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# BIOLOGICAL PERSPECTIVES

## Characterizing Viral Infection by Electron Microscopy

## *Lessons from the Coronavirus Disease 2019 Pandemic*

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The severe acute respiratory syndrome coronavirus 2 pandemic has infected millions of individuals in the United States and caused hundreds of thousands of deaths. Direct infection of extrapulmonary tissues has been postulated, and using sensitive techniques, viral RNA has been detected in multiple organs in the body, including the kidney. However, direct infection of tissues outside of the lung has been more challenging to demonstrate. This has been in part due to misinterpretation of electron microscopy studies. In this perspective, we will discuss what is known about coronavirus infection, some of the basic ultrastructural cell biology that has been confused for coronavirus infection of cells, and rigorous criteria that should be used when identifying pathogens by electron microscopy. *(Am J Pathol 2021, 191: 222–227; https://doi.org/10.1016/j.ajpath.2020.11.003)*

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) pandemic, causing coronavirus disease 2019 (COVID-19), has presented many challenges and spurred intense investigations into the pathogenesis of this disease. In addition to respiratory disease, many patients with SARS-CoV-2 infection are experiencing systemic illnesses, including kidney failure, heart failure, liver injury, neurologic dysfunction, and skin manifestations (eg, COVID toe). The etiology and pathogenesis of these sequelae are the current focus of intense research and speculation. A fundamental question is whether the extrapulmonary disease processes encountered in COVID-19 patients result from direct infection of target organs or indirect injury, resulting from initially localized infection in the lungs and upper respiratory tract and subsequent systemic responses, such as cytokine release/cytokine storm. Viruses come in all shapes and sizes but are invariably small and require an electron microscope to resolve the morphology of individual particles.[1] With the emergence of SARS-CoV-2, we are witnessing a renaissance in the use of electron microscopy (EM) to help identify virally infected cells and uncover the pathogenesis of this disease. Several articles have used EM to propose direct evidence of infection of the kidney[2–5] and other tissues[4,6–14] by SARS-CoV-2. These reports have fueled speculation that direct infection of tissues throughout the body contributes to the morbidity and mortality of COVID-19.

Unfortunately, many of these studies are fraught with confusion over differentiating virus from normal structures within cells, leading to an explosion of misinformation. Indeed, published articles claiming to provide direct evidence of SARS-CoV-2 virus infection in kidney cells and endothelial cells have provoked letters to the editor challenging these claims.[15–20] In this perspective, we will discuss what is known about coronavirus infection and some of the basic ultrastructural cell biology that has been confused for coronavirus infection of cells (namely, the

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**Table 1** Subcellular Structures That Can Be Confused with Viral Particles

| Subcellular structure* | Virus mimic* |
|---|---|
| Perichromatin granules | Small icosahedral viruses |
| Improperly fixed chromatin | Nucleocapsids |
| Nuclear pores | Herpesvirus nucleocapsids |
| Melanosomes | Poxvirus |
| Cilia and microvilli | Enveloped viruses |
| Microtubules | Viruses with helical nucleocapsids |
| Secretory vesicles and granules | Enveloped viruses |
| Multivesicular bodies and exosomes | Enveloped viruses |
| ER/Golgi and coatomer-coated vesicles† | Enveloped viruses |
| Clathrin-coated vesicles† | Enveloped viruses |
| Granules and glycogen | Small icosahedral viruses |

*Personal observations and references.[1,22]
†Protein coats can be misinterpreted as spike proteins.
ER, endoplasmic reticulum.

machinery that controls endocytosis and exocytosis and membrane transport within cells).[21]

## Electron Microscopy of Viral Infections

Understanding the biology of viruses is essential to accurately identify viral particles by EM because certain cellular organelles that can mimic the structure of viral particles (Table 1).[1,22] The location inside the cell and the type of membrane-bound organelles with which viral particles are associated can be important clues to identifying the virus. Accurate interpretation of electron micrographs requires integration of morphology and biology. This is especially important for studies that may be compromised by low resolution and poor tissue preservation, which is common in autopsy material.

Viral DNA or RNA genomes are contained within a protein coat (capsid). The nucleic acid together with the protein coat forms the nucleocapsid, which can be membrane bound (enveloped viruses) or without a membrane (naked viruses). The coronavirus is an enveloped RNA virus that infects cells after it binds to cell surface enzymes that serve as receptors, such as angiotensin-converting enzyme 2 for SARS-CoV and SARS-CoV-2, and is internalized in endocytic vesicles.[23] The S-protein of the virus is cleaved and activated, the viral envelope fuses with the vesicle membrane, and the nucleocapsid is released into the cytoplasm, where the replicative stage of the viral life cycle begins. Observing viral infection in cultured cells has provided much detail about the steps in coronavirus replication, which include the formation of double-membrane vesicles that constitute the site for synthesis of viral replicase proteins and genomes (the viral replication transcription

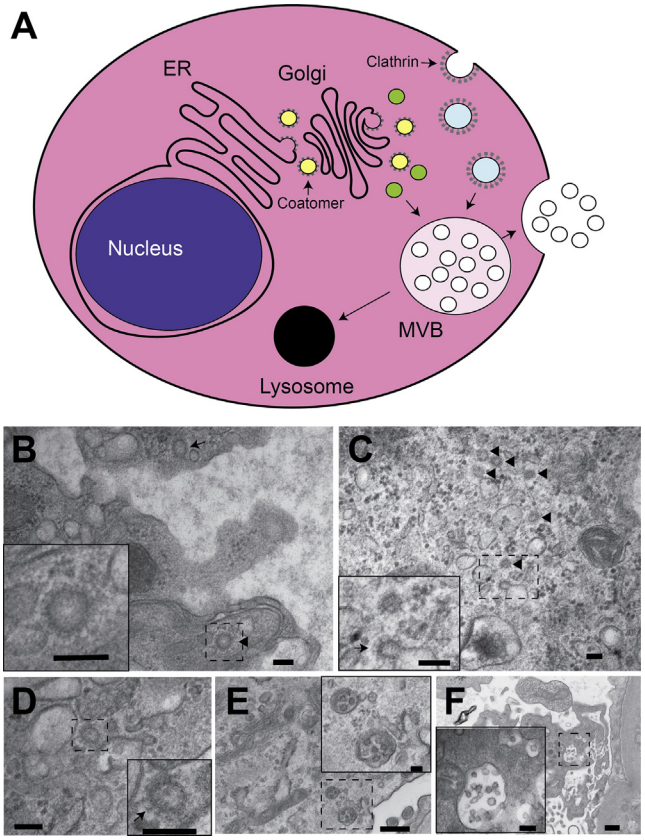


**Figure 1** Subcellular mimics of coronaviruses. **A:** Diagram of a cell with some of the intracellular structures that have been mistaken for coronavirus. Coatomer-coated vesicles (yellow) are involved in the anterograde and retrograde transport of vesicles between the endoplasmic reticulum (ER) and Golgi. Clathrin-coated vesicles (blue) are involved in endocytosis. Multivesicular bodies (MVBs) are derived from early endosomes and contain cargo (ie, destined to be degraded through fusion with lysosomes or expulsion of exosomes). **B:** Glomerular endothelial cell with coated pit (**arrow**) and vesicle (**arrowhead**), consistent with clathrin-coated pit and vesicle. **C:** Tubular epithelial cell with coated vesicles (**arrowheads**) adjacent to ER/Golgi; note vesicle budding (**arrow**; **inset**) from ER/Golgi. **D:** Glomerular endothelial cell with coated vesicles in cytoplasm that have club-shaped spikes (**arrow**). **E:** Podocyte with multivesicular bodies. **F:** Podocyte with microvilli inside an invagination of the plasma membrane, resembling a cytoplasmic vesicle. **B**–**F: Insets:** Higher magnifications of the **dashed boxed areas**. Scale bars: 100 nm (**B**–**D**, **main images**, and **B**–**F**, **insets**); 500 nm (**E** and **F**).

complex). The viral genomes and structural proteins are assembled into particles that bud into the endoplasmic reticulum (ER)–Golgi intermediate compartment.[23] Viral particles are identifiable by EM within infected cells in structures that resemble ER, Golgi, and larger vesicles and vacuoles, as well as outside of the cells.[24] An elegant series of electron micrographs from a nasal mucosal biopsy depicts coronavirus infection of epithelial cells during a naturally acquired infection.[25]

The putative virions detected in the kidney renal tubular epithelial cells, podocytes, and endothelial cells described in several recent publications appear as free particles in the cytoplasm,[2,3,6,7] a location that would not be expected for

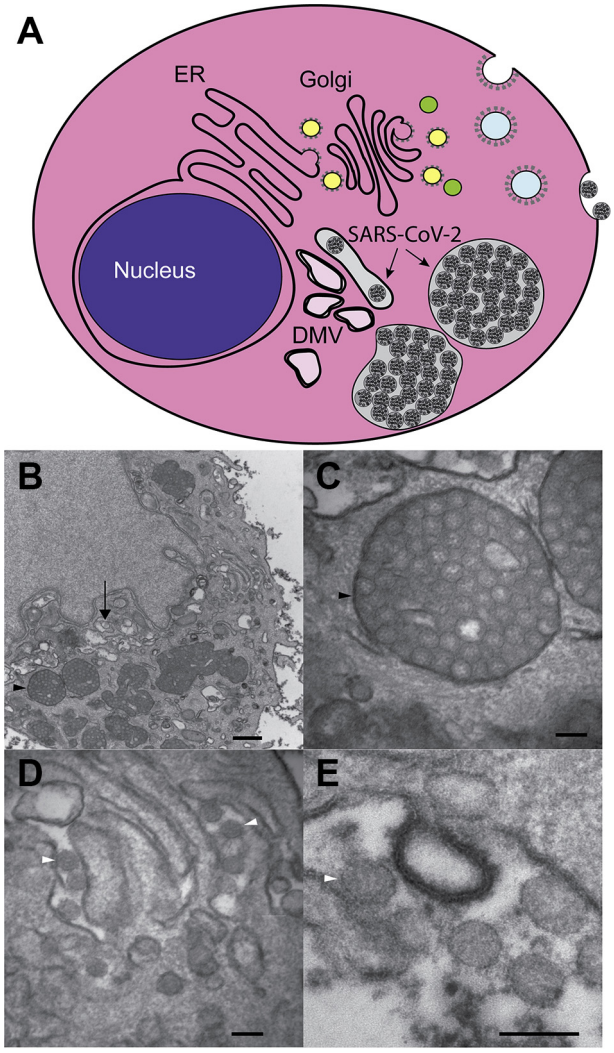


**Figure 2** Coronavirus infection in cells. **A:** Diagram of cell demonstrating structures that are associated with coronavirus infection. Double-membrane vesicles (DMVs) are found near the nucleus and represent the site of viral genome replication. Coronavirus particles bud into the cisternae of the endoplasmic reticulum (ER)/Golgi and accumulate in cytoplasmic vesicles that fuse with the plasma membrane and release virus particles into the extracellular space. **B:** A severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)—infected HBEC3-KT cell showing perinuclear DMV (**arrow**) and enlarged vesicles (**arrowhead**) filled with viral particles. **C:** Higher-magnification image of viral particles in cytoplasmic vesicles (**arrowhead**). **D:** Viral particles (**arrowheads**) within cisternae of ER/Golgi; particles have characteristic electron-dense dots corresponding to the helical nucleocapsid within the envelope. **E:** Viral particles (**arrowhead**) at the cell surface. The particles have an average envelope diameter of 64 nm and a range of 56 to 75 nm. The spikes are vague and not a prominent morphologic feature in transmission electron microscopy images. Scale bars: 500 nm (**B**); 100 nm (**C**—**E**).

coronavirus. *In vitro* studies and the rare examples of *in vivo* coronavirus infections reported before the current pandemic,[25–27] as well as recent reports of *in vitro* studies and human infections for the current pandemic,[12,28] all demonstrate coronavirus within membrane-bound organelles, or outside of cells. Similar problems lie with proposed virus detected in multiple cell types in the chorionic villi of the placenta,[9,10] endothelial cells within the lung,[6] endothelial cells within the skin,[11] and cardiomyocytes and interstitial cells in the heart.[13,14] These reports do not discuss alternative explanations for the identified structures or why SARS-CoV-2 infection of human tissues would break the existing paradigm for coronavirus infection. This raises important questions about the interpretation of the micrographs.

## Cellular Structures Mistaken for Virus

Cells have many organelles comparable in size to the coronavirus, with varying degrees of electron-dense material inside and surrounding them. Cells contain numerous small vesicles that are important for moving membranes and cargo between different compartments within the cell, and into and out of cells (Figure 1A). Notable examples include clathrin-coated and non—clathrin-coated vesicles. Clathrin-coated vesicles help bring cargo into cells via receptor-mediated endocytosis and move cargo between the trans-Golgi network and endosomes.[29] Non—clathrin-coated vesicles include the coatamer [coat proteins (COP)I and COPII]-coated vesicles that sort cargo between the ER and Golgi apparatus during retrograde and anterograde transport,[30] adaptor protein 3 (AP3)-coated vesicles involved in the biogenesis of melanosomes and platelet-dense bodies, AP4-coated vesicles involved in sorting cargo between the trans-Golgi network and endosomes, as well as the basolateral membrane,[29] and caveolin-coated vesicles involved in endocytosis, transcytosis, regulation of membrane lipids, and signaling.[31] Cellular vesicles can be difficult to classify on the basis of morphology alone but can be deduced from their relationship with other membranes in the cell. Vesicles seen budding from the plasma membrane that are about 60 to 100 nm in diameter, surrounded by an electron-dense coat, and appear spiculated, are likely clathrin-coated (Figure 1B).[32] Vesicles that measure approximately 60 to 100 nm in diameter, have similar spiculated electron-dense coats, are found in the vicinity of ER and Golgi, and bud from these organelles are likely coatamer-coated (Figure 1, C and D). Other coated vesicles identified in the cell cytoplasm can be difficult to classify on the basis of ultrastructural morphology alone (Figure 1D).

Multivesicular bodies are also involved in the endocytic and exocytic functions of cells.[33,34] Early endosomes pinch off molecules destined for removal or degradation into intraluminal vesicles, forming multivesicular bodies. The multivesicular bodies may fuse with autophagosomes or lysosomes to degrade the contents, or with the plasma membrane to expulse exosomes. The intraluminal vesicles found within larger vesicles (Figure 1E)[18,19,32] have been confused with SARS-CoV-2 particles.[8] Microvilli captured in plasma membrane invaginations can also mimic multivesicular bodies and be confused for viral particles (Figure 1F).

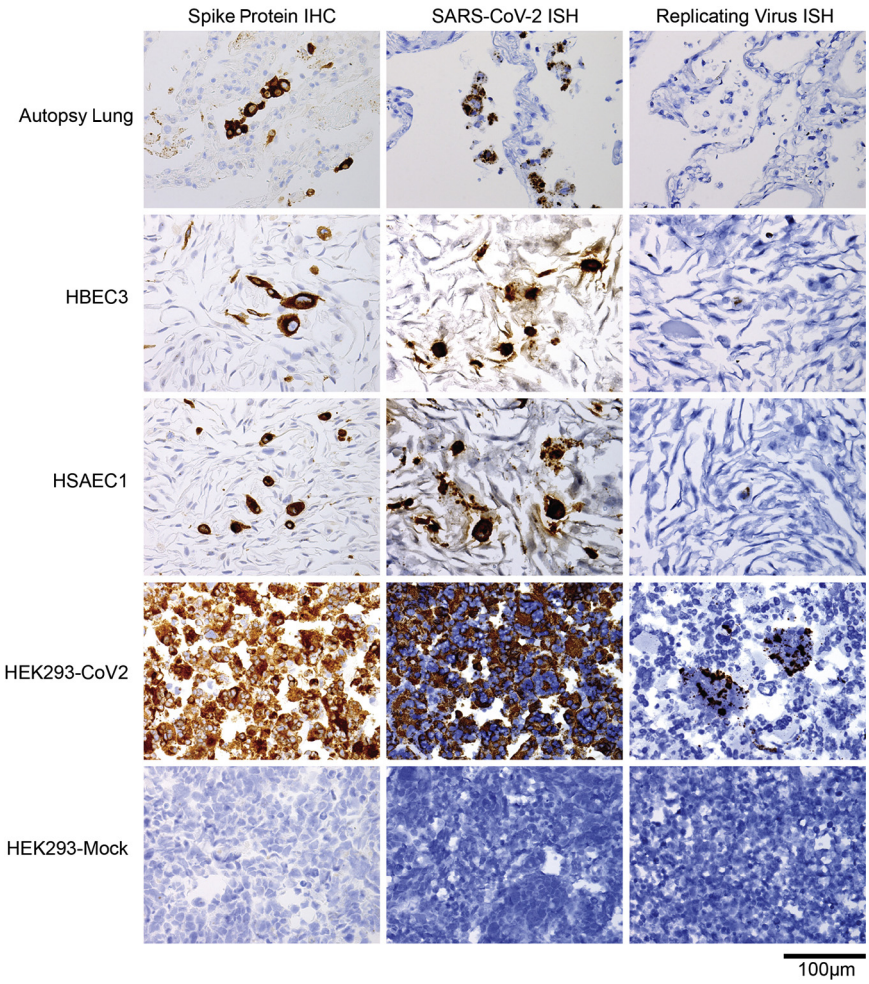


**Figure 3** Detection of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection of cells with immunohistochemistry (IHC) and *in situ* hybridization (ISH). The figure shows tissues and cells that have been stained with anti-spike antibody (SARS-CoV/SARS-CoV-2 spike antibody; chimeric monoclonal antibody; 40150-D001; Sino Biological, Wayne, PA; **left panels**), and probed with anti-spike gene probe that recognizes intact virions (RNAscope Probe - V-nCoV2019-S; ACD Biosciences, Newark, CA; **middle panels**) or replicating virus (RNAscope Probe - V-nCoV2019-S-sense; ACD Biosciences; **right panels**). The rows from top to bottom are lung tissue from a patient who died of coronavirus disease 2019 (COVID-19), HBEC3-KT cells infected with SARS-CoV-2 (HBEC3), HSAEC1-KT cells infected with SARS-CoV-2 (HSAEC1), HEK 293 angiotensin-converting enzyme 2 (ACE2) cells infected with SARS-CoV-2 (HEK293-CoV2), and mock-infected HEK 293 ACE2 cells (HEK293-Mock). The HBEC3-KT and HSAEC1-KT cells are immortalized human bronchial epithelial and small airway and cell lines, respectively (ATCC, Manassas, VA); the HEK 293 ACE2 cells are HEK293T cells that are stably transformed with the human *ACE2* gene. The anti-spike IHC and the intact virion ISH show similar staining patterns in all of the samples. The replicating virus ISH was undetectable in autopsy lung tissue, and positive in a small fraction of cells in the other samples. All images are the same magnification. Scale bar = 100 μm.

## Proposed Criteria for Identification of Viral Infection of Tissues by Electron Microscopy in COVID-19 and Future Pandemics

To ensure the rigor and reproducibility for the identification of viruses in tissues by electron microscopy, we propose that the following four criteria be met. **Structure**: morphologic features of the viral particles should conform to prior knowledge of the virus, including size and uniformity, formation of higher-order structures (aggregates/arrays/inclusions), the absence or presence of a clearly discernible membrane, and the qualities of internal (eg, nucleocapsid) and external (eg, peplomers/spikes) electron densities. If prior knowledge is lacking or incomplete, the structure of the viral particles should be established with an appropriate model system, such as electron microscopy of *in vitro* infected cells. For coronavirus, Goldsmith and Miller[19] note that coronavirus spikes are often difficult to visualize in thin sections using transmission EM, and are usually less obvious than clathrin coats. In addition, the nucleocapsid within the membrane of the viral particle has characteristic dot-like electron densities that are typically absent from cellular vesicles (Figure 2).[19] The reported diameter of the virus is approximately 80 nm.[35] However, in our studies, the SARS-CoV-2 viral particles had an average diameter of 64 nm (range, 56 to 75 nm) (Figure 2). Tissue preservation is also critical, and poor preservation, as is common for autopsy material, compromises objective interpretation of electron micrographs and the ability to conclusively identify viral particles. **Location**: viral particles should be present in sites that conform with the known biology of viral replication; strong supporting evidence is required when attempting to identify viral particles in tissues with suboptimal preservation, necrosis, and autolysis to differentiate these particles from normal cellular structures. Coronavirus particles are found inside the cisternae of the ER-Golgi and secretory compartment, as well as outside of cells (Figure 2). **Independent evidence to corroborate EM findings**: additional validated tests, such as PCR, immunohistochemistry, *in situ* hybridization, and immunoelectron microscopy, should be performed independently to confirm viral infection and further support the interpretation of the EM findings (Figure 3). **Expertise:** electron microscopy

should be performed and interpreted by experienced individuals and aided by appropriate controls and bona fide images of the virus sought. Experience with electron microscopy for diagnosis of kidney diseases alone is not sufficient to accurately discern subcellular organelles from novel viruses, and appropriate experience should be gained or sought.

## Conclusions

Early reports on the identification of novel pathogens during a pandemic leave a lasting impression. If erroneous, they have the potential to misdirect other researchers, clinicians, and the general public. Adherence to rigorous criteria for the identification of pathogens by electron microscopy will help to establish with confidence critical information needed to better understand the biology of the disease and achieve effective treatments for the current and future pandemics.

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