 doi:10.20944/preprints202201.0087.v1

Communication

Exploring Mitochondrial Localization of SARS-CoV-2 RNA by Padlock Assay. A Pilot Study in Human Placenta.

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Abstract: The ongoing COVID-19 pandemic dictated new priorities in biomedicine research. Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the causative agent of COVID-19, is a single-stranded positive-sense RNA virus. In this pilot study, we optimized our padlock assay to visualize genomic/subgenomic regions using formalin-fixed paraffin-embedded placental samples obtained from a confirmed case of COVID-19. SARS-CoV-2 RNA was localized in trophoblastic cells. We also checked the presence of the virion by immunolocalization of its glycoprotein spike. In addition, we imaged mitochondria of placental villi keeping in mind that the mitochondrion has been suggested as a potential residence of the SARS-CoV-2 genome. Indeed, we observed a substantial overlapping of SARS-CoV-2 RNA and mitochondria in trophoblastic cells. This intriguing linkage correlated with an aberrant mitochondrial network. Overall, to our knowledge, this is the first study that provides the evidence of a co-localization of the SARS-CoV-2 genome and mitochondria in SARS-CoV-2 infected tissue. These findings also support the notion that SARS-CoV-2 infection could reprogram mitochondrial activity in highly specialized maternal/fetal interface.

Keywords: COVID-19; SARS-CoV-2 RNA; mitochondria; placenta; padlock

1. Introduction

Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), the infectious agent that causes COVID-19 disease, is a type of single-stranded RNA virus that belongs to the beta coronavirus family [1, 2]. Two-thirds of the viral genome include genes encoding for large polyproteins, which are then processed into 16 non-structural proteins (NSPs) by proteolytic cleavages. The remaining one-third of the genome consists of Open Reading Frames (ORFs) for structural proteins like spike, envelope, membrane, and nucleocapsid proteins [2].

We aimed to evaluate the efficiency of our published padlock assay in visualizing SARS-CoV-2 RNA in formalin-fixed paraffin-embedded (FFPE) tissues. In fixed cells, padlock probes can target a selected RNA with a near-single-molecule resolution [3-5]. This method provides intracellular localization maps of transcripts with high spatial

 doi:10.20944/preprints202201.0087.v1

resolution and sequence specificity. For our goal, we took advantage of an archival FFPE tissue of the placenta from a pregnant woman with Covid-19. Contextually, we focused on placental mitochondria keeping in mind that increasing evidence points to the mitochondrion as an attractive target for both maintaining pregnancy and SARS-CoV-2 life cycle [6-8].

Recently, an innovative biochemical approach showed a physical and functional interaction between SARS-CoV-2 and host mitochondria [9]. However, the absence of imaging approaches, supporting this notion, remains a critical gap.

Here, we showed that padlock assay is a robust method to map the distribution of the SARS-CoV-2 genome in infected tissues, and, most importantly, for the first time we provided images visualizing the *in situ* co-localization of SARS-CoV-2 RNA and mitochondria in host cells. Furthermore, in the context of pregnancy complications, mitochondrial dysfunction appears a critical feature in the pathogenesis of preeclampsia [10]. To note, pregnant women with COVID-19 are more likely to develop preeclampsia [11]. Collectively, this study opens a new scenario for SARS-CoV-2 investigations.

2. Materials and Methods

2.1. Placental tissue samples

Archival FFPE tissue samples from two placentas at term were included in this pilot study. One of the placentas was from a 30-year-old woman who gave birth in 2021 and in which SARS-CoV-2 infection was diagnosed two months before labour. The other placenta was from a 36-year-old woman who gave birth in 2018, i.e., before the COVID-19 pandemic (https://www.who.int/emergencies/disease-outbreak-news/item/2020-DON229), and so not tested for SARS-CoV-2 infection. No other clinical data were available. Four-◎m thick sections obtained from the paraffin blocks loaded onto positively charged glass slides were used for the immunofluorescence studies. The study protocol conformed to the Declaration of Helsinki and its later amendments and was approved by the internal Institutional Review Board (Ethical Committee of Sapienza University of Rome, Policlinico Umberto I, approval number: 6536).

2.2. Antibodies and reagents

Anti-HSP60 mouse monoclonal antibody (Santa Cruz Biotechnology; work dilution for immunofluorescence, 1:100); anti-COX IV rabbit polyclonal antibody (Millipore; 1:200); anti-spike mouse monoclonal antibody (Santa Cruz Biotechnology; 1:100); anti-Vimentin rabbit monoclonal antibody (Abcam; 1:150). Alexa-Fluor-488 or Alexa-Fluor-594-conjugated secondary antibodies (Life Technologies; 1:200).

2.3. Immunofluorescence Analysis

Paraffin-embedded sections were dewaxed by 2 changes of xylene, 30 minutes each. After hydratation in graded ethanol solutions (100%, 95%, 90%, 80%, 70%, 50% and 30% ethanol, for 2 minutes each), sections were incubated for 30 minutes at 95°C with antigen retrieval solution (10mM Sodium Citrate, 0.05% Tween 20, pH 6.0). Slides were rinsed in PBS/0.1% Tween-20, blocked with 1% BSA in PBS for 1 hour at room temperature and then incubated at 4 °C overnight using the appropriate primary antibodies, washed three times in PBS/0.1% Tween-20 and then incubated with the appropriate secondary antibodies. Slides were mounted with ProLong with Dapi (Thermo Fisher Scientific) and examined by an epifluorescence microscope (Olympus BX53; Milano, Italy). Images were captured by a SPOT RT3 camera and elaborated by IAS software.

2.4. Padlock assay

Paraffin-embedded sections were dewaxed by treatment with xylene overnight. Sections were then hydrated in 100%, 95%, 90%, 80%, 70%, 50% and 30% ethanol for 2 minutes each. Sections were fixed in 4% formaldehyde for 20 minutes and then incubated

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for 30 minutes at 80 °C in the unmasking solution (10 mM Sodium Citrate, 0.05% Tween 20, pH 6.0). After a rinse in PBS, the slides were processed for the padlock assay as described [3]. For details see Supplementary material online. When padlock assay was combined with protein immunostaining, slides were further subjected to immunofluorescence analysis as described above. Slides were mounted and imaged by fluorescence microscope, as reported above.

Oligos used in this study are indicated in *Supplementary material.*

3. Results and Discussion

Exploring SARS-CoV-2 RNA in host cells assumes not only a diagnostic significance but also carries out the opportunity to critically contribute to the advance in the knowledge of the interaction of SARS-CoV-2 and human cells and, more in general, on COVID-19. Viral RNA can be visualized on FFPE tissues using *in situ* hybridization (ISH) methods [12,13]. We recently used padlock probes, combined with rolling circle amplification (RCA) strategy, to visualize subcellular locations of selected transcripts [3-5]. In our experience, padlock assay is more performant in identifying specific transcripts in cellular compartments, compared to a canonical fluorescence *in situ* hybridization (FISH). Here, we aimed to test and propose our padlock assay as an alternative approach to map SARS-CoV-2 localizations in infected tissues. SARS-CoV-2 genome consists of a positive-sense RNA with a size of about 30 kb [2]. We choose to target the viral RNA sequence encoding NSP7 viral protein that has been successfully tested in our experimental systems (*data not shown*). Next, we took advantage of an archival FFPE sample of a placenta at term from a woman in which SARS-CoV-2 infection was diagnosed two months before labour. For comparison we also evaluated additional FFPE samples of a placenta at term from a woman who gave birth before the COVID-19 pandemic. Although transient, the placenta is a fascinating organ that allows mother-fetus communication during pregnancy [14]. Studies focusing on COVID-19 in pregnancy have shown the presence of SARS-CoV-2 genome/proteins within the placental compartment [15,16]. A placental tropism seems justified by the expression of SARS-CoV-2 entry factors [17]. As schematically illustrated in figure 1A, deparaffinized sections of both placentas were subjected to padlock assay and then imaged by a high-resolution fluorescence microscope. Figure 1B shows a simplified view of placental villi and their by-layered epithelium consisting of cytotrophoblast and syncytiotrophoblast cells that line a connective tissue core in which foetal blood vessels are placed [14]. By fluorescence microscope, placental villi were easily identified by fluorescent background signal originating from tissue as well as by nuclei staining. Blood erythrocytes were detectable thanks to their peculiar strong autofluorescence. The presence of SARS-CoV-2 RNA was revealed by RCA fluorescent dots (figure 2A, *white* and *red* in black & white and coloured images, respectively; figure S1). Several dots were detectable in trophoblastic cells. This observation was in line with previous reports [15]. To note, padlock probe specificity was verified by monitoring a SARS-CoV-2-negative placenta (figure 2B; figure S1). In parallel, we also checked the distribution of the spike protein, which is required for the binding of coronavirus to target cells (figure S2). These findings not only validate the feasibility/efficiency of padlock assay in detecting RNA of interest in FFPE tissues, but also provide an alternative and robust method to map SARS-CoV-2 RNA in infected tissues.

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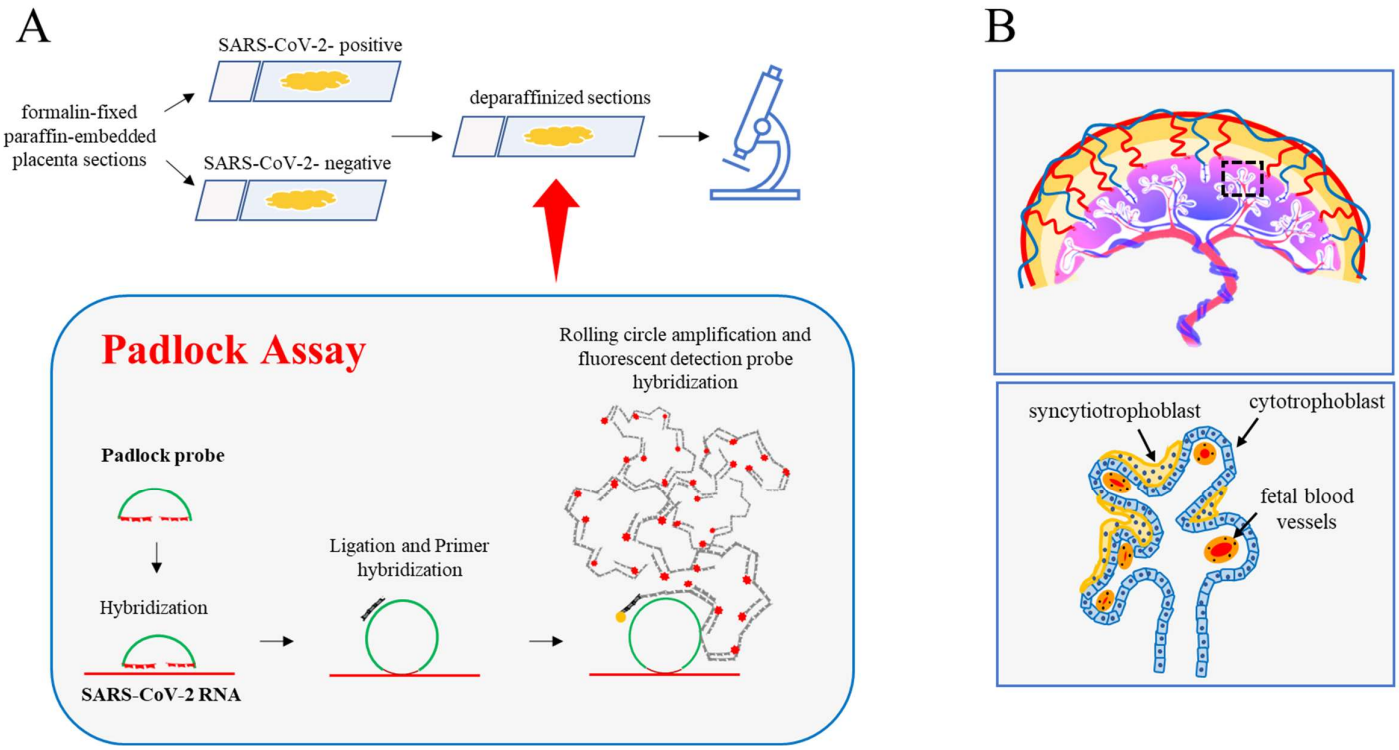

Figure 1. Padlock Assay. (**A**) Diagram illustrating main experimental steps of the padlock assay carried out in deparaffinized sections from the human placenta. Formalin-fixed paraffin-embedded sections (4 μm thickness) from the SARS-CoV-2-positive and SARS-CoV-2-negative placentas are first deparaffinized and then processed for padlock assay. A padlock probe is designed with the 5′- and 3′-terminal bases complementary to a selected sequence of SARS-CoV-2 RNA. The padlock probe is ligated and circularized with its complementary RNA template. A short DNA primer is used for initiating rolling circle amplification (RCA). In this way, the target sequence is converted in a long DNA amplicon with several copies of the padlock probe. Finally, amplicons become detectable by hybridization with the fluorophore-labelled probe. Sections are then analysed by a fluorescence microscope. (**B**) (**top**) A schematic model of the anatomy of human placental villi. (**bottom**) Enlarged detail illustrating the major cellular components of the villus.

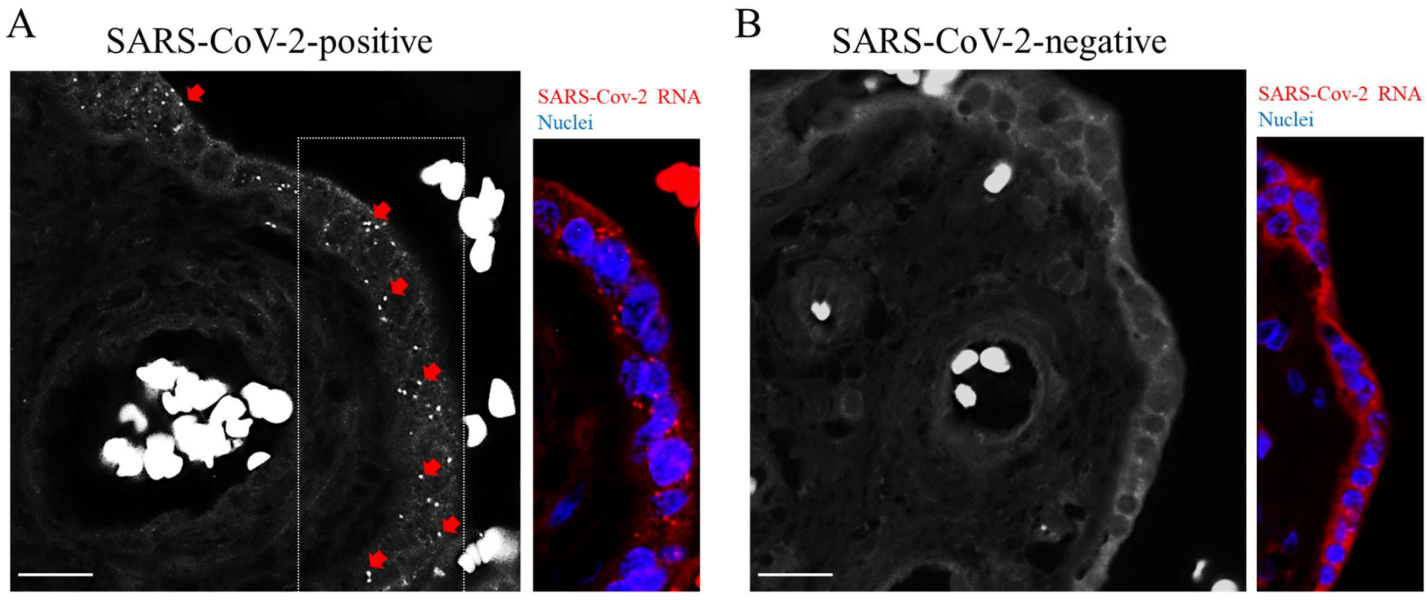

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Figure 2. Detection of SARS-CoV-2 RNA. (**A,B**) Representative images of padlock assay targeting the SARS-CoV-2 RNA in sections from the placenta. (**A**) Genomic/subgenomic regions of SARS-CoV-2 are detectable following fluorescence microscopy in the SARS-CoV-2-positive placenta (red arrows, and red dots in a selected area of the corresponding color panel). Nuclei were labelled with DAPI (*blue*). (**B**) No specific signals are detectable in sections from the SARS-CoV-2-negative placenta. Scale bar, 20 μm.

In pregnant women, a specific clinical manifestation due to SARS-CoV-2 infection remains unclear [16]. Only sporadic cases of vertical transmission have been described [17]. However, identifying molecular changes occurring in maternal/fetal interface in pregnant women with COVID-19 represents a major challenge. It is known that host cell mitochondria play a critical role in the SARS-CoV-2 life cycle. It has been suggested that mitochondrial "hijacking" by SARS-CoV-2 might be a key component in the pathogenesis of COVID-19 [18]. On the other hand, placental mitochondria play a crucial role in maintaining pregnancy. Proper dynamics regulating mitochondrial fission/fusion cycle prevent cell injury. Interestingly, cytotrophoblast and syncytiotrophoblast cells exhibit distinct mitochondrial populations with different susceptibility to damage [19]. Cytotrophoblast mitochondria are relatively larger with lamellar cristae, whereas syncytiotrophoblast mitochondria are small, spherical, with tubular cristae [20]. This may imply a different mitochondria susceptibility to damage between the different villous trophoblastic cell populations. Given the critical role of placental mitochondria, we were interested in imaging mitochondria reticulum in a placenta with COVID-19. Mitochondria were labelled by using two specific markers: an anti-HSP60 and an anti-COX IV, monoclonal and polyclonal antibodies, respectively. In combination with HSP60 antibody, we used an anti-vimentin polyclonal antibody that marks stromal and endothelial cells. Interestingly, we found an aberrant mitochondrial reticulum in the SARS-CoV-2-positive placenta. As shown in figure 3 (*SARS-CoV-2-positive*), mitochondria exhibited a polarized distribution and appeared strongly fused. Moreover, several fragmented mitochondria were also present. Conversely, SARS-CoV-2-negative placental villi exhibited a more regular mitochondrial reticulum. In this case, mitochondrial dynamic switched to a fission rather than a fusion event, coherently with the morphological changes occurring in the aging placenta [21]. We obtained similar results with both the mitochondrial markers (HSP60 and COX IV). In this context, it is important to mention that SARS-CoV-1 was found to induce mitochondrial fusion, and this event was linked to the ability of the CoV to prevent apoptosis [8,22].

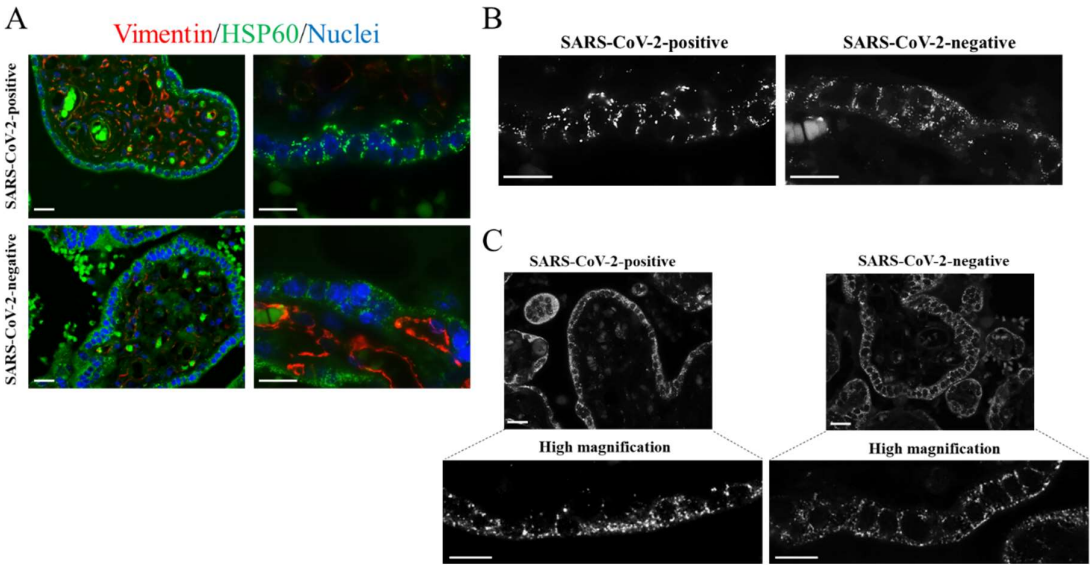

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Figure 3. Immunostaining of placental mitochondria. (**A,B,C**) Representative images of the immunofluorescence analysis showing mitochondria in placental villi of the SARS-CoV-2-positive and SARS-CoV-2-negative placentas. The images were acquired with 40x or 100x oil objectives as indicated. (**A**) Placental sections were immunolabelled with the mitochondrial marker, HSP60 (*green*). Vimentin immunostaining identifies endothelial cells of blood vessels (*red*). Nuclei were labelled with DAPI (*blue*). (**B**) Representative images, acquired with 100x oil objective, visualizing the mitochondrial marker, HSP60, in villous trophoblastic cells. (**C**) Representative images, acquired with 100x oil objective, showing placental mitochondria immunolabelled with anti-COX IV. Scale bar, 20 μm.

Genomic/subgenomic regions of SARS-CoV-2 were predicted to localize in mitochondria of the host cell [19,23]. Even if crucial, this notion remains a prediction since experimental images of this event are so far not provided. The availability of a placenta from a woman with COVID-19, prompted us to explore this intriguing aspect. We carried out an immunofluorescence combined with the padlock assay. In our experience, this combined approach provides high-quality co-localization images even though reduces the efficiency of both the immunofluorescence and padlock assay. As shown in figure 4, several dots corresponding to SARS-CoV-2 RNA clearly overlapped with placental mitochondria. Remarkable, we provide for the first time, images highlighting a physical contact between the SARS-CoV-2 RNA and host cell mitochondria. It is fascinating that this first evidence has been produced in a unique organ, such as the placenta. In this regard, images on the mitochondrial engagement by SARS-CoV-2 RNA, could provide a mechanistic/clinical interpretation of preeclampsia in women with COVID-19. This pilot study prompts us to further explore the relationship between the SARS-CoV-2 RNA and mitochondria as well as to further upgrade our imaging approach, based on padlock probes, to contribute to the advance in the knowledge of the pathogenesis of COVID-19.

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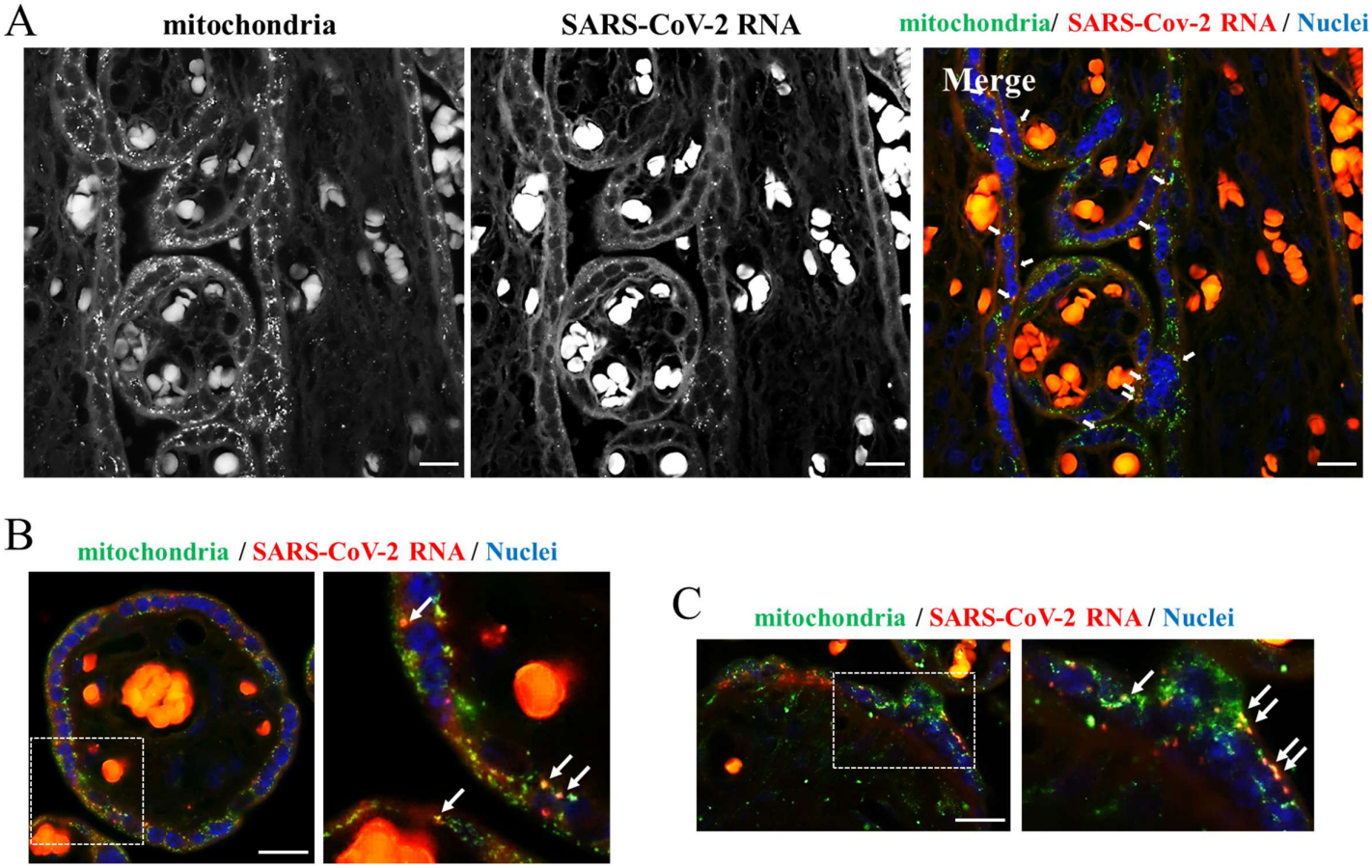

Figure 4. Dual staining of placenta sections. (**A**,**B**,**C**) Sections from the SARS-CoV-2-positive placenta were subjected to a combination of padlock and immunofluorescence assay to visualize the SARS-CoV-2 RNA (*red* in the colour images) and mitochondria (*green* in the colour images). In panel **A** and **B**, HSP60 was used as mitochondrial marker. In panel **C**, COX IV was used as mitochondrial marker (*green* in the colour panels). SARS-CoV-2 RNA (*red* in the colour panels) results partially overlapped with both mitochondrial markers in the *Merge* panel (*yellow* dots). In panel B and C, a higher magnification of a selected region is shown. Scale bar, 20 μm.

Supplementary Materials: The following are available online: Supplementary Materials and Methods: Padlock Assay; Table S1: Oligos used in this study; Figure S1: SARS-CoV-2 RNA detection by Padlock Assay; Figure S2: Immunostaining of spike protein.

Author Contributions: FG: performing experiments, writing—review and editing; CB, NC: writing—review and editing; MF, CP (Carla Petrella): data analysis, editing; MdV, AG, GF, MR: resources; IP, CDG, FP: review and editing; AC, RB: critical reading, review and editing; CP (Claudio Passananti): supervision, methodology, funding acquisition; AM: supervision, funding acquisition; MGDC: supervision, conceptualization, writing—original draft preparation. All authors were involved in writing the paper and had final approval of the submitted version.

Funding: This research received no external funding. Fellowship to F.G. is supported by Lazio Innova Project # 15286 to C.P. (Claudio Passananti).

Institutional Review Board Statement: The study protocol conformed to the Declaration of Helsinki and its later amendments and was approved by the internal Institutional Review Board (Ethical Committee of Sapienza University of Rome, Policlinico Umberto I, approval number: 6536, date: 20/10/2021).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The authors confirm that the data supporting the findings of this study are available within the article.

Acknowledgments: We thank Dr Annalisa Onori for the precious technical support.

Conflicts of Interest: The authors declare no conflict of interest.

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