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Early View

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Identification of coronavirus particles by electron microscopy: a complementary tool for deciphering COVID-19

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We thank Dittmayer and Laue for giving us the opportunity to clarify issues regarding the identification of coronavirus (CV) particles by electron microscopy (EM) demonstrated in our recent publication [1]. We would like to respond to the authors' statements, as follows:

Having a significant experience in EM for more than 20 years, we are well aware of the ultrastructural features of all cellular organelles and their potential distortion by artifacts and also of the ultrastructural characteristics of viruses, including those of coronaviruses in infected cells and tissues as they have been extensively described [2-5].

Regarding the experimental approach followed in our investigation [1], tissue samples applied for EM were selectively removed, under the guidance of haematoxylin and eosin stained section, from formalin-fixed paraffin embedded (FFPE) autopsy lung tissue blocks. Subsequently, they were deparaffinized and re-embedded in epoxy resins according to standard EM procedure. Indeed, the ultrastructural features of the tissue were not well preserved, as foreseen, due to the fact that tissue samples underwent the process of embedding in paraffin and additional treatment during deparaffinization, dehydration, infiltration and finally re-embedding in epoxy resins [2]. Another fact that influences the proper evaluation of the ultrastructural features in the current setting stems from the limited availability and rareness of the examined paraffin-embedded COVID-19 lung

autopsies, that did not allow neither repetition of the procedure, as often happens in EM, nor directly embedding of the autopsy material in epoxy resins.

In this context, the ultrastructure of CV particles might also be affected and may not reveal all the established morphological features, as also supported by Bullock et al 2021 and Krasemann et al. 2022 (preprint version). In our study, in Fig1C (iv) and (v), the CV particles, in the proximity of endoplasmic reticulum (ER) and in vacuole, respectively, have a partially visible biomembrane, while a surrounding fuzz is apparent in all CV particles (Fig.1C iv-vi), which is suggestive of peplomers or spikes. Actually, spikes are better visualized by thin sectioning of formalin or glutaraldehyde fixed tissue for EM when *en bloc* staining with tannic acid and uranyl acetate precedes tissue embedding in epoxy resins [2, 6]. Moreover, without any doubt, the CV particles are definitely located in the proximity of ER (Fig1C iv) and in vacuoles (Fig1C v, vi) and although the poorly preserved ultrastructure, they are not misinterpreted with other artificially altered structures, such as swollen mitochondria or a deteriorated multivesicular body, as is for instance evident in Fig.1C (v) above the vacuole with two CV particles. A criterion that is not met in the depicted cell is the presence of numerous, similarly shaped CV particles [2, 6]. However, this might be due to the low viral load of the particular cell. According to Krasemann et al. 2022 (preprint version), in SARS-CoV-2 infected type 2 pneumocytes of formalin-fixed autopsy lung processed for EM (but not deparaffinized), a wide range of intracellular virus load per sectioned cell profile was observed (range: 4-620 intracellular particles), “rendering a huge difference in the likelihood of particle detection”. In our study, the particular depicted cell was selected in order to be in correspondence with the demarcated cell in the semi-thin section (Fig.1C i). Following additional examination of the same sample in another AT2 infected cell, similarly shaped CV particles grouped in vesicles, adjacent to the cell surface, were identified (Figure). They meet all the ultrastructural criteria of deparaffinized and re-processed tissue for EM, such as electron-dense appearance, partially visible biomembrane (Figure, arrowheads), surface projections in some of them (Figure, arrows), rendering their identification indisputable.

However, the main message of the study of Evangelou et al. [1] is the SARS-CoV-2-induced cellular senescence correlated with a proinflammatory phenotype, COVID-19 disease outcome and viral mutagenesis. The use of EM was a redundant experimental approach that was conducted complementary to the main methodology for SARS-CoV-2 detection, with a prospect to identify CV particles, despite the considered technical limitations. The validity of the scientific concept has been extensively proven at the experimental level, with molecular (RT-PCR), *in situ* (immunohistochemistry) and *in vitro*

assays (Vero cells and alveospheres infection), providing overall novel insights of the underlying cellular mechanisms occurring in COVID-19 disease. In our opinion, our work should be regarded under the latter conceptual prism.

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Legend

Figure. Electron micrograph of a SARS-CoV-2 infected AT2 cell of a paraffin-embedded lung autopsy which was deparaffinized and re-embedded for electron microscopy. CV particles with electron-dense appearance are gathered in vesicular formations (white arrows) near the cell surface. Inset: higher magnification of a group of virions. Biomembrane is partially visible around some of the particles (arrowheads), while surface projections, indicating spikes, are observed occasionally around others (black arrows). MV: microvilli