JAGIELLONIAN UNIVERSITY
IN KRAKOW

Krakow, 9th of November 2020

Malopolska Centre of Biotechnology

Dear Editor,

We warmly thank you for the reviews of our manuscript entitled: "Visualization of the SARS-CoV-2 by immuno RNA-FISH.", which were very helpful. You will find enclosed a rebuttal letter that addresses each of the editorial and peer review comments individually. We have incorporated all recommended changes and feel that the manuscript is improved as a result.

We hope that you will find our manuscript suitable for publication in Journal of Visualized Experiments and look forward to hearing from you.

Yours Sincerely,

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ANSWERS TO THE EDITORIAL COMMENTS

Comment 1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Our manuscript has been proofread by the Bioedit editing service (www.bioedit.com).

Comment 2. Please revise the following lines to avoid previously published work: 104-109,

An Immuno-RNA FISH technique presented in this manuscript was used in the study described in the Journal of Virology (Milewska A, Kula-Pacurar A et. al., 2020, DOI: 10.1128/JVI.00957-20), and that is why we included this our citation in the present manuscript.

Comment 3. For in-text formatting, corresponding reference numbers should appear as numbered superscripts after the appropriate statement(s).

We changed the reference numbering, as suggested by the Editor.

ANSWERS TO THE REVIEWERS' COMMENTS

Reviewer #1:

Manuscript Summary:
In this paper Kula-Pacurar and collaborators describe a method to simultaneously visualize SARS-CoV-2 RNA, by fluorescence in situ hybridization (RNA FISH), and a protein of interest, by immunofluorescence. They propose two variants of the method, one for lab cells on coverslips and one for human airway epithelium (HAE) cultures.
I have no major concerns. The protocol is clear and very well detailed, with useful caution alerts and note.

We thank the reviewer for these nice comments.

Minor Concerns:
The authors could add an additional paragraph just before the protocol with a list of all lab equipment needed as well as basic reagents.

As suggested by the reviewer, we included list of equipment to the already existing file containing list of reagents.

Reviewer #2:
Manuscript Summary:
Although the protocol highlights a technique that can be of value to study host-viral interaction, there

are some major steps that needs further explanation for readers to be able to understand and replicate the protocol.

Major Concerns:
Comment 1. Why is probe not be added to the well of 12 well plate like other steps? Taking coverslip out is very difficult and can even lead to breaking of coverslip. Furthermore, drying is a possibility in overnight incubation with 30- 50ul probe on coverslip with parafilm.

We thank the reviewer for this important comment. This is our standard procedure that allows us to decrease the amount of expensive reagents such as probes, amplifiers, and antibodies for IF. Importantly, the incubation in humidified chamber prevents the cells from drying out. We included a note information in the new version of our manuscript:

« NOTE: As an alternative to placing 30–50 µl portions onto parafilm and placing under coverslips for incubation, 300 µl of probe solution can be added directly to coverslips in a 12-well plate. The latter procedure is simpler but required substantial amounts of reagents. «

Comment 2. Day2 amplification step has sudden mention of hairpin. Assuming the author mean "HCR amplifier B1" by hairpin, it needs to be mentioned and clarified in section 2.3.1, line 166.

We agree with the reviewer that the text was unclear. We clarified this point in the section 2.3.1 by adding the following sentence: “An HCR amplifier comprises metastable HCR hairpins h1 and h2.”

Minor Concerns:
Comment 1. Is there a specific reason why authors do not grow mammalian cells the same way as HAE cells? if so, authors need to explain as having both cultures grown in similar conditions/surfaces increases reliability and reproducibility of the protocol.

Preparation of HAE 3D cultures requires a different and laborious protocol than simply culturing mammalian cells on a coverslip. The protocol starts with the isolation of primary human epithelial cells from conductive airways resected from transplant patients. Cells are mechanically detached from the tissue after protease treatment and cultured on plastic in bronchial epithelial growth medium (BEGM). Subsequently, cells are transferred onto permeable Transwell insert supports and cultured in BEGM. After the cells reach full confluence, the apical medium is removed, and the basolateral medium is changed to an air-liquid interface (ALI). Cells are cultured on the interphase of air and media for 4 to 6 weeks, when they differentiate to form pseudostratified mucociliary epithelia.

In our manuscripts, we cited a previously published protocol that describes how to prepare HAE cultures.

“Fully differentiated HAE cultures were prepared as described [18] on permeable Transwell insert supports with a membrane (diameter = 6.5 mm) and cultured in bronchial epithelial growth medium until reaching full confluence.”

[18] Fulcher, M. L., Gabriel, S., Burns, K. A., Yankaskas, J. R. & Randell, S. H. Well-differentiated human airway epithelial cell cultures. *Methods Mol Med* **107**, 183-206, doi:10.1385/1-59259-861-7:183 (2005).

Comment 2. 1hr vs overnight fixation in PFA can make a difference in results as over-fixation can cause background and nonspecific binding.

We agree and apologize for this confusion. We removed “the overnight incubation” as it was simply our mistake.