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Method Article

Simultaneous detection and quantification of spike mRNA and protein in SARS-CoV-2 infected airway epithelium.

Kailey Jerome[1], Sarah Sattar[1], Masfique Mehedi*

Department of Biomedical Sciences, University of North Dakota School of Medicine & Health Sciences, Grand Forks, ND 58202, USA

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ABSTRACT

Visualizing and quantifying mRNA and its corresponding protein provides a unique perspective of gene expression at a single-molecule level. Here, we describe a method for differentiating primary cells for making airway epithelium and detecting SARS-CoV-2 Spike (S) mRNA and S protein in the paraformaldehyde-fixed paraffin-embedded severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infected airway epithelium. For simultaneous detection of mRNA and protein in the same cell, we combined two protocols: 1. RNA fluorescence-based in situ hybridization (RNA-FISH) based mRNA detection and 2. fluorescence-based immunohistochemistry (IHC) based protein detection. The detection of mRNA and proteins in the same cell also allows for quantifying them using the open-source software QuPath, which provides an accurate and more straightforward fluorescent-based quantification of mRNA and protein in the microscopic images of the infected cells. Additionally, we can achieve the subcellular distribution of both S mRNA and S protein. This method identifies SARS-CoV-2 S gene products' (mRNA and protein) degree of expression and their subcellular localization in the infected airway epithelium. Advantages of this method include:

- Simultaneous detection and quantification of mRNA and protein in the same cell.
- Universal use due to the ability to use mRNA-specific primer-probe and protein-specific antibodies.
- An open-source software QuPath provides a straightforward fluorescent-based quantification.

Specifications table

Subject area	*Immunology and Microbiology*
More specific subject area:	*Virology*
Name of your method:	*Simultaneous detection & quantification of mRNA and protein*
Name and reference of original method:	*N/A*
Resource availability:	*Table*

* Corresponding author.
E-mail address: masfique.mehedi@und.edu (M. Mehedi).
[1] These authors contributed equally to this work.

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REAGENT or RESOURCE	SOURCE	Catalog #
Antibodies		
Anti-SARS/SARS-CoV-2 S rabbit polyclonal antibody (1:100 dilution)	Thermo Fisher Scientific	Cat# PA5–81,795;
Goat anti-rabbit IgG, Alexa Fluor® 647 (1:100 dilution)	Thermo Fisher Scientific	Cat# A-21,245;
Anti-Acetyl-α-Tubulin rabbit monoclonal antibody (1:1000 dilution)	Cell Signaling Technology	Cat#5335;
Goat anti-Mouse IgG, Alexa Fluor® 647, (1:100 dilution)	Thermo Fisher Scientific	Cat# A21–235
Chemicals, Peptides, and Recombinant Proteins		
Airway Epithelial Cell Medium	PromoCell	C-21,060
Airway Epithelial Cell Medium SupplementMix	PromoCell	C-39,165
PureCol®, Bovine Collagen	Cell Systems	5005
TrypLE™ Express Enzyme	Thermo Fisher Scientific	12,604,021
Penicillin-Streptomycin	Thermo Fisher Scientific	15,140,122
Amphotericin B	Thermo Fisher Scientific	15,290,026
PneumaCult™-ALI Medium	Stemcell Technologies	05,001
Hydrocortisone stock	Stemcell Technologies	07,926
Heparin Solution	Stemcell Technologies	07,980
NucBlue™ fixed cell stain ReadyProbes	Thermo Fisher Scientific	R37606
ProLong™ Gold anti-fade Mountant	Thermo Fisher Scientific	P36930
16% Formaldehyde (Methanol free)	Polysciences	18,814–10
Goat Serum Blocking Solution	Vector Laboratories	S-1000–20
Rhodamine Phalloidin (Amanita phalloides)	Cytoskeleton Inc.	PHDR1
Histo-clear	Fisher Scientific	5,032,950
Ethanol 190 Proof	Decon Labs. Inc.,	2801
Triton X-100	Sigma-Aldrich	X-100
Tween-20	Sigma-Aldrich	P1379
Trypan Blue dye	Stem Cell Technology	07,050
1X DPBS	Sigma Life Science	D8537
1X PBS pH 7.4	Prepared in Lab	N/A
Probe V-nCoV2019-S	Advanced Cell Diagnostics	848,561
Positive Control Probe Hs-PPIB	Advanced Cell Diagnostics	313,901
Experimental Models: Primary cells		
Primary normal human bronchial epithelial (NHBE) Cells (healthy adult, deidentified, $N = 2$)	Provided by Dr. Kristina Bailey laboratory	N/A
Software		
LASX with 2D and 3D Deconvolution	Leica Microsystem	https://www.leica-microsystems.com/
QuPath	Quantitative Pathology and Bioimage Analysis	[1]
IMARIS	Oxford Instruments	N/A
PRISM	GraphPad	N/A
Other		
Falcon tissue culture dish 100 mm dish	Corning	353,003
6.5 mm Transwell® with 0.4 μm Pore Polyester Membrane Insert	Corning	3470
Superfrost Plus Microscope slides	Fisher Scientific	22–037–246
Immedge Hydrophobic Barrier Pen	Vector Laboratories	H-4000
Coverglass 12 mm	Carolina	633,029
Leica DMi8 Inverted Microscope	Leica Microsystem	N/A
RNAscope Multiplex Florescent Reagent kit V2	Advanced Cell Diagnostics	323,100
HybEZ oven	Advanced Cell Diagnostics	N/A
HybEZ humidity control tray and HybEZ slide rack	Advanced Cell Diagnostics	N/A

Method details

A. Primary NHBE cell propagation and maintenance

Note**:** This will take 4–7 days

1. Before seeding cells on cell culture dish, coat the dish with a surface coating reagent, PureCol (4% solution in sterile water). Coat 100 mm cell culture dish with 5 mL PureCol for 20 min at room temperature (15 - 25 ˚C). We recommend coating the cell culture dish just before the seeding cells on to them. Keep the coated plates at room temperature (15 - 25 ˚C). Alternatively, a pre-coated cell culture dish can be used.
2. Thaw cryopreserved primary early-passage NHBE cells (around 10ˆ6 cells) for a minute in a water bath set at 37 ˚C before use.
3. Using a sterile pipette, add 1 mL of new cAEC medium into the cryovial drop by drop to reduce cell damage by the temperature different. Immediately, transfer cells from the cryovial into a 15 mL conical tube.
4. Spin the cells at 330x*g* at 4 ˚C for 5 min in a benchtop centrifuge. Carefully remove the supernatant from the tube and resuspend the cell pellet in 2 mL of cAEC medium
5. For cell passaging, count cells and add desired cell to the precoated 100 mm dish containing around 10 mL of cAEC medium and gently swirl the dish for homogeneous distribution of the cells.
6. Incubate the cells at 37 ˚C and 5% CO2 until the cell monolayer appears to be near fully confluent (4 – 6 days).
7. For maintenance, replace 10 mL cAEC medium every 48 h.

8. Once cells are confluent (around 90%), they can be passaged further (cell split ratio at 1:3) for maintenance or use for cell differentiation.

Note: While primary NHBE cells can be passaged many times, it is not recommended to use cells at more than passage 4 for differentiation due to the unreliability of proper differentiation in higher passaged cells.

B. Differentiating primary NHBE cells for obtaining bronchial airway epithelium

Note**:** This will take 4–5 weeks.

1. Before seeding cells on to the apical side of the Transwell® plate inserts, coat with a surface coating reagent PureCol (4% solution in sterile water). Coat Transwell® plate inserts (e.g., 6.5 mm) at the apical side with 100 µl PureCol™ for 20 min at room temperature (15 - 25 ˚C). Remove PureCol™.
2. Aspirate the old medium from a confluent NHBE culture (passage 4) and wash with 5 mL of sterile 1x DPBS. Add 5 mL of TrypLE™ Express Enzyme (room temperature 15 - 25 ˚C stored) and incubate for 5 min at 37 ˚C.
3. Transfer the cells into a 15 mL conical tube and spin at 330x*g* for 5 min at 4 ˚C. Carefully remove supernatant and resuspend cell pellet in 2 mL of cAEC medium.
4. Count cells, e.g., manual count using trypan blue dye and hemocytometer. Use 1in 2 dilution (e.g., 10 µm trypan blue dye + 10 µm cells).
5. Seed around 50,000 cells in 100 µl of cAEC medium on the apical side of a Transwell. Add 500 µl of cAEC medium on the basal side.
6. Incubate cells at 37 ˚C for 24–48 h until the cells have formed a 100% confluent monolayer. Remove the cAEC medium from both apical and basal sides of the Transwell. Immediately, add 500 µl complete PneumaCult-ALI medium on the basal side of the Transwell; but, the apical side was left liquid less for resembling airway.
7. For differentiation, incubate Transwell at 37˚ for 3 to 4 weeks with complete PneumaCult-ALI medium change every 48 h on basal sides.
8. For removing extensive mucus, add 200 µl of sterile 1x DPBS on the apical side of the Transwell and incubate for 30 min at 37 ˚C. Carefully remove the DPBS by placing a sterile pipette tip just above the cell layer without touching the cells. Mucus washing step can be done once a week since day 14 differentiation. This can be increased to every other day when excessive mucus production is visible.

Note: Collagen-coated Transwells can be purchased from commercial vendors. The NHBE-driven airway epithelium in the air-liquid interface starts producing mucus from around the 14th day of differentiation, and ciliary function is also visible around that time. We recommend using primary NHBE cells at passage 4 or earlier for differentiation [4,5]. We recommend using TrypLE Express Enzyme instead of trypsin because it is a milder reagent than generic trypsin and no need for any trypsin neutralizing reagent. For achieving highly differentiated airway epithelium, the medium change has been done every other day.

C. Viral infection and formalin-fixation

Note: This will take 1–5 days.

1. Infect pseudo-stratified bronchial airway epithelium in Transwell with SARS-CoV-2 (nCoV-WA1–2020; GenBank accession number MN985325.1) at a multiplicity of infection (MOI) of 0.1 for four days at 37 ˚C and in a 5% CO2 humidified incubator.
    a. Prepare virus inoculum in 100 µm 1x DPBS.
    b. Add virus inoculum (virus in 100 µl cAEC medium) at the apical side of the airway epithelium.
    c. Incubate the Transwel for two hours at 37 ˚C and in a 5% CO2 humidified incubator.
    d. Remove virus inoculum, wash apical side 2x with 100 µm 1x DPBS.
    e. Carefully remove DPBS to keep the airway epithelium dry.
2. After viral infection for four days, wash apical and basal sides of the Transwells twice with 1x PBS (200 µl for apical and 500 µl for basal).
3. For fixation of mock-infected or SARS-cov-2-infected airway epithelium, add freshly prepared 4% paraformaldehyde (PFA) solution in 1x DPBS (prepared by using 16% formaldehyde, which eliminates the step of depolymerization paraformaldehyde prior to use) to both apical and basal sides of the Transwells (200 µl for apical and 500 µl for basal).
4. Incubate the Transwells for 18 – 24 h at room temperature (15 - 25 ˚C).
5. Remove PFA and wash and keep the Transwells submerged in 1x PBS for a week for short-term storage.

Note: We recommend using a four-week differentiated pseudostratified intact bronchial airway epithelium for infection experiment. Specific biosafety regulation needs to be followed based virus, e.g., SARS-CoV-2 infection should be performed at a high-containment facility. The virus-infected Transwells should be PFA-fixed around 12 h or overnight before transporting them safely from the high biocontainment facility.

D. Paraffin-embedding and sectioning

1. For embedding bronchial airway epithelium, first, carefully take-out polycarbonate membrane from the Transwell using a sterile scalpel. For convenience, the membrane can be place in a Petri dish containing 1x PBS.
2. Second, carefully cut the membrane into at least two small pieces to fit into a Paraffin cassette, which has a smaller diameter than the total membrane. Place a tiny piece of membrane into a Paraffin cassette containing 1xPBS.

Pause point: Paraffin cassette containing 1xPBS can be stored at 4 ˚C for a few days.
For processing, the membrane in the cassette undergoes following incubation steps:

Reagent	Time (minutes)
1xPBS	5
50% EtOH	5
70% EtOH	5
95% EtOH	5
95% EtOH	5
100% EtOH	5
100% EtOH	5
Histo-Clear	5
Histo-Clear	5

1. Three, 30-minute paraffin infiltration steps are required in a vacuum oven with a temperature setting of 58 ˚C +/- 2 ˚C under pressure for enhanced paraffin infiltration.
2. Embed the membrane on the edge and the paraffin block can be stored at 4 ˚C. The paraffin block can be stored at room temperature or at 4 ˚C for a year.
3. Cut sections about 5 μM thick using a rotary microtome and mount onto Superfrost plus slides. Sectioned slides can be stored at 4 ˚C for several months to a year.

E. Deparaffinization and fluorescence-based Immunohistochemistry (IHC)

Note: This will take 1–2 days and no antigen retrieval step is necessary for FFPE NHBE cells. This protocol has not been optimized for FFPE tissue sample.

1. Deparaffinize membrane on section slides by incubating them in coplin jars containing in following solutions:

Reagent	Time (minutes)
Histo-Clear	5
Histo-Clear	5
100% EtOH	5
100% EtOH	5
100% EtOH	5
95% EtOH	5
70% EtOH	5
Distilled water	5
Distilled water	5

2. Remove slides from coplin jar and put on a tray (e.g., HybEZ^{TM} Slide Rack in a HybEZ^{TM} Humidity Control Tray). With an Immedge Hydrophobic Barrier Pen, draw a square around each tissue. This step keeps reagents localized on the tissue section.
3. Add 0.5% (v/v) Triton X-100 solution in 1xDPBS on the slide to submerge the sample. Incubate 30 min at room temperature (15 - 25 ˚C).
4. Perform three 5-minutes washes with 1x PBST 0.1% PBST (phosphate buffer saline, Tween 20, Sigma-Aldrich).
5. Add 100 μL of blocking buffer (10% goat serum, Vector Laboratories, in 1x DPBS) to each tissue section. Incubate the slides at 4 ˚C for 2 h.
6. Prepare solution for primary antibody incubation in 0.1% PBST.
7. After blocking, remove the blocking buffer by gently wicking away excess solution with a KimWipe.
8. Add 100 μL primary antibody solution onto each slide. SARS-CoV2 S specific rabbit polyclonal (1:100 dilution, in 1x DPBS). Incubate the slides 12 h or overnight at 4 ˚C in a HybEZ humidity control tray.
9. Remove primary antibody solution from the slides and wash three times for five minutes with 0.1% PBST.
10. Prepare secondary antibody solution (secondary antibodies should correspond to the primary antibodies) at 1:100 0.1% PBST, e.g., goat anti-rabbit AF647 (1:100 dilution, 1x DPBS) corresponds with SARS-CoV2 S specific rabbit polyclonal antibody.

Note: Avoid or reduce light exposure to the sample in the next steps.

11. After the final wash step, using a fresh KimWipe, gently wick away as much PBS as possible. Then, add 100 μL of the appropriate secondary antibody in 1x DPBS to each tissue section. Incubate slides in secondary antibody in 1x DPBS for at least 2 h at 4 ºC or 45 min at room temperature (15–25 ºC) in the HybEZ humidity control tray.
12. Perform three 5 min washes with 1X PBST.

F. RNA fluorescence-based in situ hybridization (RNA-FISH) using RNAscope.

Probe incubation

1. Prepare RNAscope materials by preheating the HybEZ oven (should be set to 40ºC) and warming the RNAscope probe for 10 min at 40 ºC in a water bath. After the 10-minute incubation, allow the probe to cool to room temperature (15–25 ºC) before use.

2. Rinse slides (from step 5 s) twice for fifteen seconds in RNAse free water. Remove excess water with a fresh KimWipe.
3. Add 1 drop Protease Plus to cover the entire tissue section. Incubate slides for 30 min in the HybEZ oven set to 40°C.
4. After permeabilization with Protease Plus, wash the slides twice for two minutes with RNAse free water. This is done by applying the water directly onto the tissue section and incubating the slides in the HybEZ slide tray at room temperature (15 - 25 ˚C).
5. Remove excess water with a KimWipe. Then, apply just enough of the appropriate probe to cover the tissue section (~2 drops). For this specific protocol, RNAscope Probe V-nCoV2019-S was used.
6. Incubate slides in HybEZ oven for 20 min at 40 °C. Remove the humidity control tray from the oven and leave the slides 12 h or overnight at room temperature (15 - 25 ˚C).
7. Wash each of the slides 2 times with 200 µl of wash buffer by adding the buffer dropwise and incubate at room temperature (15–25 °C) for 2 min.

AMP signal amplification

1. Add RNAscope Multiplex Fl v2 AMP 1; close the tray and insert into the HybEZ oven for 30 min incubation at 40°C.
2. Wash 2 times with 100 ul of 1X wash buffer for 2 min at room temperature (15 - 25 ˚C).
3. Add RNAscope Multiplex Fl v2 AMP 2; close the tray and insert into the HybEZ oven for 30 min incubation at 40°C.
4. Wash 2 times with 100 ul of 1X wash buffer for 2 min at room temperature.
5. Add RNAscope Multiplex Fl v2 AMP 3; close the tray and insert into the HybEZ oven for 15 min incubation at 40 °C.
6. Wash each of the slides 2 times with 100ul of 1X wash buffer for 2 min at room temperature.

Developing HRP- C1 Signal

1. Add RNAscope Multiplex FL v2 HRP- C1; close the tray and insert into the HybEZ oven for 30 min incubation at 40 °C.
2. Wash each of the slides 2 times with 100 µl of 1X wash buffer for 2 min at room temperature.
3. Add 150 µl of prepared Opal 570 dye (1:750) in TSA buffer to each slide and incubate for 30 min at 40 °C.
4. Wash each of the slides 2 times with 100 µl of 1X wash buffer for 2 min at room temperature.
5. Add RNAscope Multiplex FL v2 HRP blocker to entirely cover each slide; close the tray and insert into the HybEZ oven for 15 min incubation at 40 °C.
6. Wash each of the slides 2 times with 100 µl of 1X wash buffer for 2 min at room temperature (15 - 25 ˚C).

Note: Choose appropriate HRP channel for specific probe, e.g., for C1, C2 or C3 probe follow HRP-C1, HRP-C2, or HRP-C3, respectively for developing signal.

Counterstain and mounting

1. Add 1 drop of DAPI (RNAscope) to each slide so tissue is completely covered and incubate for 1 min at room temperature (15 - 25 ˚C).
2. Wash with 100 µl of 1X wash buffer for 2 min at room temperature (15 - 25 ˚C).
3. For mounting, add 8 - 10 µl of gold antifade mounting medium on each of the slides and seal with a square coverslip. Dry the slides in the dark.
4. Seal the coverslip with nail polish for several minutes to dry.
5. Store slide at 4 °C until imaging. Image the slide immediately to avoid fluorescence signal deterioration. However, the slides can be stored for a month for a short-term storage (six months). The images can be taken under a Leica DMi8 epifluorescence microscope. For the figure prep ration, Z-stack imaging was done, and images were processed through Leica LASX software.
6. *Quantification using Qu Path program.*

Uploading the image (IMG, JPEG, TIFF, and PNG)

1. Open the Qu Path program. Select “file” in the top left corner of the screen then “Open”.
2. Select image type as “Brightfield (other)”. Click the line icon labeled “line” on the top left of the screen.

Set scale

1. Zoom in on the scale bar on the image and draw a line over the scale bar.
2. Under image tap on the left-hand menu: double click red “unknown” and enter line length as the length of the scale bar.

Color deconvolution

1. Select the square icon on the top left of the screen and draw a square over a nucleus being sure that it only contains the blue color.

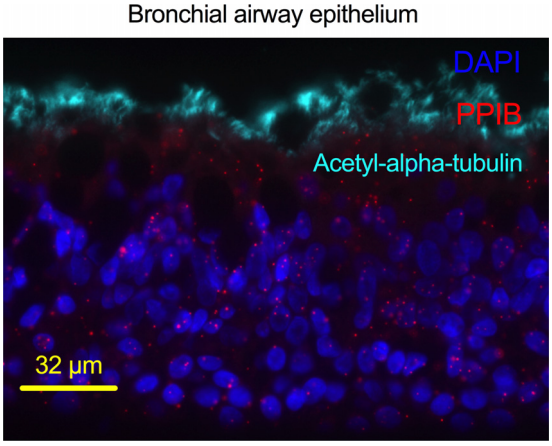

Fig. 1. Simultaneous detection of cellular cilium and cellular PPIB mRNA. The airway epithelium was fixed with paraformaldehyde (PFA), paraffin-embedded, sectioned, stained, and imaged under a Leica DMi8 epifluorescence microscope using 63x oil-objective. PPIB mRNA was detected by RNAscope using PPIB mRNA specific probe and Opal 570 dye (shown in red) and cilia was detected by immunofluorescence using acetyl-alpha tubulin specific mouse monoclonal antibody and corresponding goat anti-mouse AF647 secondary antibody (shown in cyan).

2. Under image tab on left-hand menu: Double click “hematoxylin”. Select “Yes” then Select “Okay”.
3. Repeat this process for the TxRED channel- double click “DAB”.
4. Repeat this process for the Cy5 channel- double click “Residual”.

Subcellular spot detection

1. Once cells have been detected to the best degree of accuracy, change the image type to “Fluorescence”, and under the image tab on left-hand menu: double click “brightfield (other)”, and change to “Fluorescence”.
2. Select “analyze” then “cell detection” then “subcellular detection”.
    a. Change Channel 1 and 2 to a baseline threshold of 5
    b. Select “Channel 1″ set it as “red” and “mRNA”
    c. Select “Channel 2″ set it as “green” and “protein"
    d. Keep channel 3 at −1
3. Select “split by intensity” (can also “split by shape” if desired). Be sure “include clusters” is selected as well.
4. If the signal has noise “smooth before detection” can be checked.
5. Adjust threshold levels until a good degree of accuracy is acquired.
6. Select “Measure” then “show detection measurements”.
7. Copy data to clipboard and paste data into prism or excel.

Data analysis

1. Once the data has been transferred into excel, delete all the data in the columns besides the column labeled “Subcellular spot: Channel #: num spots estimated”.
2. All the rows besides the rows labeled “PathCellObject” can be deleted as well.

Note: Each row represents a single cell, while the columns represent the number of spots found.

3. The data can then be copied and pasted into prism to generate a graph.

RESULTS & DISCUSSION

This optimized protocol combines IHC-based protein detection and RNAscope-based mRNA detection. Both IHC and RNA-FISH assays are known for their specificity [7,8]. Thus, simultaneous detection and quantification of mRNA and corresponding protein can be obtained at a single molecule level in a cell. For validation and optimization, we initially detected cellular peptidylprolyl isomerase (PPIB) mRNA using an RNAscope probe (Hs-PPIB), and cilia were detected by using an antibody specific to acetyl-alpha tubulin (Fig. 1) in the 5 µm thick section of FFPE airway epithelium. Here we showed the detection and quantification of S mRNA and S protein in the 5 µm thick section of FFPE of SARS-CoV-2 infected airway epithelium using an image taken under an epifluorescence microscope (Fig. 2) or a confocal microscope (Fig. 3). One of the significant findings using this protocol was a simultaneous detection of both S mRNA and S protein in the infected cell nucleus. Apparently, the nuclear translocation of S mRNA and S protein is a novel feature of SARS-CoV-2 infection [6]. Based on a brightfield observation, we could show S mRNA and S protein in the SARS-CoV-2

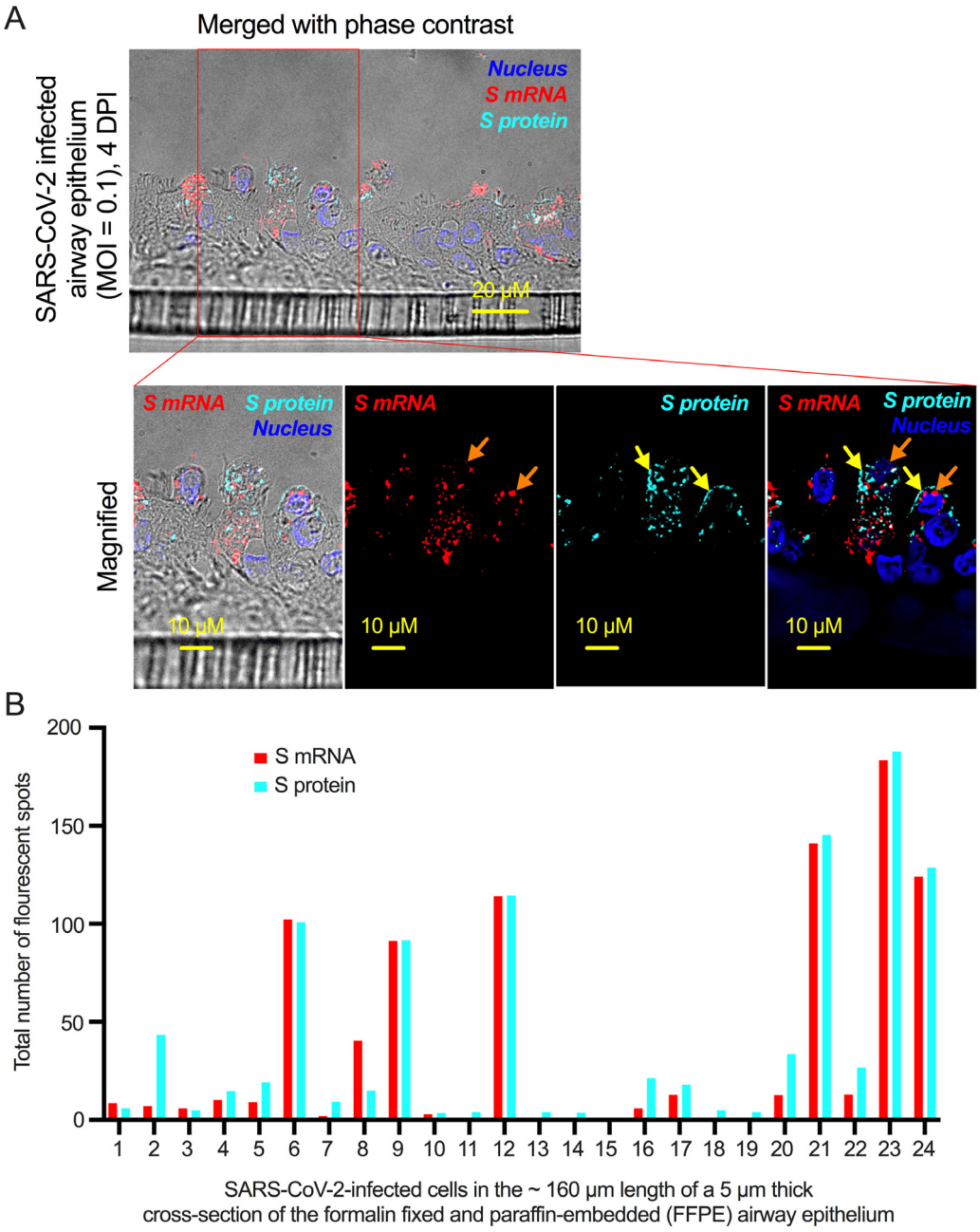

Fig. 2. Simultaneous detection and quantification of both Spike (S) mRNA and S protein near single molecule level in the SARS-CoV-2 infected airway epithelium. A pseudostratified airway epithelium obtained by four-week differentiating normal human bronchial epithelial (NHBE) cells of healthy adult (deidentified) was infected with SARS-CoV-2 (USA/WA-CDC- At four days post-infection, the airway epithelium was fixed with paraformaldehyde (PFA), paraffin-embedded, sectioned, stained, and imaged under a Leica DMi8 epifluorescence microscope using 63x oil-objective. S mRNA was detected by RNAscope using SARS-CoV-2 S mRNA specific probe and Opal 570 dye (shown in red) and S protein was detected by immunofluorescence using SARS-CoV-2 S protein specific rabbit polyclonal primary antibody and corresponding goat anti-rabbit AF647 secondary antibody (shown in cyan). A. Both S mRNA and S protein of SARS-CoV-2 can be detected in the infected airway epithelium (top image). A section of the top image magnified to show the simultaneous detection of S mRNA and S protein (bottom images). Orange and yellow arrows indicate S mRNA and S protein, respectively in the same cell. B. The number of S mRNA and S protein per cell plotted. Both S mRNA and S protein were quantified not only at a single molecule but also at a single cell level. The data represent two independent SARS-CoV-2 infected airway epithelium experiments, each used independent healthy adult donor (deidentified).

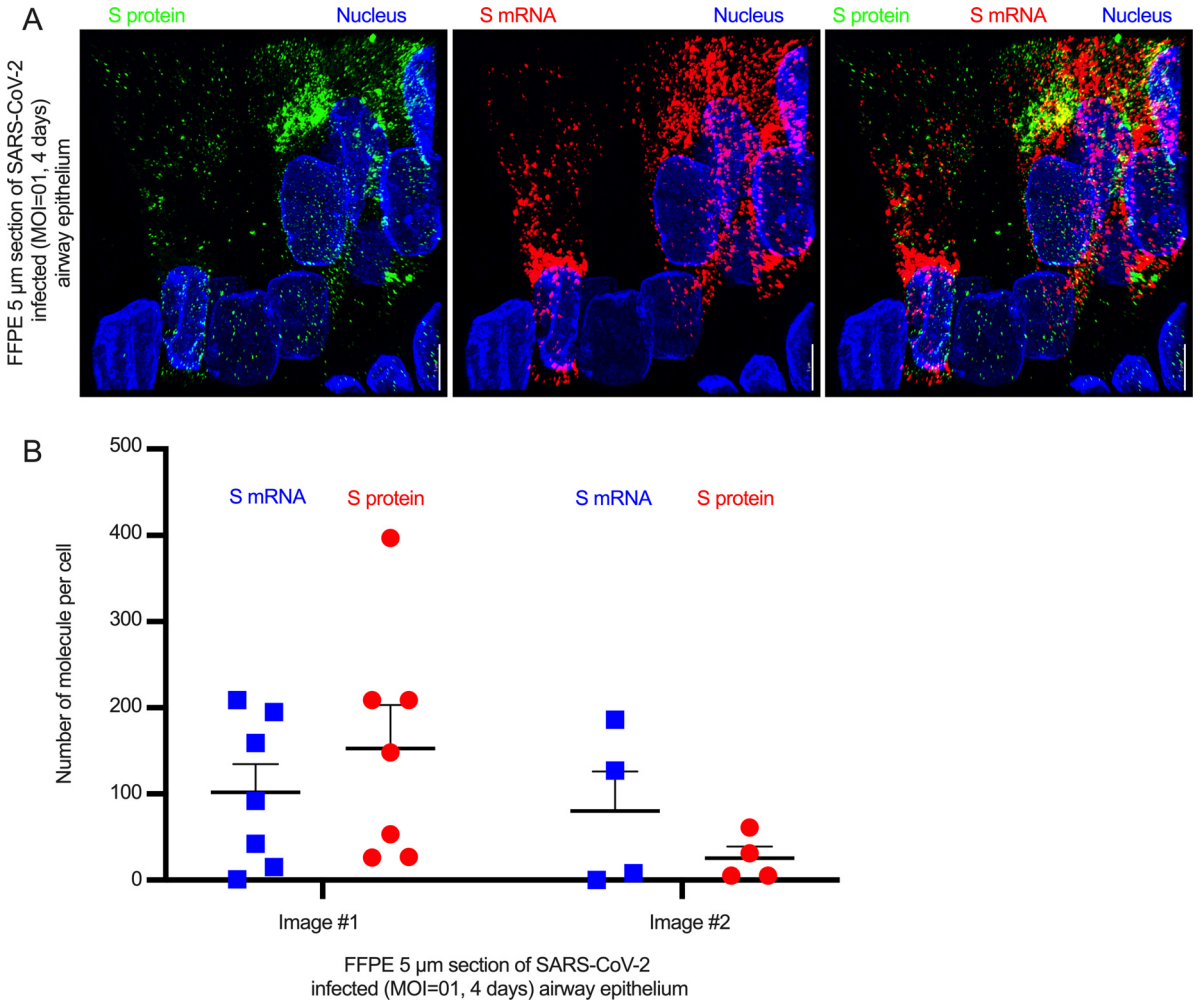

Fig. 3. Convenient quantification of S mRNA and S protein from confocal microscopy images obtained by imaging an FFPE 5 µm thick section of SARS-CoV-2 infected airway epithelium. A. The FFPE section was imaged under a Leica Stellaris confocal microscope (Leica Microsystem) using a 63x oil objective. The images were then deconvolved using Huygen Essential deconvolution software (Scientific Volume Imaging). The surface rendering function of Imaris image processing software (Oxford Instruments). Scale bar 5 µm. B. The number of S mRNA and S protein per cell quantified by Qu-path and plotted from two independent images (#1 and #2) from one experiment (described in Fig 2). The error bars represent SEM.

infected cells, including in the shedded-infected cells (Fig. 2). A high-resolution imaging by a confocal microscope followed by an image deconvolution step provides a convenient detection & quantification; however, the signal saturation was one of the limitations for an accurate detection at the single molecule level (Figs. 2 and 3). While stimulated emission depletion (STED) microscopy can provide higher separation between fluorescence signals [3], it may not achieve the required less than 10 nm resolution to separate individual dots. Thus, a series of dilutions for each fluorescent signal can be utilized to detect and separate accurate signals. However, further optimization is necessary for detecting at the single molecular level. This protocol can be used on the older FFPE archived samples, as FFPE block retains macromolecules at native state at least 11 years when tested [2]. Importantly, this protocol has several advantages over generic PCR-based detection of SARS-CoV-2 in the FFPE tissue section. First, this protocol gives simultaneous spatial detection of mRNA and corresponding protein and quantifies to provide possible correlation between them. Second, the probe for a viral mRNA (e.g., S mRNA) is specific. Thus strain-specific SARS-CoV-2 detection is possible. It also reduces the non-specificity inherently associated with PCR-based detection. Third, we can adopt this protocol for the simultaneous detection of multi-protein & multi-mRNA.

Ethics statements

Not applicable.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Kailey Jerome: Methodology, Writing – original draft. **Sarah Sattar:** Methodology, Visualization, Writing – original draft. **Masfique Mehedi:** Project administration, Visualization, Methodology, Conceptualization, Supervision, Writing – original draft, Writing – review & editing.

Data availability

Data will be made available on request.

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