Abstract: This manuscript details a stringent protocol for the in situ detection of severe acute respiratory syndrome coronavirus 2 (SARS-CoV2) RNA and 4 different viral proteins: envelope, spike, membrane, and nucleocapsid. Key aspects of the protocol are: (1) analysis of adjacent (serial) sections for viral RNA and at least 2 viral proteins; (2) cytologic alterations in the cells scored as virus positive based on an hematoxylin and eosin stain; (3) in situ demonstration of a host response in the cells scored as virus positive; (4) co-labeling experiments that show that the viral RNA and/or proteins co-localize with each other and the angiotensin converting enzyme 2 (ACE2) receptor; and (5) lack of signal in equivalent tissues obtained before the pandemic. Optimization conditions for the four viral proteins as well as the ACE2 receptor were each antigen retrieval in an EDTA solution which facilitates co-expression analyses. It is recommended not to use either electron microscopy or qRTPCR as methods to corroborate in situ SARS-CoV2 detection. This stringent protocol, that relies on sequentially labeled serial sections and can be completed in one working day, demonstrated the following: (1) infectious SARS-CoV2 is abundant in the lung in fatal coronavirus disease-2019 and is seen primarily in macrophages and endothelial cells; (2) circulating viral capsid proteins (spike, envelope, membrane without RNA) are evident in multiple organs including the skin and brain where it is endocytosed by ACE2+ cells and induce an endothelialitis; (3) both the infectious virus and circulating spike protein induce complement activation and cytologic changes in the viral positive cells.

Key Words: ACE2, COVID-19, in situ, immunohistochemistry, SARS-CoV2

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The coronavirus disease-2019 (COVID-19) pandemic has of this writing infected over 150 million people with over 3 million deaths. Although several vaccines have been approved, it will take many months for herd immunity to be achieved assuming a steady and widespread access to the vaccines in combination with natural immunity. qRTPCR has been considered the gold standard to detect infectious virus, both for diagnosing people capable of spreading the virus through oral/nasal aerosols and in documenting which specific tissues, often formalin-fixed, paraffin embedded tissues, harbor the virus. Some of these studies have suggested that, although the nasopharynx and lung are the epicenters of infectious severe acute respiratory syndrome coronavirus 2 (SARS-CoV2), viral RNA, presumably infectious, can also be found in many sites including the blood, placenta, brain, liver, heart, and kidney. This has led some to hypothesize that severe/fatal COVID-19 represents systemic infection that often involves the endothelial cells of microvessels.

Multiple studies have reported on the in situ detection of either SARS-CoV2 RNA and/or proteins and, often times, the results are contradictory. For example, different investigators have reported that the placenta can harbor a high viral load of SARS-CoV2 whereas other studies, examining similar populations of women, have indicated that neither viral RNA or proteins are found in the placenta or are rarely detected. Some studies have reported that, although the nasopharynx and lung are the epicenters of infectious severe acute respiratory syndrome coronavirus 2 (SARS-CoV2), viral RNA, presumably infectious, can also be found in many sites including the blood, placenta, brain, liver, heart, and kidney. This has led some to hypothesize that severe/fatal COVID-19 represents systemic infection that often involves the endothelial cells of microvessels.

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typically the nucleocapsid or spike protein, is used as the primary test to document the virus. This data is then combined with qRTPCR and/or EM data to arrive at the conclusion that infectious virus is present at the site studied. However, EM detection of SARS-CoV2 may have a high false positive rate and qRTPCR requires the obligatory destruction of the tissue which negates the ability to determine which specific cell type(s) contain the virus and also cannot differentiate between infectious virions in the bloodstream versus the tissue per se. The purpose of this manuscript is to present a standardized stringent protocol for the in situ detection of SARS-CoV2 RNA and proteins which should help facilitate inter-laboratory reproducibility.

MATERIALS AND METHODS

Formalin-fixed, Paraffin Embedded Human Brain Samples

Autopsy lung, skin, and brain tissues were available from 10 people who died of COVID-19. They ranged in age from 36 to 92 (mean 71; 6 men and 4 women). The lung and brain tissues from 5 aged matched cases from people who died before 2016 served as negative controls. Five additional lung samples from patients who were diagnosed with idiopathic pulmonary fibrosis before 2011 served as additional controls. Also studied were 5 placentas from women who delivered while infected with SARS-CoV2 and 5 matching pre-COVID controls; each of the deliveries were unremarkable.

Immunohistochemistry

Immunohistochemistry was done as per a previously published protocol. In brief, optimal conditions for each antibody were determined by testing various dilutions and pretreatment conditions. Table 1 lists the antibodies used in this study, including the source/catalog number/and optimal conditions. Note that the optimal pretreatment in most cases was 30 minutes with the Leica EDTA antigen retrieval solution which facilitated co-expression analyses.

In brief, the immunohistochemistry protocol used the Leica Bond Max automated platform with the specific conditions listed in Table 1. Both the Fast red (DS 9820) and the DAB (DS 9800) detection kits from Leica Biosystems (Buffalo Grove, IL) were used and gave equivalent results. The horseradish peroxidase conjugate from Enzo Life Sciences (Farmingdale, New York, NY) was used in place of the equivalent reagent from Leica in the DAB kit as this has been shown to reduce background for some but not all primary antibodies. The horseradish peroxidase conjugate from Enzo Life Sciences (Farmingdale, New York, NY) was used in place of the equivalent reagent from Leica in the DAB kit as this has been shown to reduce background for some but not all primary antibodies. Immunochemical detection of activated caspase 3, interleukin (IL)6, and tumor necrosis factor-α (TNFα) were used to document the host response to SARS-CoV2 infection as previously published.

Detection of SARS-CoV2 RNA was done using the ACD RNAscope (Newark, CA) probe (Cat No. 848561-C3) using the manufacturers recommended protocol as previously published. Detection of the Enzo SARS-CoV2 RNA probe in situs was as listed in Table 1. The latter probe assay using Loop-RNA probes labeled with biotin which allowed the use of either the Enzo SAView horseradish peroxidase (brown signal) or SAView AP (blue signal) conjugate for probe detection.

Co-expression and Statistical Analyses

Co-expression analyses were done using the Nuance/InForm system whereby each chromogenic signal is separated, converted to a fluorescence-based signal, then mixed to determine what percentage of cells were expressing the 2 proteins of interest as previously described.

Other Pathologic Considerations

Serial sections (4 µm apart) were made on 10 sequentially numbered slides which allowed the study of the same groups of cells in subjacent sections since most cells range from 10 to 40 µm in size. Hematoxylin and eosin staining was included in the analyses to correlate the histologic features associated with viral detection.

RESULTS

Initially, serial sections of 10 lung tissues from 10 people who died of COVID-19 and 10 control lungs (5 unremarkable cases and 5 idiopathic pulmonary fibrosis) were studied for SARS-CoV2 RNA by in situ hybridization in a blinded manner. Under the optimal conditions described in Table 1, no signal was evident in the 10 non-COVID-19 cases whereas as signal was evident in 8/10

| Reagent | Source/Catalog # | Dilution/Pretreatment |
|---------|-----------------|-----------------------|
| SARS-CoV2 RNA probe | ACD/848561-C3 | RTU/AR and protease* 1:3000 protease* |
| SARS-CoV2 RNA probe | Enzo/LOOP-RNA probe | 1:25,000/AR EDTA |
| ACE2 Ab | ProSci/3215 | 1:6000/AR EDTA |
| SARS-CoV2 Spike S1 Ab | ProSci/9083 | 1:12,000/AR EDTA |
| SARS-CoV2 Spike S2 Ab | ProSci/9123 | 1:4000/AR EDTA |
| SARS-CoV2 Spike RBD Ab | ProSci/9087 | 1:13,000/AR EDTA |
| SARS-CoV2 Spike ENV Ab | ProSci/3521 | 1:800/AR EDTA |
| SARS-CoV2 Spike MEM Ab | ProSci/3527 | 1:6000/AR EDTA |
| SARS-CoV2 Spike NC Ab | ProSci/9099 | 1:1200/AR EDTA |
| Caspase 3 Ab | ABCAM/ab184787 | 1:7000/AR EDTA |
| IL6 Ab | ABCAM/ab6672 | 1:5000/AR EDTA |
| TNF alpha Ab | ABCAM/ab270264 | 1:25000/AR EDTA |

*As per ACD recommended protocol.

ACD indicates advanced cell diagnostics, Newark CA; ACE2, angiotensin converting enzyme 2; AR, antigen retrieval; AR EDTA, 30 minutes with Leica EDTA antigen retrieval solution; Enzo, Enzo Life Sciences (Farmingdale NY); IL6, interleukin; ProSci, Poway CA; protease, 4 minutes in proteinase K; RTU, ready to use; TNF, tumor necrosis factor.
COVID-19 pneumonias (Fig. 1). In 3 of the 10 positive cases, many cells (>100/cm²) were evident in a given tissue whereas in the other SARS-CoV2 RNA positive lungs, <100 positive cells were noted/cm² (Fig. 1). The viral RNA localized to 3 main cell types: (1) endothelial cells, (2) alveolar macrophages, and (3) alveolar pneumocytes. In the 3 cases with >100 infected cells/cm², the majority of the infected cells were the endothelial cells lining the alveoli as documented by CD31 co-expression whereas the alveolar macrophage (co-expressing both CD68 and CD206, also known as the mannose receptor) was the second most common infected cell with alveolar pneumocyte (pan cytokeratin positive) the least common infected type (Fig. 1). In the COVID-19 infected lungs with few infected cells, most of the viral positive cells were alveolar macrophages (Fig. 1) or pneumocytes. Equivalent results were evident with the ACD and Enzo SARS-CoV2 RNA probes (Fig. 1).

Next, serial sections from 2 of the 3 lungs with the high viral load were analyzed for the following SARS-CoV2 proteins: envelope, membrane, spike (S1, RBD of spike, and S2) and nucleocapsid using the optimal conditions outlined in Table 1. These cases were tested concurrently with three lung controls and the results were read blinded to the clinical information. Representative results are provided in Figure 1. Note that each of the viral proteins showed the same distribution as the viral RNA in the serial sections of the infected lung tissue. No signal was seen in the lung samples obtained before 2019. Co-localization was done with the S1 subunit of the spike protein and ACE2. As seen in Figure 1, all cell types positive for the spike protein expressed ACE2.

Co-expression experiments were done with the Nuance software which will demonstrate co-localization of any 2 molecules if they are within 150 nm of each other in 3 dimensional space. Since the infectious SARS-CoV2 virus is between 70 and 90 nm in size, one should see a nearly 1:1 co-localization of any 2 viral molecules after co-expression analyses assuming that the signal intensity is equivalent for each. Co-expression was done in the lung tissues for viral RNA and the S1 protein, as well as between the spike S1 subunit and either the envelope or membrane proteins. Representative data is shown in Figure 2. Note the strong and nearly 1:1 co-localization of the spike protein with the viral RNA as well as between the spike and the viral membrane proteins.

The histologic correlates to the viral infection were addressed next. In the lung tissues with high viral copy number, there was a strong correlation between histopathologic changes and the detection of infectious virus. Areas with high viral load showed a paucicellular inflammation in which the alveolar wall was thickened and there was marked degenerative changes in the endothelia and epithelia (Fig. 2); microthrombi and red blood cell extravasation were commonly present in such areas.

FIGURE 1. Detection of severe acute respiratory syndrome coronavirus 2 RNA and multiple viral proteins in serial sections in coronavirus disease-2019 (COVID-19)-associated pneumonia. A and B, Show the in situ detection of severe acute respiratory syndrome coronavirus 2 RNA in fatal COVID-19 pneumonia with the ACD and Enzo Life Sciences probes, respectively. Note the 2 different patterns of viral RNA distribution commonly seen in COVID-19 pneumonia: (1) signal primarily in the endothelial cells of septal capillaries and associated macrophages (circles, A) and the signal localized mostly to alveolar macrophages (B). Note that the viral RNA is evident in areas of severe lung damage. In comparison, viral RNA was not evident in the adjacent histologically normal lung (C). Note the similar distribution of the viral protein Spike S2 subunit (D), and S1 subunit (E). Co-expression of the S1 protein (fluorescent red) and ACE2 viral receptor (fluorescent green) shows a strong co-localization pattern as fluorescent yellow (F). ACE2 indicates angiotensin converting enzyme 2.
Adjacent areas of the lung with no detectable virus showed unremarkable alveoli (Fig. 1).

The next criterion for accurately documenting SARS-CoV2 infection was to analyze for a concomitant host response since it is well established that active coronavirus in humans is associated with a myriad of cellular responses.\textsuperscript{1-5,23,24} In this manuscript, the focus was on three proteins that have been associated with severe COVID-19: activated caspase 3, TNF$\alpha$, and IL6.\textsuperscript{5,21}

As seen in Figure 3, activated caspase 3 was evident in the lung pneumonia and was most prominent in areas with a high viral load and associated alveolar degeneration. SARS-CoV2 induces a microangiopathy in which different components of the complement cascade are activated which, in turn, is a major factor in the pathophysiology of COVID-19 associated pneumonia.\textsuperscript{21}

As seen in Figure 3, there was strong co-expression between the viral envelope protein with both C5b-9 and C4d. The SARS-CoV2 capsid proteins have been detected in the ACE2$^+$ endothelia of the microvessels in the brain and subcutaneous fat.\textsuperscript{23} Analyses of serial sections of brain tissue did show an equivalent distribution of the spike protein with IL6, caspase 3, and the viral membrane protein (Fig. 3). Interestingly, viral RNA was not evident in these analyses (Fig. 3).

Next, a series of lung, brain, and placental tissues obtained between 2010 and 2018 that served as negative controls were analyzed for background. Using the conditions as outlined in Table 1, no background was evident in the various tissues (Fig. 4). However, many of the SARS-CoV2-related antibodies used for immunohistochemistry showed a relatively narrow window between signal and background. For example, the Spike S2 antibody showed no background at a dilution of 1:12,000. However, at dilutions of 1:4000 or 1:6000, the normal, pre-COVID lungs did show color changes that could be misinterpreted as signal. As seen in Figure 4, the background was evident in many cell types, notably the epithelia in small airways, which were uniformly negative for the virus with in situ hybridization (Fig. 4). Note in Figure 4 that the serial sections of the normal lung were negative when tested with the S2 spike at the optimal concentration and the nucleocapsid proteins. Further note that the bronchial epithelia were cytologically normal and showed no evidence of cytokine expression (data not shown). Each of the viral-related antibodies tended to show background when the dilution was about 2X as listed in Table 1. The ACD RNA probe was more likely to show background than the Enzo RNA probe and, thus, required carefully monitoring if done manually. Figure 4 also shows a typical pattern for background in which the entire lumen of the microvessel showed a strong color reaction, in this case for IL6 though this pattern was typical for background for the other antibodies tested. This is also evident in Figure 4 for a pre-2019 normal placenta tested for the spike S1 protein at too high a concentration. Note the loss of the background when

FIGURE 2. Utility of co-expression analyses and histologic examination for documenting severe acute respiratory syndrome coronavirus 2 infection. A, Shows the distribution of the viral RNA in fatal coronavirus disease-2019 pneumonia; note the high viral load and the degenerative changes. The corresponding hematoxylin and eosin (B) shows a microangiopathy in which the septal capillaries are markedly expanded and disrupted with degenerative changes in the lining cells. Co-expression analysis for the S1 subunit of the spike protein (fluorescent green) and viral membrane protein (fluorescent red) was done with the Nuance software which separates the 2 signals (C, S1 subunit and D, membrane protein) and then merges them (E, with fluorescent yellow denoting co-localization). Note in the merged image the near 1:1 co-localization of the 2 viral proteins. F, Shows the equivalent results for the co-localization of the viral RNA and the S1 spike protein.
the optimal spike antibody concentration was used and that the placenta was ACE2 negative, another indicator that the viral result was a false positive.

Figure 5 shows the co-expression of viral proteins (spike and membrane) with the cytokine TNFα as well as the viral envelope protein and ACE2 in the central
nervous system and skin. Note the near 1:1 ratio of the viral capsid protein and TNFα. However, note that whereas the viral envelope protein is always present with ACE2, there are ACE2+ areas that lack the viral protein.

Figure 6 shows the recommended protocol for maximizing the specificity of the in situ detection of SARS-CoV2 RNA or proteins.

DISCUSSION

The in situ detection of SARS-CoV2 RNA and proteins provides essential data for understanding the pathophysiology of the viral infection causing the pandemic. This study provides a protocol that would allow for standardized demonstration of viral infection among different investigators. The bases of the protocol are (1) a given positive in situ result for viral RNA or protein is corroborated by demonstrating in serial sections at least 2 additional viral proteins in the same distribution; (2) co-expression analyses of 2 or more SARS-CoV2 molecules showing a near 1:1 ratio, making the results much more likely to represent true viral infection since it is unlikely that the in situ background profile of 2 or more proteins/RNA would be identical;22 (3) co-localization of the primary cellular target of SARS-CoV2, ACE2, with viral proteins and/or RNA; (4) degenerative cytologic changes in the infected cells; (5) demonstration of the host cell response in the same area as the infected cells; (6) lack of signal in pre-COVID tissues. It should be stressed that the optimal conditions for the antibodies used in this study may differ in other laboratories because of, for example, different automated platforms, detection kits, or other sources of the antibodies. The key point is to optimize each antibody using the overall strategy outlined in this paper.

There are many contradictory studies regarding the distribution of the infectious virus in COVID-19. Many of these studies are based, at least in part, on detection of the viral RNA using qRTPCR and EM as corroboration of the 1 in situ result.1–5 These studies have suggested that the nasopharynx and lung are where the viral infection begins and that the infectious virus often, in severe disease, becomes systemic and can be found in the blood, placenta, brain, liver, heart, and kidney where fatal COVID-19 then ensues. This has led some to hypothesize that severe/fatal COVID-19 represents systemic infection that often involves the endothelial cells of microvessels.1–4

A related point is that there is much variability in the reports of different laboratories regarding the distribution of SARS-CoV2 in different tissues. Some have reported that the placenta can harbor a high viral load of SARS-CoV2 suggesting that horizontal viral spread can occur. Other studies, examining similar populations of women, have indicated that neither viral RNA or proteins are found in the placenta or are rarely detected.9–11 Analyses of the skin,14–18 brain,25–27 and heart28,29 have also reported much variability in the data ranging from detection of many infectious viral particles, to viral protein alone, to no virus in these disparate sites.

EM has been reported to show many false positive results for SARS-CoV2.20 In our experience, one reason may be that clathrin coated vesicles can mimic the appearance of the spike protein in EM. With regards to qRTPCR, we have previously shown that RT in situ PCR and in situ hybridization for high copy viral RNA targets, like SARS-CoV2, give equivalent results.30 However, we did not perform qRTPCR in these experiments as this method cannot localize the signal to a specific cell type. Enzo’s loop-RNA probes, coupled with their nanopolymer detection reagent

FIGURE 5. Co-expression analyses of severe acute respiratory syndrome coronavirus 2 and host related proteins. A, Shows the light microscopy image after co-expression of the viral spike protein and tumor necrosis factor-α (TNFα) in the CNS from a fatal coronavirus disease-2019 case. Note the perivascular edema around the microvessel. The Nuance system isolates the spike signal as fluorescent green (B), the TNFα signal as fluorescent red (C), then merges the 2 signals where co-localization is seen as fluorescent yellow (D). E–H, Shows co-localization of the viral membrane protein and ACE2 (E and F) as well as TNFα and viral envelope protein (G and H), respectively, in a case of thrombotic retiform purpura of the skin in a fatal coronavirus disease-2019 case. Note in E and F that the viral membrane protein (red) always co-localizes with ACE2 (green) but that the latter is evident at times without the viral capsid protein. ACE2 indicates angiotensin converting enzyme 2; CNS, central nervous system.
(POLYVIEW), offer high sensitivity. These probes offer unbiased signal amplification from the loop in the probes (nonhybridizing biotin-labeled regions). The ease of use of the probes, compatibility with existing biotin-based detection assays, and lack of specialized equipment provide several advantages over existing technologies. Importantly, Loop-RNA probes do not require lengthy protocols to aid the formation the signal structures required in branched DNA (bDNA) probe technology that in turn tend to increase background.

The reasons for background with any in situ based test are many, and include incorrect pretreatment conditions, too high a probe/primary body concentration, and the use of either primary and/or secondary antibodies that tend to show nonspecific staining.22,30 This simple but important observation is the basis of why using several SARS-CoV2-related molecules and co-localization analyses are important parts of a standardized protocol. This is because it is unlikely for 2 or more probes/primary antibodies to show the same background profile as defined by the types of cells which show background and the cyto logic distribution of the background.22,30 In many of the papers that do in situ testing for viral RNA and/or protein, one viral molecule alone is tested. It is hoped that a more rigorous, standardized protocol as presented in this manuscript will help establish more uniformity among different laboratories and, thus, provide a more accurate representation of the in situ distribution of both infectious virus and pseudovirions (viral protein minus the RNA) in SARS-CoV2 associated disease.

Finally, with regards to some technical tips, the RNAscope and Enzo LOOP-RNA system each have excellent sensitivity. However, they are each susceptible to background, more so the RNAscope assay, which may reflect the three amplification steps in the assay. With regards to the different viral protein antibodies, we found that the spike, membrane, and nucleocapsid specific antibodies had a wider signal to background ratio compared with the RBD and envelope proteins and thus preferred these antibodies for most of our testing.

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