons (BSE) was used as, even at rather low magnification, a diffuse contrast is visible if the Ag concentration is high enough. It must be specified that the AMG staining seems to be much more sensitive than SEM contrast. With EDX, the detection limit of Ag in biological matrix is about a few tenths of mass %. It appeared to be insufficient to localize dispersed Ag content.

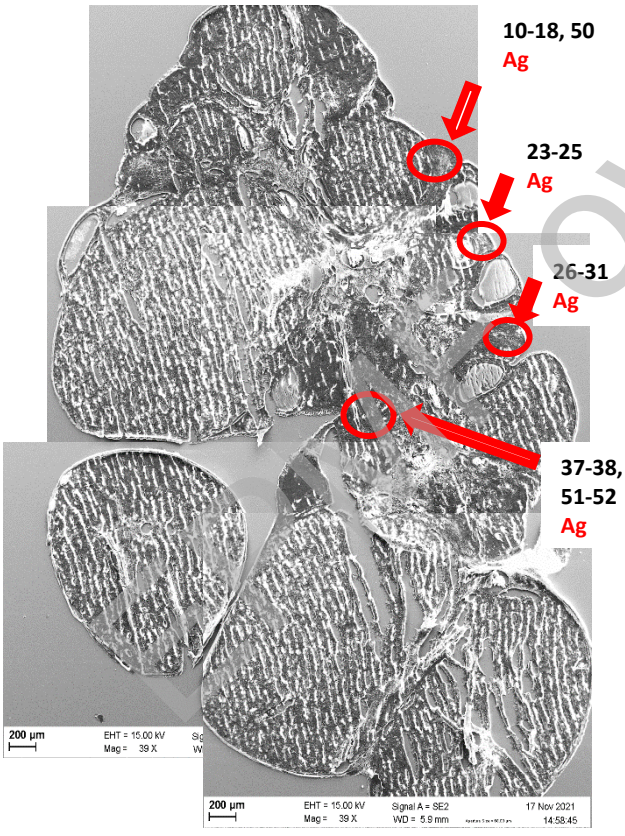
The results of SEM-EDX are thus related, for each slide, to a few numbers of areas in which the presence of Ag appeared clearly. In many other zones, Ag was not detected, even when the AMG staining indicated its presence.



4F – 137



4F - 138



4F – 139 (Plus Gold support)

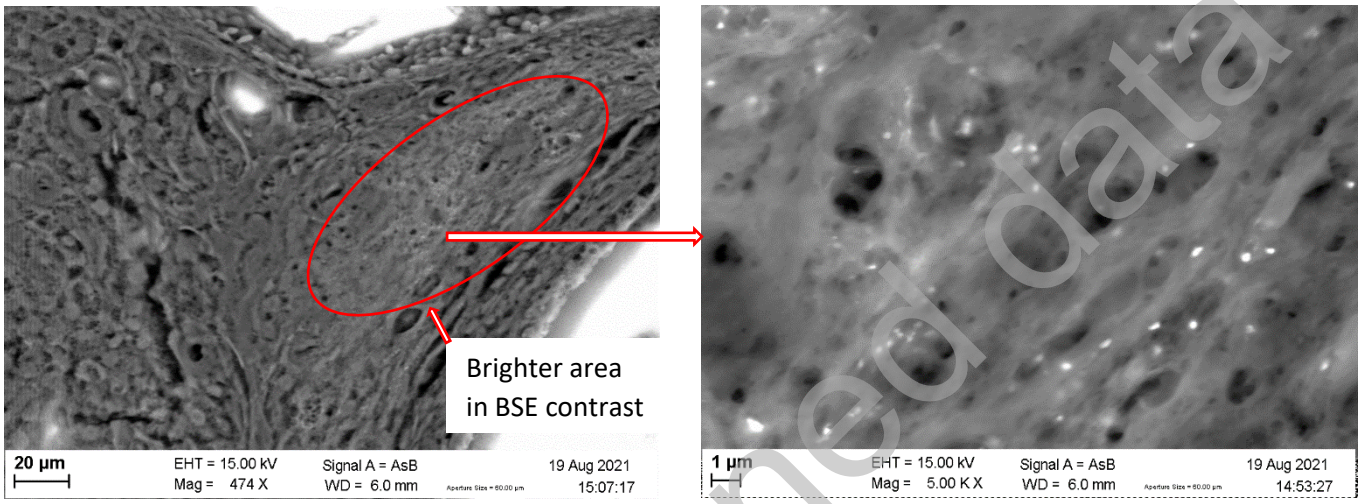
Figure 1. *In the three slides, location of areas where Ag was detected by BSE, with reference to indexation of micrographs. Areas marked in bold are Ag-rich structures, giving a diffuse BSE contrast at low magnification.*

2. Aspect of Ag deposits

In areas marked in bold in Figure 1, the presence of Ag is associated with strings of nanoscale structures, well highlighted by atomic number contrast, and they appear bright on the micros obtained with the BSE signal (Figure 2). Their Ag content was confirmed by EDX analysis while pointing the electron beam on individual structures.

a. Low-magnification of Ag-rich area (4F-138 micro 26)

b. Higher magnification of a (4F-138 micro 24)



c. 4F-137 micro 22

d. 4F-139 micro 11

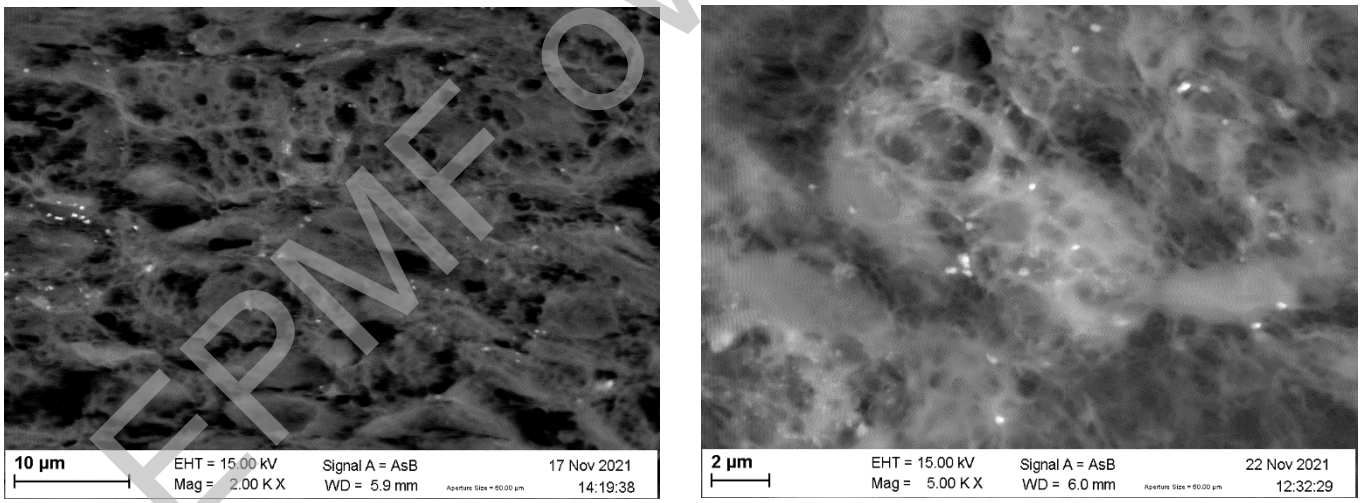


Figure 2. *Strings of Ag-rich nanoscale structures revealed by BSE contrast*

Similar Ag-rich structures were also found in isolated locations, marked as non-bold in Figure 1. The detection of such individual nanostructures is much more delicate, as they are dispersed and only visible at high magnification (Figure 3).

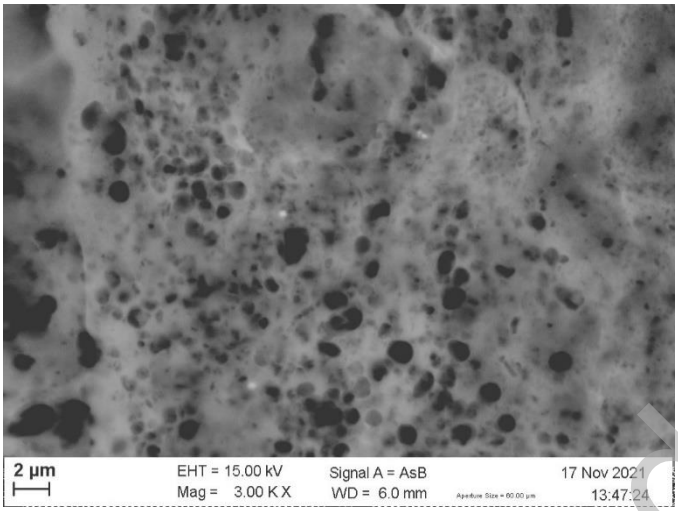


Figure 3. *Isolated Ag-rich structures in 4F-137 micro 16*

Among all observed nanostructures, some of them were identified to be Fe-rich instead of Ag-rich.

3. Composition of the nanoscale structures

EDX analyses were carried out in different modes, i.e. with a beam scanning a small area or with a fixed beam.

The first acquisition mode allows the exploration of larger areas, and thus a faster detection of areas containing Ag. The second mode is used to amplify the intrinsic nanostructures signal, while pointing the electron beam on one individual particle. However, EDX signal collected with a fixed focused beam comes from a volume of several cubic microns (on this type of sample and a beam voltage of 15 kV). As a result, the signal of one individual nanostructure cannot be completely separated from the signal of the surrounding material (biological and glass)

a. Ag-S-Se

In some areas, the analysis of the nanostructures in fixed beam mode shows that Ag is associated with Se, and with an enrichment in S (Figure 4). The Se/Ag mass ratio is 5 to 15%, with a fairly large margin of uncertainty due to the very small size of the precipitates. Concerning the S content of the Ag nanostructures, the EDX quantification model does not allow it to be dissociated from that of the matrix. However, as the intensity of the S signal increases by a factor of 2 to 3 in the spectra targeting the Ag nanostructures compared to the average of the spectra obtained in the areas not containing Ag, it can be concluded that the Ag nanostructures contain a significant amount of S. No other element seems to be systematically associated with Ag. Again, the small size of the precipitates must be considered, because of which minor elements could go unnoticed.

Note: the intensity of the Si, Na... signals that originate (largely) from the glass support varies from one place to another in the section depending on the density of the biological tissue and its effective thickness after drying.

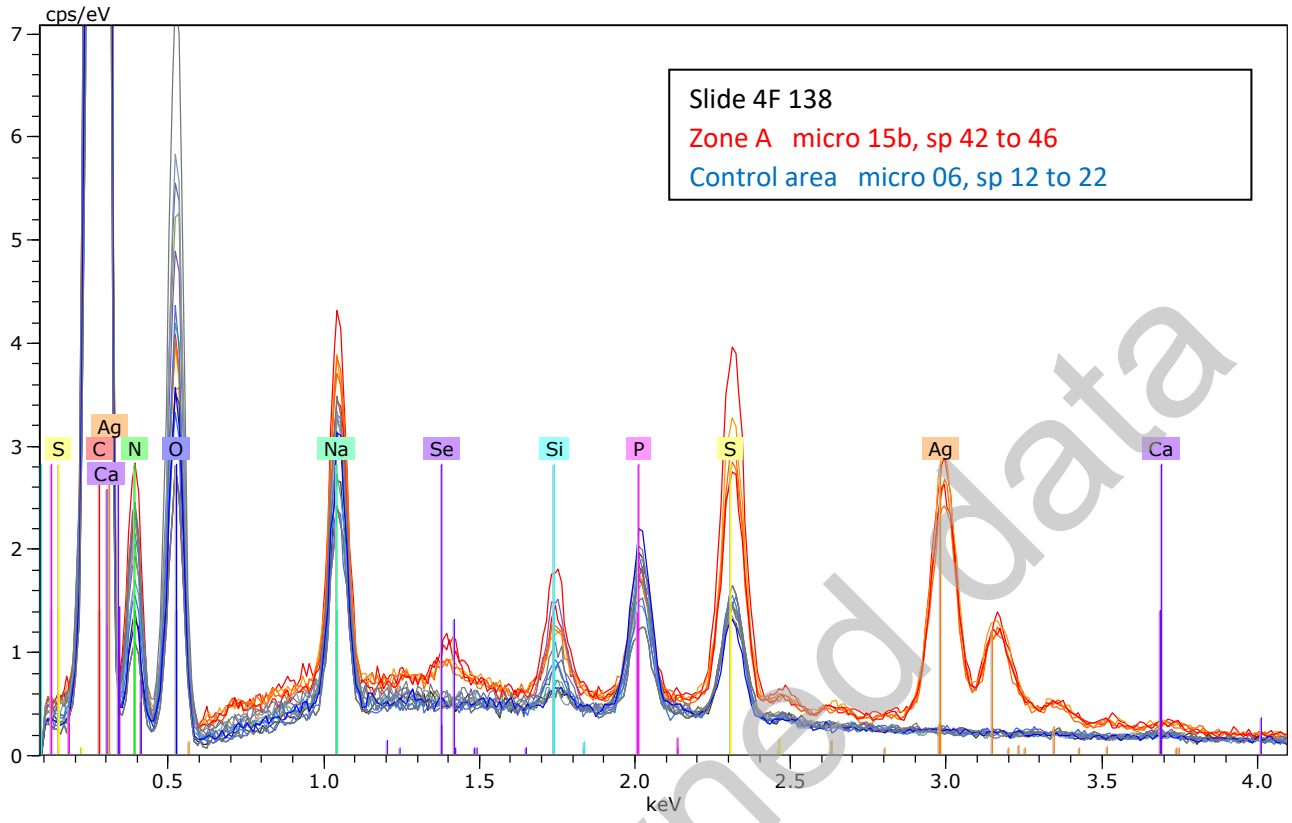


Figure 4. *EDX spectra recorded from slide 4F-138, with a fixed beam on a series of nanostructures in zone A (in red) compared to the signal recorded in a control area (in blue).*

Similar composition is analyzed on nanoscale structures in slides 4F-137 and 4F-139.

b. Ag-S

In other areas, fixed beam analysis of the nanostructures reveals an Ag and S rich composition, without associated Se. The Se-L signal is below the significance threshold, the quantification of the spectra giving a Se/Ag mass ratio of 0-4% within the deconvolution uncertainty range (Figure 5).

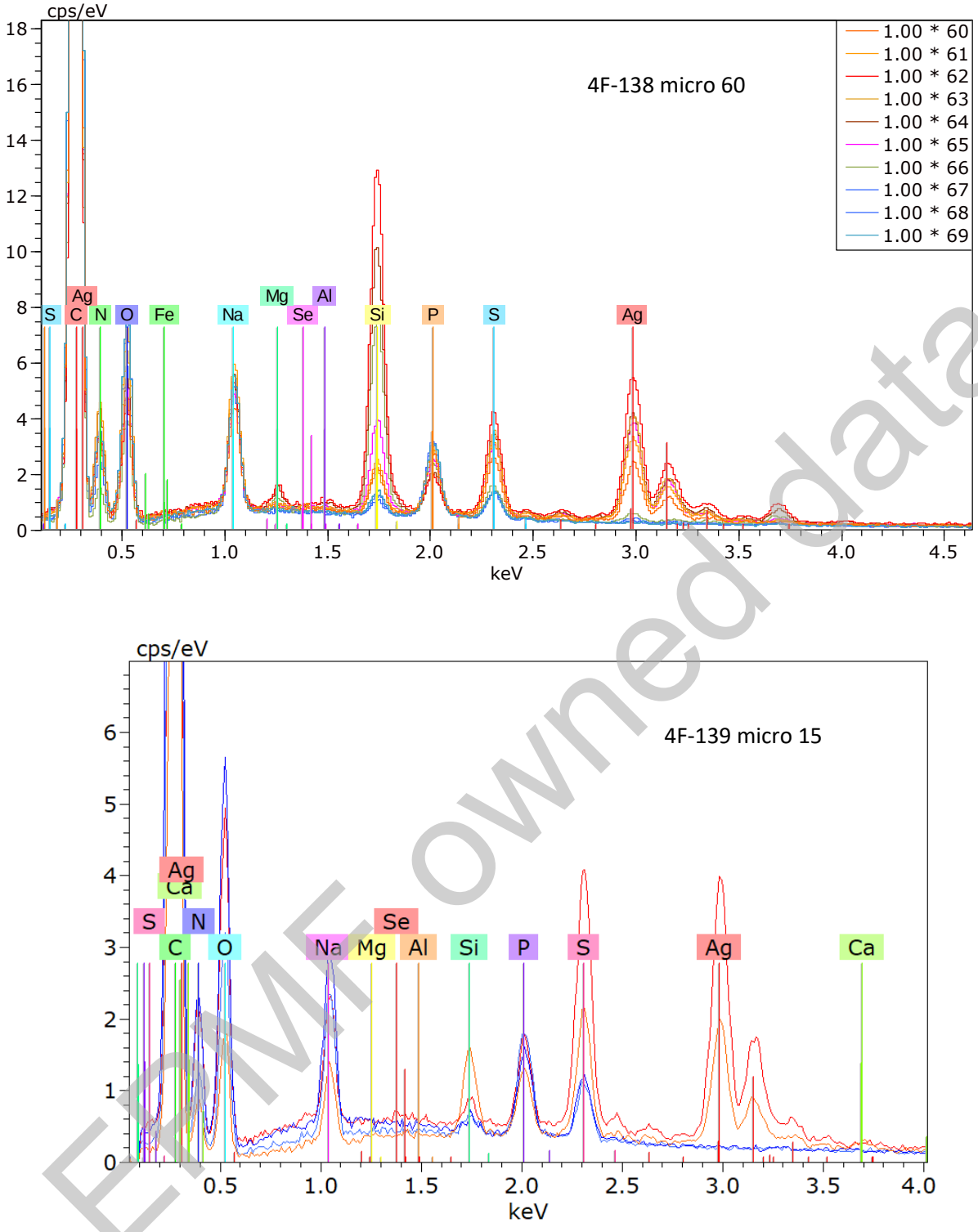


Figure 5. *EDX spectra collected in slide 4F 138 in the area of micro 60 (a) and in slide 4F-139 micro 15 (b)*

c. Fe-O

Some of the particles observed, often of a slightly larger size and more irregular morphology, are composed of Fe in oxidized form (Figure 6). The accumulation of this type of particle could explain the enrichment of Fe in the Ag-containing area.

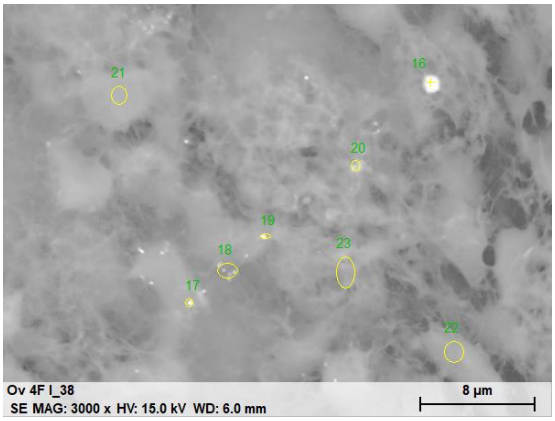
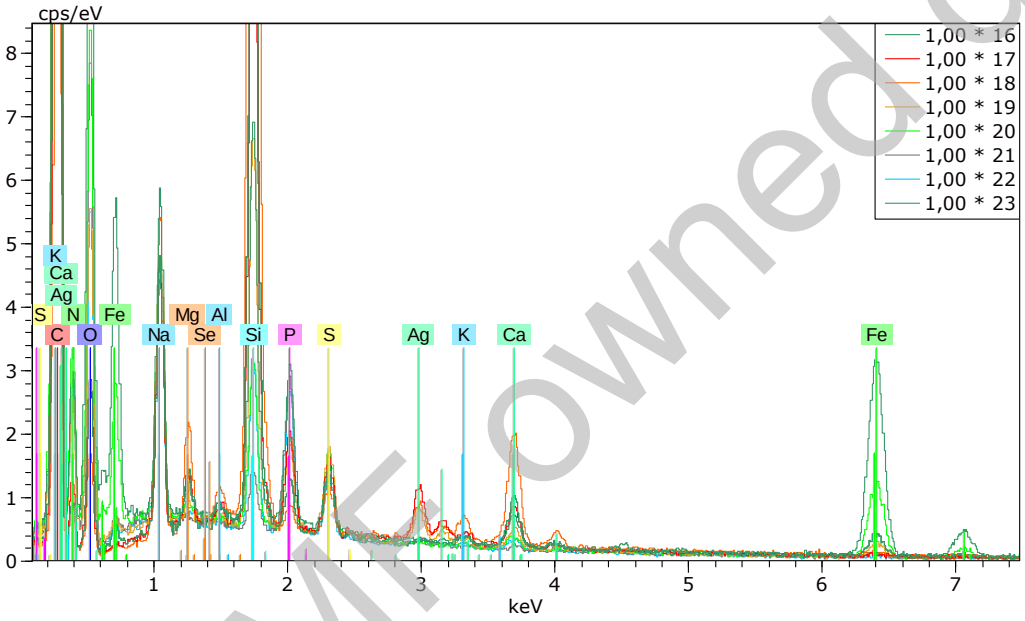


Figure 6. *Slide 4F-138 micro 38 : analysis of nanostructures and surrounding tissue*



d. Element mapping on Ag-rich zones

Feature mapping was used to observe the extent of Ag content. This is more difficult to analyze on the basis of SEM micros as the variation in the diffuse BSE contrast is rather low, and as the nanostructures are not detectable at low magnification.

In 4F-138 zone A (Figure 7), the presence of Ag extends over a range of about 170 µm long and 35 µm wide. The presence of Ag is associated with an S enrichment. The presence of Se is more difficult to interpret because the signal level is low, and in the ranges where the signal of the glass support is visible (Si, Na, Mg, Al...). The Se-L lines are poorly deconvoluted from the nearby Mg-K and Al-K lines.

In this zone, the element mapping also shows an enrichment in Fe of the biological tissue in the Ag-containing area.

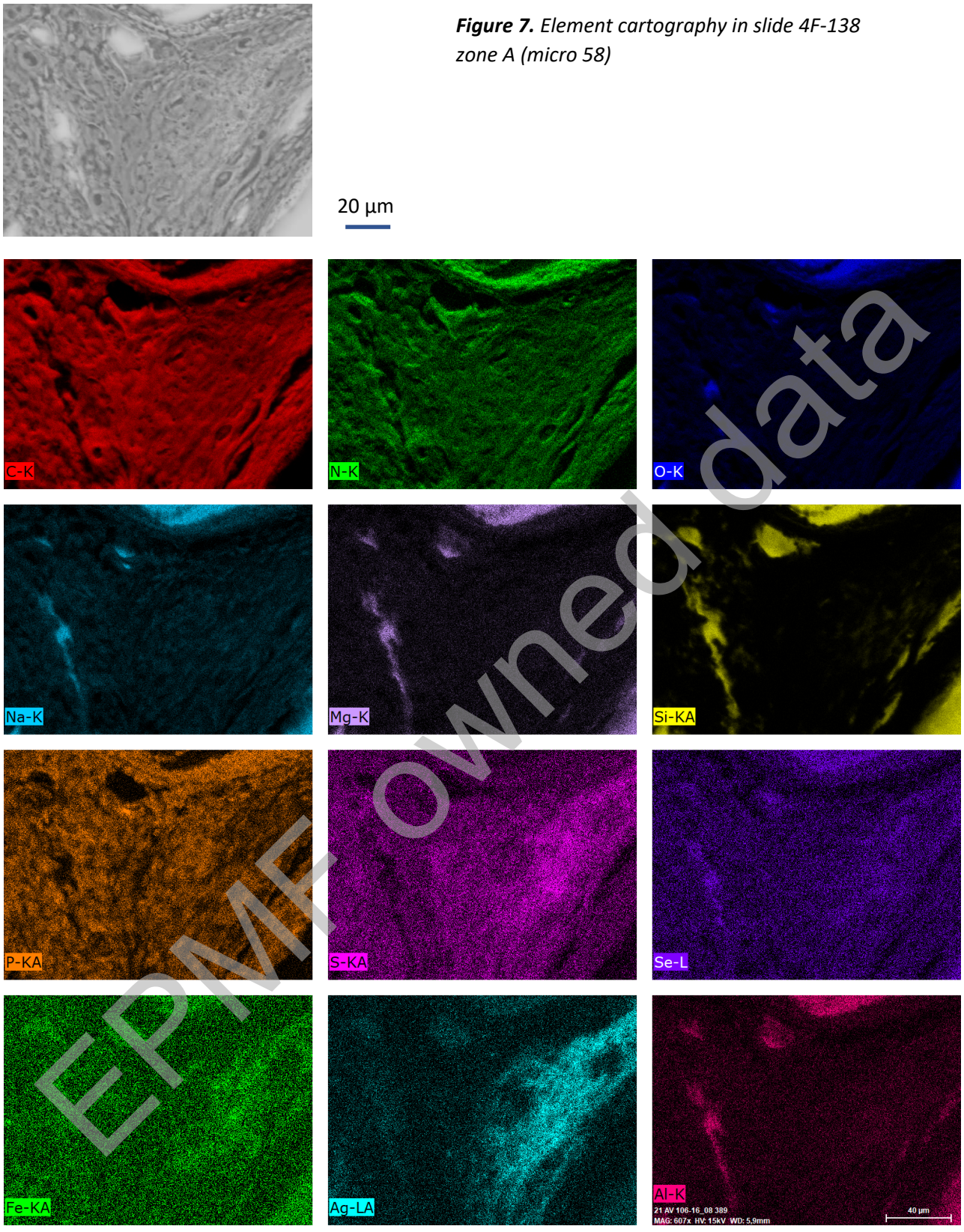


Figure 7. *Element cartography in slide 4F-138 zone A (micro 58)*

Figure 8 shows element maps obtained at high magnification, under intense beam conditions and with a long acquisition time (45 minutes) in order to obtain a high signal-to-noise ratio.

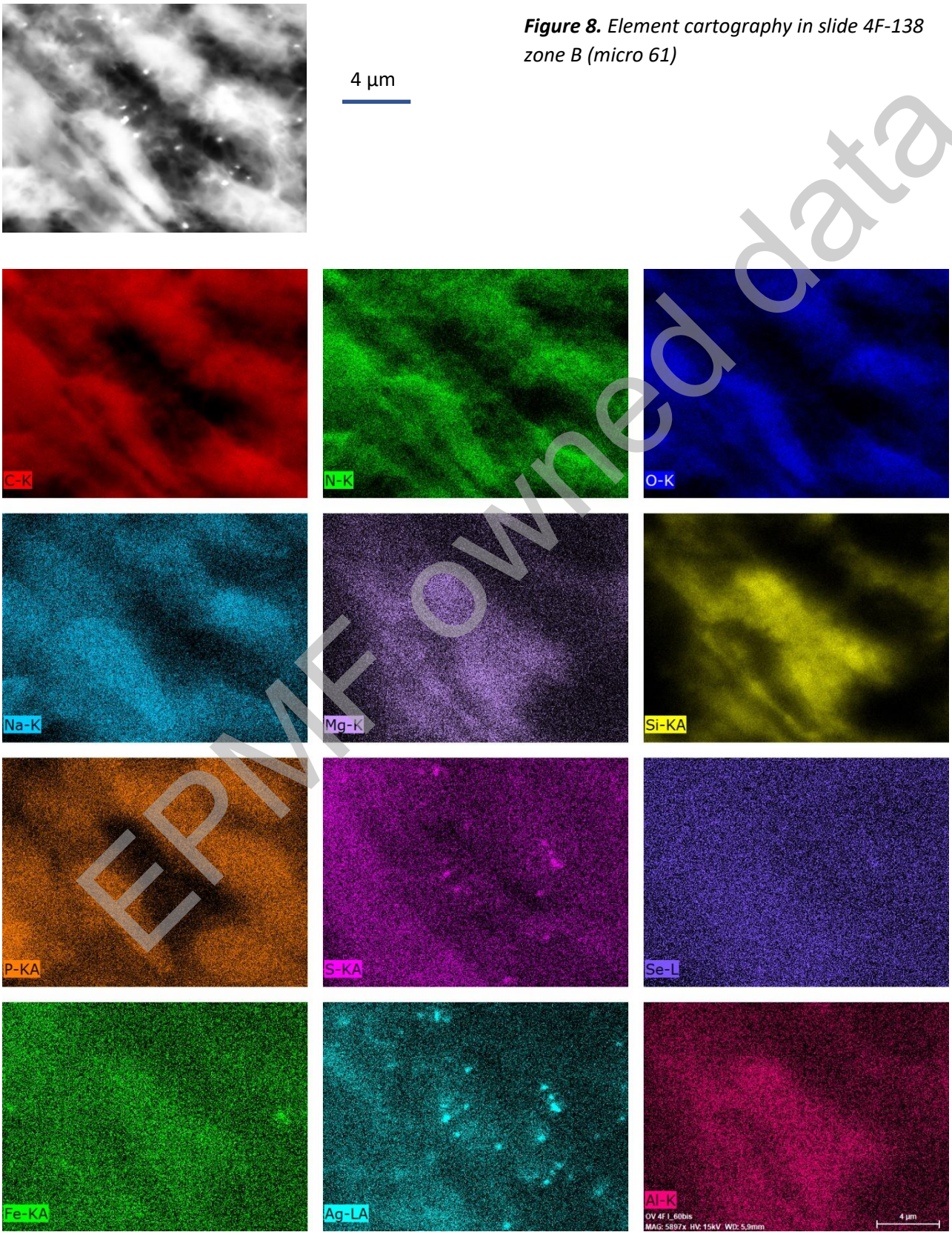


Figure 8. *Element cartography in slide 4F-138 zone B (micro 61)*

Figure 8 shows that the S enrichment is well localized on the Ag nanostructures, whereas the Fe enrichment seems more diffuse, except on the Fe oxide particles.
Again, the general interpretation of this mapping must consider the variations in thickness of the biological section and, therefore, the variable contribution of the glass slide depending on the position.

The presence of Ag associated to an enrichment in S is also visible in figure 9, obtained on slide 4F-139. Because of variation in thickness of the biological section, the signal given by the slide is more pronounced in some portions.

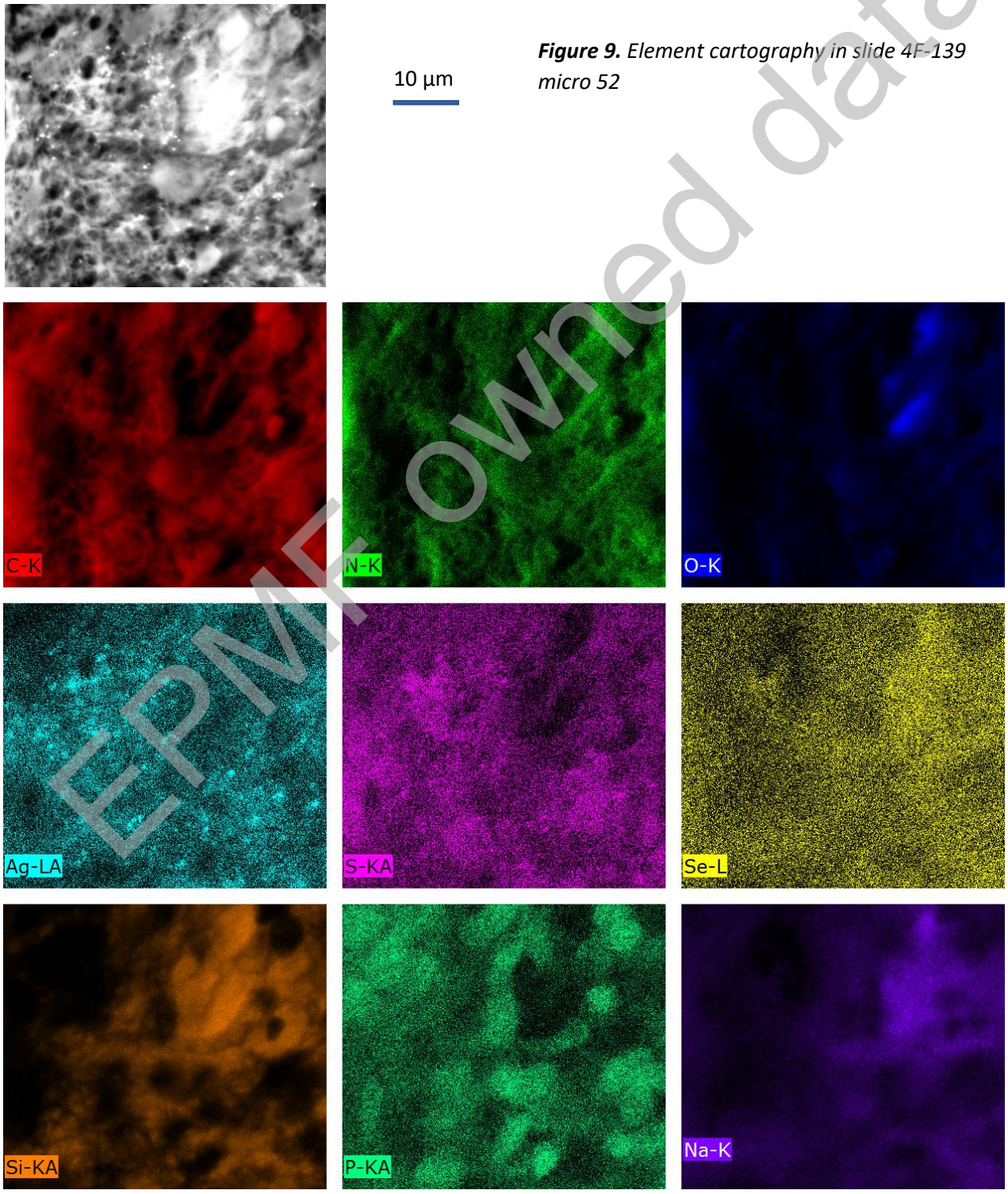


Figure 9. *Element cartography in slide 4F-139 micro 52*

Similar results were obtained one slide 4F-137, as shown in figure 10. In this area, the intensity of global signal is highly variable due to curvature of the section, impacting the analysis of results.

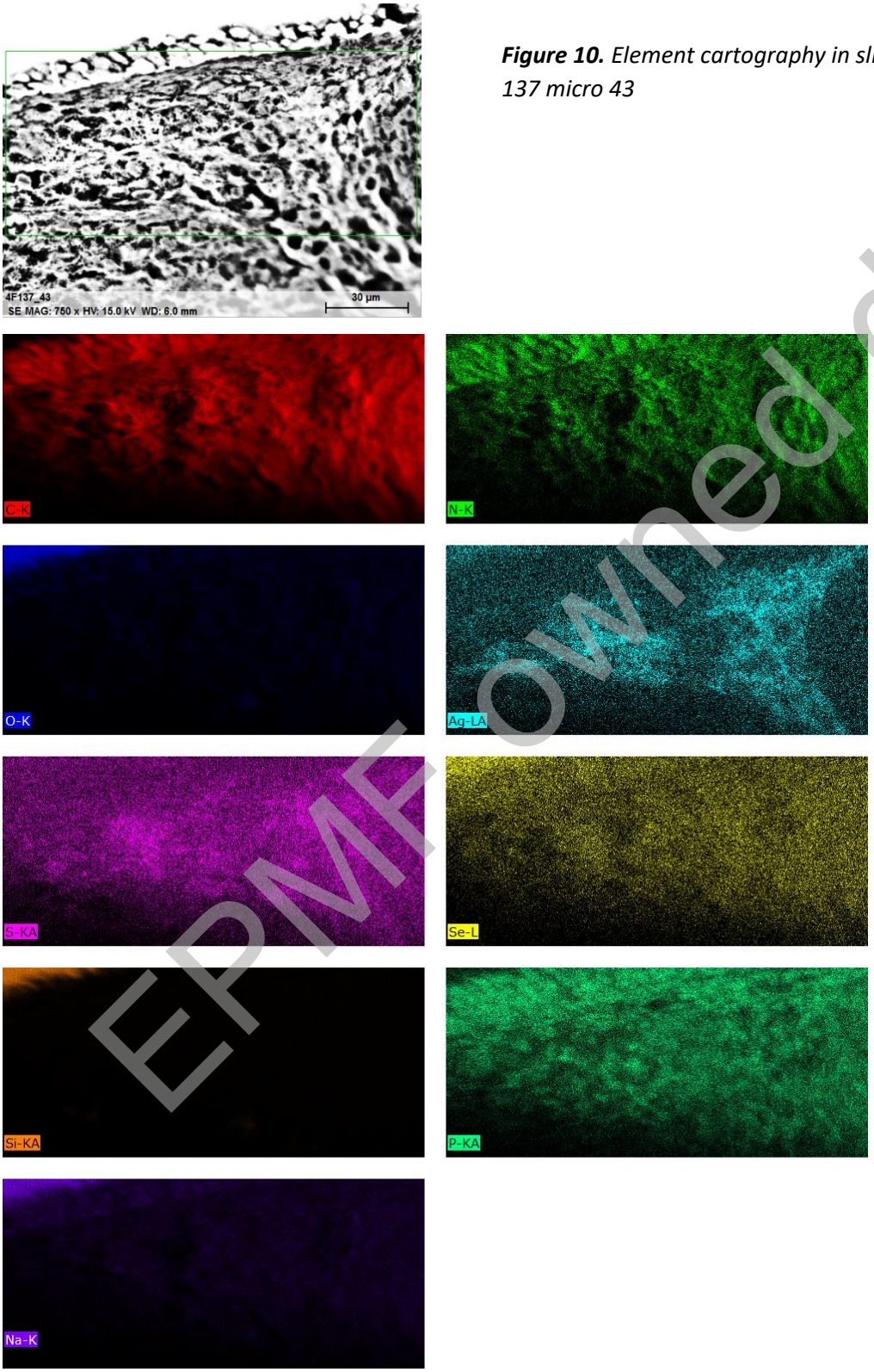


Figure 10. *Element cartography in slide 4F-137 micro 43*

CONCLUSION

The presence of Ag was observed in ovarian sections from the 4F experimental. Ag appears in the form of strings of nanoscale structures located in certain areas of the section. These 250 to 500 nm particles are composed of Ag and S. A weak Se signal is observed in association with Ag in some of the investigated areas; in another areas, no significant presence of Se was identified.
Fe enrichment of the biological tissue was observed in the Ag-containing areas, as well as Fe oxide precipitates.
All the results, SEM micrographs and EDX analyses, are available separately.

A general overview of information gained through the study in summarized in Figures S1, S2 and S3 (in annex).

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Figure S1 : overview of slides used in this study for rat F4-137

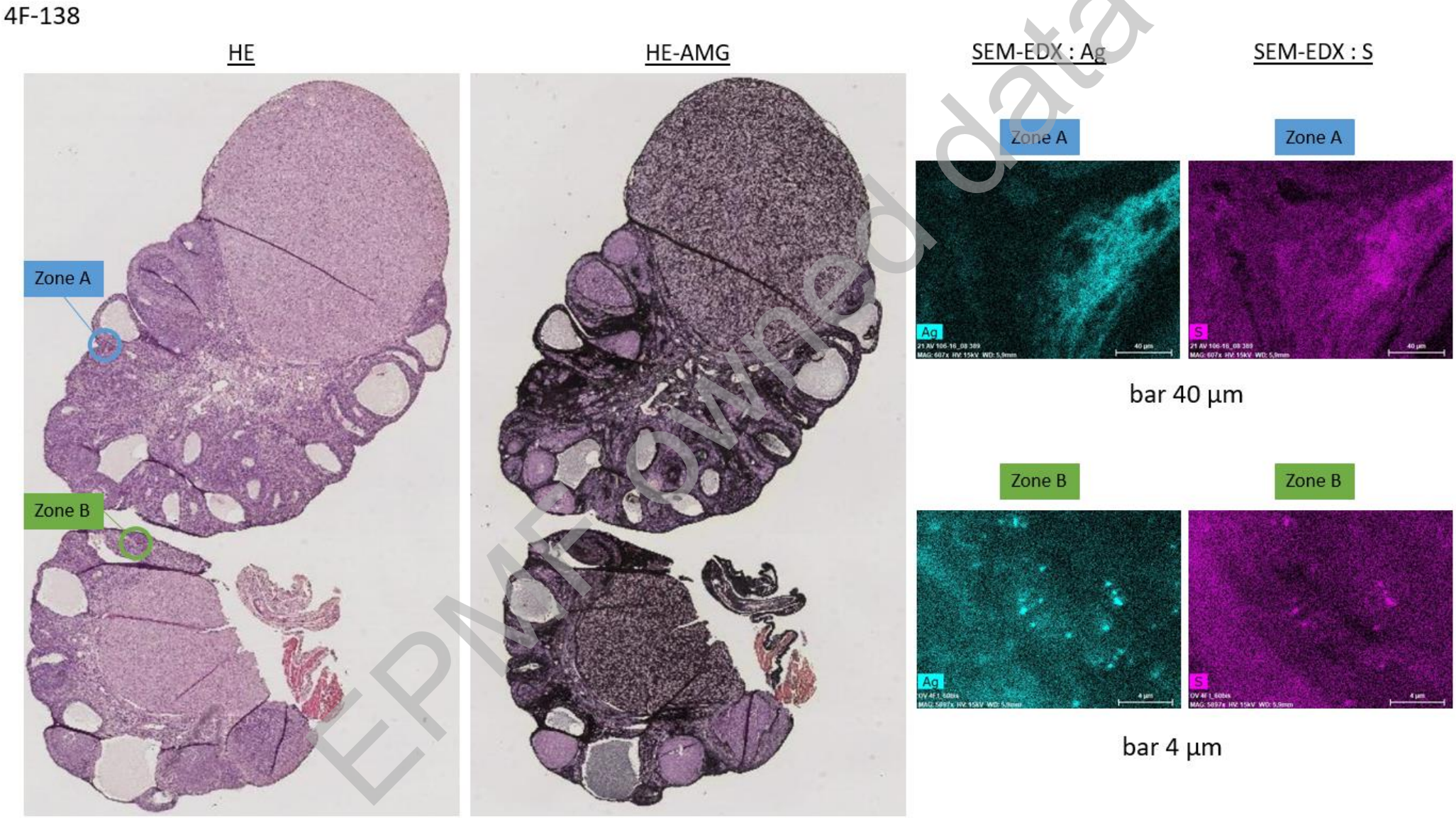

Figure S2 : overview of slides used in this study for rat F4-138

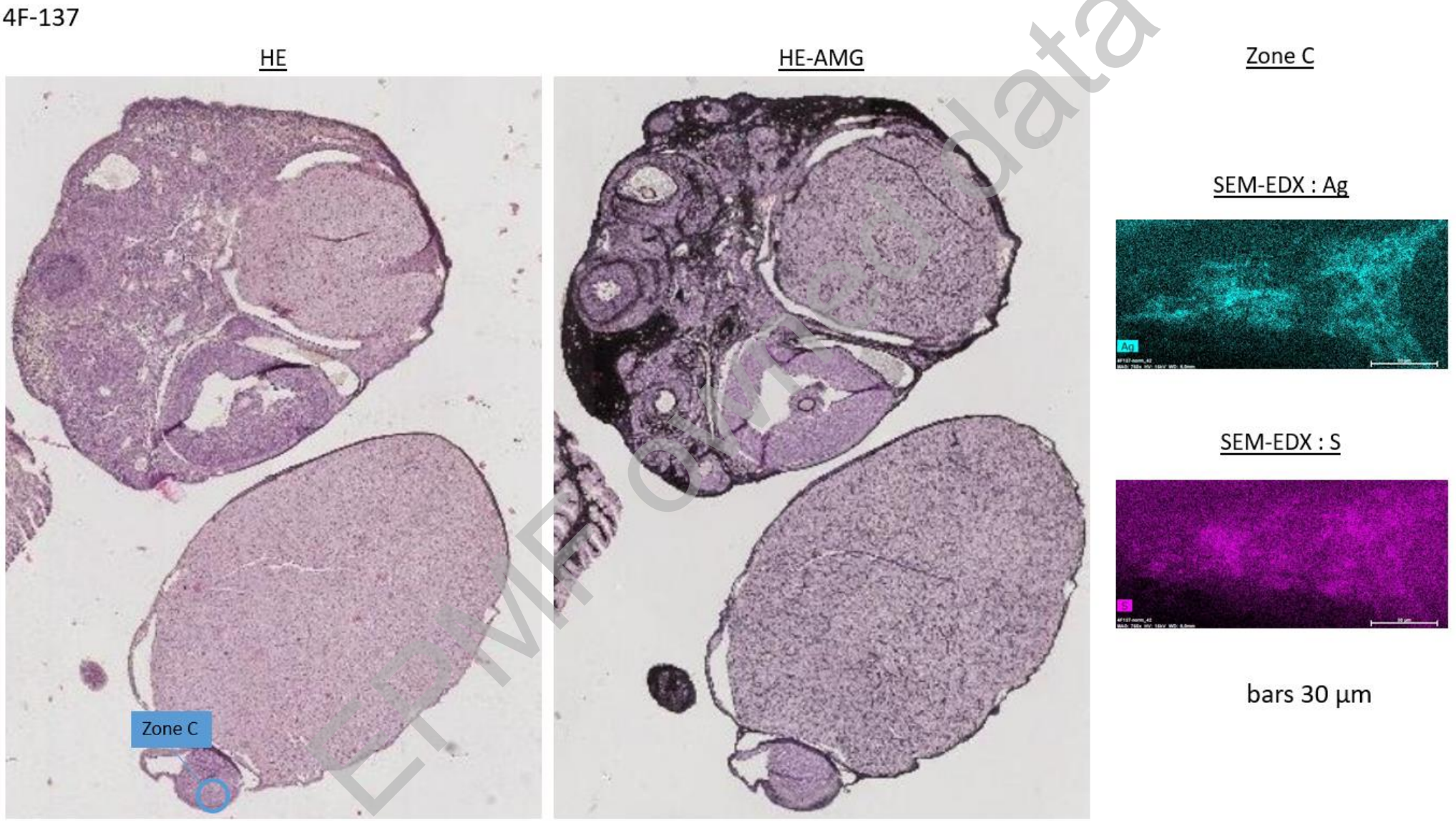

Figure S3 : overview of slides used in this study for rat F4-139