Dual spatially resolved transcriptomics for SARS-CoV-2 host-pathogen colocalization studies in humans

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Abstract

To advance our understanding of cellular host-pathogen interactions, technologies that facilitate the co-capture of both host and pathogen spatial transcriptome information are needed. Here, we present an approach to simultaneously capture host and pathogen spatial gene expression information from the same formalin-fixed paraffin embedded (FFPE) tissue section using the spatial transcriptomics technology. We applied the method to COVID-19 patient lung samples and enabled the dual detection of human and SARS-CoV-2 transcriptomes at 55 µm resolution. We validated our spatial detection of SARS-CoV-2 and identified an average specificity of 94.92% in comparison to RNAScope and 82.20% in comparison to *in situ* sequencing (ISS). COVID-19 tissues showed an upregulation of host immune response, such as increased expression of inflammatory cytokines, lymphocyte and fibroblast markers. Our colocalization analysis revealed that SARS-CoV-2^{+} spots presented shifts in host RNA metabolism, autophagy, NFκB, and interferon response pathways. Future applications of our approach will enable new insights into host response to pathogen infection through the simultaneous, unbiased detection of two transcriptomes.

Introduction

Much is still unknown about how hosts react to pathogens and how pathogen infection underlies various biological processes and disease states. Although single-cell transcriptomics methods have improved the elucidation of cell-type specific effects caused by pathogens and how these relate to disease outcomes[1,2], such approaches remove pathogens and host cells from their natural environment, limiting the study of complex dynamics of localized infections. To gain insights on the localized host response to pathogen infection, technologies that allow the co-capture of both host and pathogen spatial transcriptome information are needed. Moreover, there is often the need to work with formalin-fixed paraffin embedded (FFPE) tissue blocks to neutralize the pathogen and, when studying human infectious diseases, to access biobanks where samples are deposited. For instance, in light of the COVID-19 pandemic, human and SARS-CoV-2 transcriptome information was spatially captured from FFPE lung tissues to study human host response to SARS-CoV-2 viral infection in the lung[3–9], providing new insights into the heterogeneous viral distribution and host response to infection. However, although there are currently some FFPE compatible spatially-resolved transcriptomics methods available[10–18], such methods are limited by either providing only a partial view of the full transcriptome[11–14] or having low tissue area throughput, due to either long experimental times[10,15,16] or having to rely on the selection of predefined tissue regions of interest[19].

Here, we present a spatially-resolved transcriptomics strategy to unbiasedly explore the transcriptome-wide landscape of two transcriptomes using FFPE tissues. We leveraged the high-throughput, sequencing-based commercially available spatial transcriptomic (ST) platform[20] and introduced the co-detection of a second transcriptome, that of the SARS-CoV-2 virus, to the human one. We tested the potential of such an approach through the dual capture of human and SARS-CoV-2 viral transcriptomes at 55mµm (~1-10 cells) spatial resolution in COVID-19 patient lung FFPE tissues. We validated our spatial detection of SARS-CoV-2 with targeted transcriptome technologies RNAScope[11] and *in situ* sequencing (ISS)[14,21,22]. With our approach, we identified both general immune response signatures and tissue-specific processes evoked by the virus, such as the domination of plasma cells, activated fibroblasts, and

inflammatory cytokines in COVID-19 lung tissues due to prolonged SARS-CoV-2 infection. A prominent feature of our method is the colocalization of human and viral gene expression information that allows an understanding of human tissue response to viral infection by comparing areas with and without the presence of viral RNA in the same tissue section. Such an approach uncovered several genes involved in RNA metabolism, autophagy, NFκB, and interferon-response pathways to be differentially expressed in viral active areas, potentially shedding new light on COVID-19 pathogenesis. Our strategy opens up the possibility of spatially studying host response to pathogen infections through the simultaneous, unbiased detection of two transcriptomes.

Results

Spatial Transcriptomics enables the simultaneous capture of human and SARS-CoV-2 spatial transcriptomes

To study the localized infection effects caused by SARS-CoV-2 in human lungs, we advanced the Visium Spatial Gene Expression assay for FFPE tissues[20] to simultaneously capture human and SARS-CoV-2 whole transcriptome (WT) information at a 55 μm resolution. Specifically, we analyzed 16,688 human genes and 10 SARS-CoV-2 gene transcripts in total across 13 tissue sections from 5 patient lung tissue samples, 3 from COVID-19 patients (i.e., 1C, 2C, 3C) and 2 from control patients (i.e., 4nC, 5nC) (Figure 1a, Supplementary Figure 1, Supplementary Table 1, Supplementary Table 2). First, we verified the specificity of the SARS-CoV-2 probes (S) for capturing SARS-CoV-2 transcripts only. To do so, we applied both human WT probes (H) and spike-in SARS-CoV-2 probes to control tissue sections. We did not identify any SARS-CoV-2 transcripts above background levels (see Methods), demonstrating the SARS-CoV-2 probes were specific to capture SARS-CoV-2 information (Figure 1b, Supplementary Figure 2).

To independently validate the viral detection by our set of SARS-CoV-2 probes, we compared the ST viral detection to the viral detection offered by the orthogonal imaging-based RNAScope technology[11] using consecutive sections. Specifically, we compared the S gene signal detected by ST and RNAScope across all COVID-19 and control samples. To systemically and unbiasedly analyze all our samples, we developed an automated signal detection computational pipeline across all platforms (see Methods, Supplementary Figures 3-5). Using our computational approach, we found an average specificity of the ST method of 94.92% (1C: 86.86%, 2C: 99.37%, 3C: 98.53%) (Figure 1c). Furthermore, we performed a second validation of the ST S gene detection, and a validation of the ST E gene, by using *in situ* sequencing (ISS)[14,21,22] in the sample with the highest viral load, 1C, using the same automated computational pipeline (see Methods). Despite several sections in between (~300 μm) the ST and ISS sections, we observed an overall similar viral distribution trend between ST and ISS sections and an average specificity of 82.20% (83.65% for the E gene and 80.74% for the S gene) (Figure 1c, Supplementary Figure 6), confirming that the ST method can capture SARS-CoV-2 information accurately.

Subsequently, we wanted to understand if the addition of the SARS-CoV-2 probes impacted the quality of the human gene expression information captured. To this end, we analyzed consecutive COVID-19 sections with human WT probes and spike-in SARS-CoV-2 probes versus only human WT probes. Across COVID-19 and control tissue sections, we generated a dataset consisting of a total of 37,754 spots, with an average of ~2,013 unique human genes

110 and ~3,809 unique human molecules per spot, respectively (Figure 1d). We captured very similar human gene
111 expression profiles between sample replicate sections and across most samples, both with and without SARS-CoV-2
112 probes added (r= 0.98-1, p-value < 2.2e-16) (Figure 1d-e, Supplementary Figure 7). Overall, these results demonstrate
113 highly reproducible capture of human transcriptomic information and the specificity of the SARS-CoV-2 probes in
114 detecting the SARS-CoV-2 transcriptome without interfering with the capture efficiency of the human transcripts.

115 **SARS-CoV-2 genes have different expression levels but follow a similar spatial distribution**

116 In COVID-19 sections, 9.5% of spots (i.e., 1,132 spots in total) presented SARS-CoV-2 transcriptional signal with
117 highly reproducible capture of SARS-CoV-2 gene expression between consecutive sections (r=0.98, p-value < 1.5e-
118 6, Figure 1f). Overall, we captured up to 9 different SARS-CoV-2 genes (Supplementary Table 3) with an average per
119 spot of ~1.7 unique molecules and ~1.5 unique genes, respectively. These relative low levels of viral load are likely
120 associated with the longer disease duration (13-17 days) of these patients (Supplementary Table 2) in agreement with
121 several studies that observed lower, or even undetectable, viral load in COVID-19 patients with longer survival times[3–
122 5,7,23]. By looking at the overall distribution of SARS-CoV-2$^+$ spots in COVID-19 samples, we observed a wide range
123 of percent SARS-CoV-2$^+$ spots in a COVID-19 sample section: 33.6% for 1C, 1.1% for 2C, and 1.0-1.6% for 3C
124 (Figure 1g, Supplementary Table 3). Others have observed such inter-sample viral load heterogeneity[3,4,7,9] and even
125 heterogeneous distribution within the same tissue sample[7]. In this regard, we also observed varied abundances of the
126 different SARS-CoV-2 gene transcripts across all three COVID-19 samples (Figure 1h, Supplementary Figure 8). For
127 example, across our COVID-19 sample sections, N was the highest expressed SARS-CoV-2 gene while ORF10 was
128 not detected at all, in line with previous reports of N as the most abundant subgenomic RNA (sgRNA)[24,25] and ORF10
129 as consistently either absent or the lowest sgRNA detected[24,25]. The abundance trend of the remaining SARS-CoV-2
130 genes (M, E, S, ORF1ab, ORF3a, ORF7a, ORF7b, ORF8) varied across the four COVID-19 sample sections
131 (Supplementary Figure 8) and factors driving these differences remain to be further investigated in the literature.
132 Although the SARS-CoV-2 transcripts differed in their abundances across genes, we observed a fairly even spatial
133 distribution of each gene across samples 1C and 3C, while for 2C the transcripts showed a more localized spatial
134 distribution (Figure 1i, Supplementary Figures 9-10). Variation in the SARS-CoV-2 gene abundances could be
135 influenced by the SARS-CoV-2 probes binding to both viral genomic RNA (gRNA) and subgenomic RNA (sgRNA),
136 as previous studies observed sgRNA abundance variation[24,25] and such differences could be reflected in our SARS-
137 CoV-2 transcriptomic data.

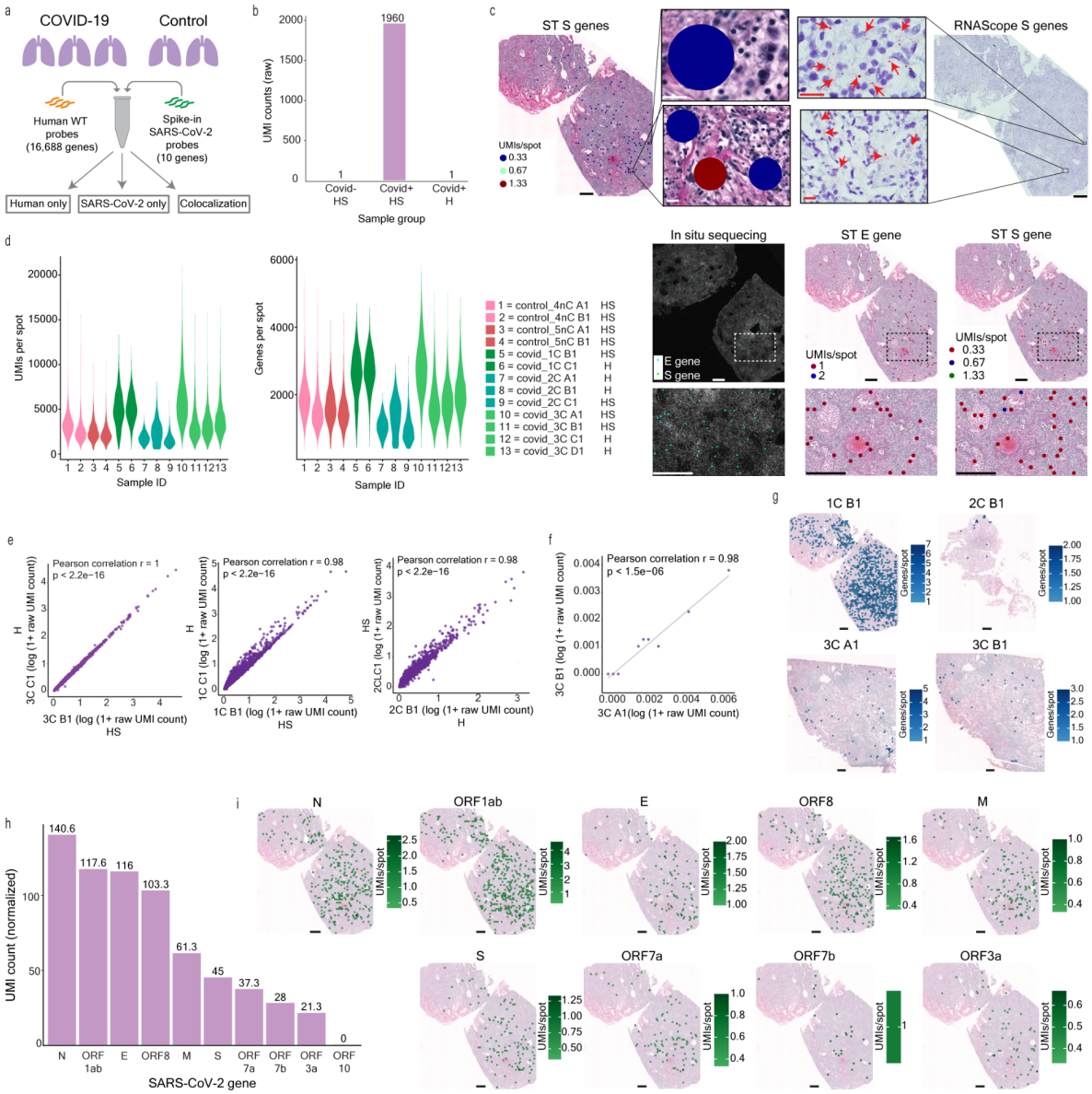

Figure 1. Dual detection of human and SARS-CoV-2 transcriptomes by Spatial Transcriptomics. (a) Overview of the study. (b) SARS-CoV-2 detection in control sections with human and SARS-CoV-2 probes added (HS), in COVID-19 sections with human and SARS-CoV-2 probes added (HS), and in COVID-19 sections with only human probes added (H). (c) Distribution of UMI and gene counts per capture spot across patient sample sections. (d) Pearson correlation of average human gene expression between consecutive sections for each sample, one section with human and SARS-CoV-2 probes added (HS) and the other with only human probes added (H). P-value < 2.2e-16. (e) SARS-CoV-2 S gene detection by ST and RNAScope in a consecutive section. SARS-CoV-2 S & E genes detection by ST and *in situ* sequencing (ISS). ST and RNAScope full tissue section scale bars are 550µm. RNAScope and ST S gene zoomed in panel scale bars are 20µm. All ISS and ST panel scale bars are 550µm. (f) Average SARS-CoV-2 transcriptome capture between consecutive sections. (g) SARS-CoV-2 genes per capture spot across each COVID-19

149 sample. Scale bars are 500µm. (h) Abundance (total normalized UMI counts) of the SARS-CoV-2 genes across all
150 COVID-19 samples. (i) The spatial distribution of UMI counts per capture spot of each SARS-CoV-2 gene for
151 COVID-19 sample 1C. Scale bars are 500µm.

152 **COVID-19 induces lymphocyte infiltration and expressional shifts in lung myeloid, fibroblast, and alveolar**
153 **epithelial cells**

154 To explore the human lung cellular landscape in response to SARS-CoV-2 infection, we performed unsupervised,
155 joint graph-based clustering of spatial transcriptome data collected from both COVID-19 and control sections and
156 found six distinct clusters (Figure 2a). Investigation into the differentially expressed (DE) genes of the six clusters
157 revealed all of them to be a mixture of different cell types, however, we identified clear DE gene signatures for clusters
158 dominated by myeloid cells (cluster 1), endothelial cells (cluster 2), B-cells/plasma cells (cluster 3), epithelial cells
159 (cluster 4), and fibroblasts (cluster 6) (Figure 2b-c, Supplementary Figure 11, Supplementary Table 4). Cluster 5 was
160 characterized by DE genes specific for endothelial, fibroblast and smooth muscle cells, where further sub clustering
161 of this group resulted in three subclusters (subcluster 1: fibroblast-dominated, subcluster 2: smooth muscle cell-
162 dominated, subcluster 3: mixture of endothelial and immune cells), in line with our previous observations
163 (Supplementary Table 5). SARS-CoV-2^{+} spots appeared throughout different morphological areas without obvious
164 enrichment in any of the spatial clusters (Figure 2a), possibly explained by the resolution of spatial transcriptomics
165 not yet reaching the single cell level, and in agreement with the uniform spatial gene expression of the SARS-CoV-2
166 genes across the same tissue section (Figure 1i, Supplementary Figure 10)

167 All three COVID-19 lung samples included in this study represented the late-phase pneumonia stage (between 13-17
168 days post-infection) and showed consistent histopathological features with organizing diffuse alveolar damage,
169 extensive fibrosis, and leukocyte infiltration, accompanied by low viral load[4,7,23] (Supplementary Figure 1). We found
170 that these substantial structural differences between the COVID-19 and control lung sections were also indicated in
171 the transcriptome data. Specifically, DE genes for COVID-19 lung sections were dominated by signatures of plasma
172 cells (IGHG3, IGKC, IGHM, JCHAIN, IGHG2, IGKV4-1, IGLV3-1, IGHA1), activated fibroblasts (COL1A1,
173 COL1A2, COL3A1), inflammatory cytokines (CXCL9, CCL18) and complement factors (C1QB, C1QC), reflecting
174 the overall tissue response to a prolonged SARS-CoV-2 infection (Figure 2d, Supplementary Table 6).

175 We did not find viral entry factors ACE2, TMPRSS2, PCK5, or PCSK7 to be differentially expressed between
176 COVID-19 and control lung sections, in line with a recent study[3]. However, we found three other known SARS-CoV-
177 2 entry factors, CTSL[26,27], CTSB[26], and NRP1[28,29] to be upregulated in the COVID-19 lungs (Supplementary Table
178 6). Previous studies[30,31] observed that CTSL and CTSB have increased expression in COVID-19 lungs, with SARS-
179 CoV-2 infection demonstrated to promote CTSL expression that in turn enhances viral infection[27].

180 Within the myeloid-cell rich cluster 1 of the COVID-19 tissue samples, we observed selective up- (CD163, F13A1,
181 CD14, LYZ, APOE, C1QA, B2M) or downregulation (PPARG, VCAN, FCN1, YAP1, FCGR3A) of certain marker
182 genes, previously used to annotate distinct monocyte-macrophage lineage subsets in single cell transcriptomic datasets

183 of COVID-19 lungs[3], implicating a shift in myeloid cell subtypes during disease progression (Supplementary Table
184 7). Besides many consensus fibrosis markers (COL1A1, COL3A1, COL5A1, SPP1, FN1, POSTN), we found the
185 CTHRC1 gene, recently described as a marker of pathological, pulmonary fibrosis-associated fibroblasts[32], to be
186 highly expressed in the fibroblast-rich cluster 6 of COVID-19 lungs (Supplementary Table 7). At the same time, we
187 identified markers of alveolar fibroblasts, thought to be a cellular source of the CTHRC1^{+} fibroblast subpopulation[32],
188 as either mildly downregulated (TCF21, PDGFRA) or upregulated (NPNT1) in the same cluster (Supplementary Table
189 7).

190 To investigate the biological changes occurring in the epithelial cell compartments, we performed further sub-
191 clustering of cluster 4 (Supplementary Table 8). In the pneumocyte-dominated subcluster 1, COVID-19 lung tissue
192 showed markedly (SFTPC, SLC34A2, MUC1, LYZ) or moderately (LAMP3, PGC, NAPSA, CEBPA, LPCAT1,
193 SDC1, NKX2-1, PIGR, ABCA3, ALPL) elevated expression of type 2 alveolar epithelial (AT2) cell markers, while
194 the type 1 alveolar epithelial (AT1) cell markers appeared to be either slightly down- (KRT7, AGER, FSTL3,
195 SCNN1G) or upregulated (CYP4B1, ICAM1, AQP4, RTKN2, EMP2, GPRC5A, AQP3, CAV1) (Figure 2e,
196 Supplementary Table 9). These changes might point at a hyperplastic expansion of AT2 cells in the diseased tissue,
197 consistent with microscopic observations made in lungs with longer disease duration[33,34] and characterized by low
198 viral load[4].

199 **Colocalization analysis of SARS-CoV-2 and human transcriptomes identifies dysregulation of RNA metabolism**
200 **and NFκB pathway activation**

201 With the possibility of separately capturing the human and SARS-CoV-2 spatial transcriptomes, our approach allowed
202 us to conduct colocalization studies and identify host gene expression changes caused by the active presence of the
203 viral mRNA in lung cells at 55 μm resolution. By comparing the human gene expression patterns in SARS-CoV-2^{+}
204 and SARS-CoV-2^{-} spots only in COVID-19 tissue sections (Supplementary Table 10), we revealed several genes
205 involved in RNA metabolism to be affected by the presence of the viral mRNA (Figure 2f, Supplementary Table 10).
206 We found SRRM2, a component of the spliceosome, to be upregulated in SARS-CoV-2^{+} spots (Figure 2f,
207 Supplementary Table 10). SRRM2 plays a central role in nuclear speckle formation and thus in the replication, splicing
208 and trafficking of Herpes Simplex Virus and Influenza A virus[35], which suggests a potentially similar role in the
209 processing of the SARS-CoV-2 virus. A study described HNRNPA2/B1, another upregulated gene involved in the
210 packaging of nascent pre-mRNA, in the formation of cytoplasmic stress granules responsible for the assembly of the
211 nucleocapsid protein and genomic RNA of SARS-CoV-2[36] (Supplementary Table 10). Another study showed that the
212 NSP1 of SARS-CoV-2 facilitates viral RNA processing and blocks effective IFNβ expression through directly binding
213 HNRNPA2/B1 and redistributing it between the nucleus and cytoplasm[37]. Direct modulation of SMMR2 and
214 HNRNPA2/B1 expression levels in the host cells might be an alternative mechanism for the SARS-CoV-2 virus to
215 ensure proper synthesis, assembly, and further spreading of viral particles. Notably, we found the RNase1 gene to be
216 downregulated in SARS-CoV-2^{+} spots, potentially blocking degradation of viral RNA in the environment of actively
217 infected cells (Figure 2f-g, Supplementary Table 10).

Many viruses, including SARS-CoV-2, have developed strategies to antagonize the autophagy pathway and thus escape host cell immunity[38,39]. Small GTPase proteins ARF1 and ARF6 act in the early steps of the autophagosome formation, and recent studies postulated that ARF6 might be bound and inhibited by the SARS-CoV-2 protein NSP15[40,41]. We have found SMAP2, a GTPase activating protein interacting with the ARF1 and ARF6 proteins, to be upregulated in SARS-CoV-2^{+} spots (Figure 2f, Supplementary Table 10). By catalyzing the GTP hydrolysis of ARF1 and ARF6 and thus rendering them in an inactive state, an increase in SMAP2 expression would mean a plausible alternative mechanism for the SARS-CoV-2 virus to block the autophagy pathway and promote viral replication and dissemination.

Previous work highlighted the NFκB pathway as a central signaling pathway in COVID-19 pathogenesis and the initiator of the so-called cytokine storm, characteristic of the disease[42,43]. In line with studies describing the direct induction of the NFκB pathway components by the SARS-COV-2 virus[44,45], we found NFKB2 and NFKBIA, along with CXCL9, CCL17 and CCL21, to be upregulated in SARS-CoV-2^{+} spots (Figure 2f-g, Supplementary Table 10), and a study showed that the ORF7 protein of the SARS-CoV-2 virus induced these genes in an NFκB-dependent manner[46]. Previous studies proposed CCL17 as a potential predictive biomarker to distinguish between mild/moderate and severe/critical disease[47], and CXCL9 to be part of a biomarker panel associated with mortality in patients with COVID-19[48]. Notably, we identified certain complement factors (C1QB, CFD, C7) and interferon response genes (IFI6, ISG15) to be upregulated in COVID-19 lungs compared to control lungs (Supplementary Table 6), in line with previous studies[3,7,49]; however, these genes were downregulated in the SARS-CoV-2^{+} spots of the infected COVID-19 lungs (Figure 2f, Supplementary Table 10), which points to spatially localized differences in host response to the virus as previously observed in terms of the interferon response genes[4]. A study proposed that the SARS-CoV-2 virus can direct a reduction in IFN response[50], such as through NSP3 cleavage of ISG15[51], which in turn might affect the subsequent activation of the complement system[52,53].

In addition, we detected a downregulation of certain immunoglobulin genes (IGKC, IGKV4-1, IGHA1, IGHG2) and extracellular matrix components (FBLN, COL1A2, COL3A1, BGN, COL1A1, SPP1) in the SARS-CoV-2^{+} spots in the diseased COVID-19 tissue samples, which may be explained by the viral infection preceding both the extensive plasma cell infiltration and fibroblast activation in time (Figure 2f, Supplementary Table 10). It is also possible, however, that areas closely resembling the healthy state of the lungs are more permissive for the replication of viral components. Finally, we found several AT2 (SFTPB, SFTPC, MUC1, SLC34A2), AT1 (GPRC5A, AGER) and the alveolar endothelial cell marker AQP1 to be downregulated in the SARS-CoV-2^{+} spots (Figure 2f, Supplementary Table 10). These differences can either represent functional impairment or increased apoptosis of alveolar epithelial cells, which are known to be the primary cellular targets of the SARS-CoV-2 in the lungs[3,49].

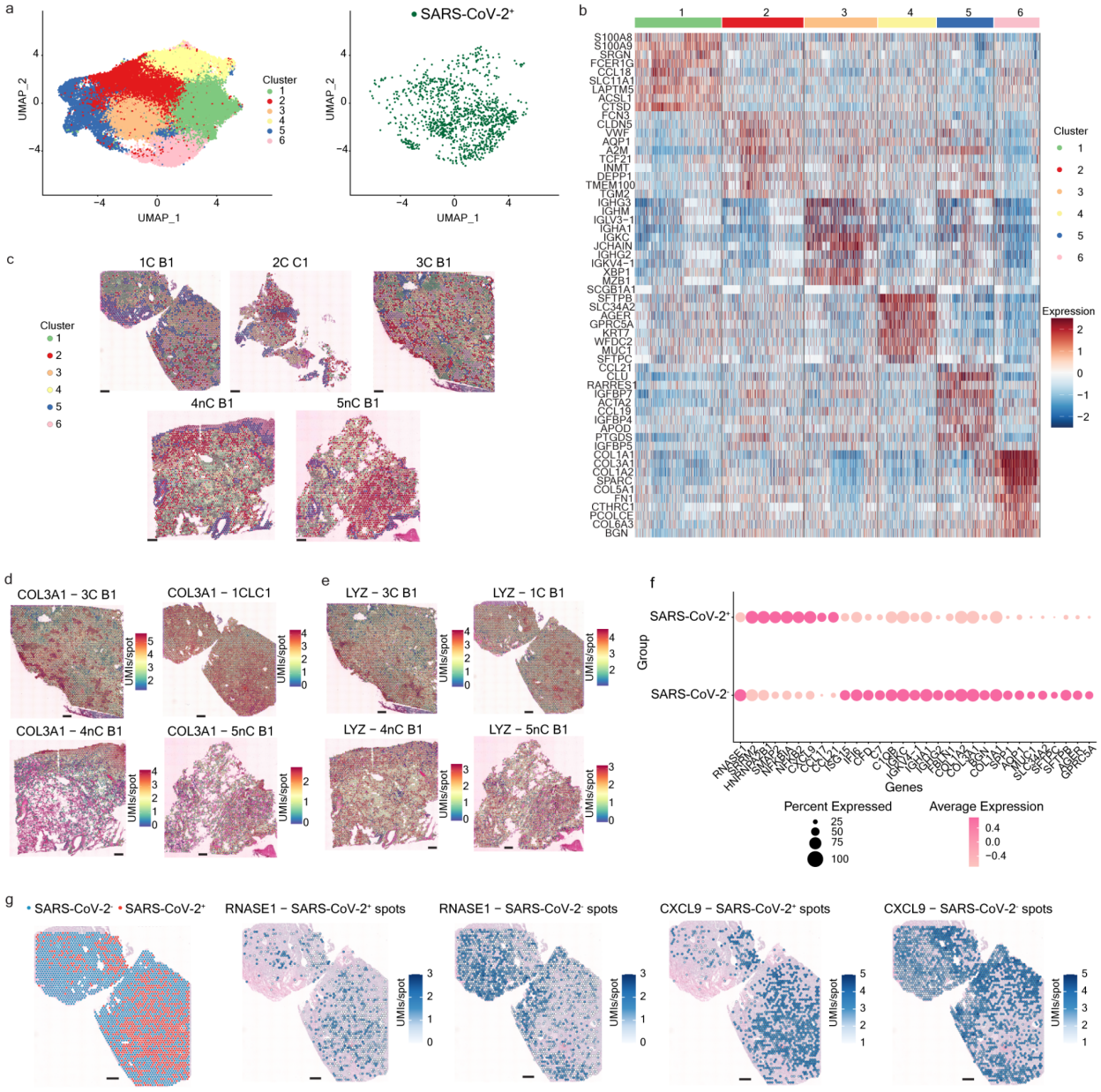

Figure 2. Human host response to SARS-CoV-2 infection. (a) Clustering of the human transcriptome data across COVID-19 and control sections reveals 6 distinct clusters with SARS-CoV-2^{+} spots distributed throughout the clusters. (b) Differential genes per cluster across COVID-19 and control sections. (c) Spatial distribution of the clusters on COVID-19 and control sections. Scale bars are 500µm. (d-e) Spatial distribution of genes upregulated in COVID-19 sections, COL3A1 (d) and LYZ (e). Scale bars are 500µm. (f) Dotplot depicting differential expression of human genes in SARS-CoV-2^{+} and SARS-CoV-2^{-} spots in COVID-19 sections. (g) Spatial distribution of RNase1, downregulated in SARS-CoV-2^{+} spots, and CXCL9, upregulated in SARS-CoV-2^{+} spots, in COVID-19 sample 1C. Scale bars are 500µm.

Discussion

Future work on SARS-CoV-2-human spatially resolved interactions could utilize the method proposed here to explore how the different SARS-CoV-2 genes regulate host gene expression through the colocalization of specific SARS-CoV-2 genes with the human transcriptome. Furthermore, additional probes that indicate if SARS-CoV-2 is actively replicating by targeting the negative strand of the viral genomic RNA (gRNA) could be developed. In terms of the general outlook on spatially resolved host-pathogen interactions, limitations of our proposed approach include the requirement of previous knowledge of the pathogen transcriptome of interest to develop targeted probes, the inability to detect different human RNA splice variants, the lack of capturing human non-coding RNA groups that may have important regulatory functions, and the inability to detect new viral variants since the viral RNA is not directly sequenced. However, probes targeting specific host RNAs of interest could be developed to overcome some of these shortcomings.

In conclusion, the proposed method enables insights into highly localized host response to pathogen infection within the spatial context of the tissue microenvironment at the whole-transcriptome level in an unbiased and high-throughput manner. The method has the potential to be applied to other human pathogens with the development of targeted probes and thus examine the interplay between host and pathogen across the multitude of human infectious diseases. Overall, our approach opens the door to new possibilities of studying infectious disease at a large scale by exploring multiple transcriptomes in a single experiment.

Author contributions

S.G. and O.B. conceived and designed the project. T.V., T.B., A.K. performed sample collection. H.S. performed spatial transcriptomics and *in situ* sequencing experiments. E.L. performed RNAscope experiments. A.J. provided assistance with spatial transcriptomics experiments. A.N. provided assistance with *in situ* sequencing experiments. A.H. designed SARS-CoV-2 probes. E.B. performed read alignments. Y.M. performed computational analysis of the spatial transcriptomics data and colocalization analysis with supervision of S.G. J.W. designed the computational approach for spatial transcriptomics, RNAscope, and *in situ* sequencing validation and performed the analysis with supervision of S.G. E.L. performed image annotation. H.S., E.L., H.D., O.B. and S.G. interpreted the results. H.S. prepared figures with input from S.G. S.G. and O.B. supervised and guided the project. H.S., E.L., O.B. and S.G. wrote the manuscript with input from Y.M. and J.W. All the authors read and approved the manuscript.

Acknowledgements

This work was supported by the Swedish Research Council as Formas grant 2017-01066 and Vetenskapsrådet grant 2020-04864 to S.G. O.B. was supported by the Center for Regenerative Therapies Dresden, the Karolinska Institute, the Swedish Research Council, the Ragnar Söderberg Foundation, the Åke Wiberg Foundation, and the LeDucq Foundation. We thank 10x Genomics for support with instruments and/or lab reagents and technical advice (Patrick Roelli and Iván Hernández). We thank Karl Frontzek for help with providing lung samples.

Conflict of interest

H.S., Y.M., S.G. are scientific advisors to 10x Genomics, Inc. that holds IP rights to the ST technology and previously acquired ReadCoor and Cartana and their accompanying intellectual property rights. S.G. holds 10x Genomics stocks. H.S., Y.M., E.B., A.H. and S.G. are co-inventors on patent filings relating to this work. E.B., A.H., A.J. and A.N. are employees of 10x Genomics and hold stock options. All other authors declare no competing interests.

Methods

Patient selection, sample collection and processing

Collection of postmortem samples from lung tissue was performed at the 2nd Department of Pathology, Semmelweis University (Budapest, Hungary) and the University Hospital Zurich (Switzerland). Autopsy cases were selected from patients who were hospitalized because of COVID-19 infection and died at the local clinical departments of the universities. Criteria for selection were: premortem positive (COVID-19 cases) or negative (control cases) SARS-CoV-2 PCR test, lack of malignancy of the lung, closed clinical documents and less than 24 hours as a postmortem interval (PMI). Autopsy was done in harmony with the World Health Organization's (WHO) recommendation for autopsy of COVID-19 cases[54]. The biopsies were fixated in formaldehyde (4%) and then went through a dehydration process overnight. Dehydrated samples were embedded into paraffin blocks and were stored at 4°C until sectioning. The use of tissue specimens collected at Semmelweis University in this study was approved by the Hungarian Scientific Research Ethics Committee (ETT TUKEB IV/3961-2/2020/EKU). Samples and data were managed anonymously. At the University Hospital Zurich, small quantities of bodily substances removed in the course of an autopsy were anonymized for research purposes without consent, in the absence of a documented refusal of the deceased persons. In accordance with the Swiss Federal Act on Research involving Human Beings, this study did not require institutional board approval. Subsequent experiments were approved by the Swedish Ethical Review Authority (2010/313-31/3, 2018/689-32). Relevant clinical parameters of the patients included in this study are summarized in Supplementary Table 2.

Sample selection - Evaluating RNA Quality

Total RNA was extracted from each formalin-fixed paraffin-embedded (FFPE) sample block with the RNeasy FFPE kit (Qiagen, Cat. No. / ID: 73504) following the manufacturer's instructions (deparaffinization was performed using xylene (#28975.291 VWR) and 96% EtOH (#20823.290 VWR) or 100% EtOH (#1.00983.1000 VWR)). The concentration of extracted total RNA was determined with the RNA HS Qubit assay (Thermo Fisher Scientific) following the manufacturer's instructions. Total RNA was diluted to between 2-5ng and RNA fragment length assessed using the Agilent RNA 6000 Pico Kit following the manufacturer's instructions. The RNA quality of the sample was evaluated by the DV200 measurement (percentage of RNA fragments longer than 200 nucleotides) as specified in the

322 Visium Spatial Gene Expression for FFPE – Tissue Preparation Guide[55]. Samples with a DV200 greater than 40%
323 were selected for Visium FFPE, RNAScope and *in situ* sequencing.

324 **SARS-CoV-2 probe design**

325 SARS-CoV-2 probes were designed as described[56], with probes designed based on the reference transcriptome
326 Sars_cov_2.ASM985889v3, Ensembl build 101 (https://covid-19.ensembl.org/Sars_cov_2/Info/Index). Probes were
327 designed to target SARS-CoV-2 genes: Surface glycoprotein (S), Envelope protein (E), Membrane glycoprotein
328 (M), ORF1ab, ORF3a, ORF7a, ORF7b, ORF8, Nucleocapsid phosphoprotein (N), and ORF10 (10x Genomics)
329 (Supplementary Tables 1,11-12).

330 **Spatial Transcriptomics**

331 Consecutive 5µm tissue sections from each sample were placed onto Visium Spatial Gene Expression slides (PN:
332 2000233, 10X Genomics) and stored overnight in a desiccator[55]. 5µm consecutive sections to the sections used for
333 Visium FFPE were placed onto Superfrost Plus microscope slides (#631-9483, VWR) and stored at 4°C until used for
334 RNAScope and *in situ* sequencing. Deparaffinization, Hematoxylin and Eosin staining, and decrosslinking were
335 performed as specified in the the Visium Spatial Gene Expression for FFPE – Deparaffinization, H&E Staining,
336 Imaging & Decrosslinking Demonstrated Protocol[57]. Spatial gene expression profiling of RNA from FFPE lung
337 samples was performed by following all steps in the Visium Spatial Gene Expression Reagent Kits for FFPE User
338 Guide[20] with the modifications: For COVID-19 samples (see Supplementary Table 2), four 5µm consecutive sections
339 per patient sample tissue FFPE block were placed on Visium Spatial Gene Expression slides (PN: 2000233, 10x
340 Genomics). For step 1.1.g, Human whole transcriptome (WT) probes (10x Genomics) were added to two consecutive
341 sections (technical replicates) with the Probe Hybridization Mix: 19.8µL Nuclease-free water, 77.0µL FFPE Hyb
342 Buffer, 6.6µL LHS Human WT probes, and 6.6µL RHS Human WT probes, per sample. Human WT and spike-in
343 custom probes targeting SARS-CoV-2 genes (10X Genomics) were added to the remaining two consecutive sections
344 (technical replicates) with the Probe Hybridization Mix: 14.5µL Nuclease Free water, 77.0µL FFPE Hyb Buffer, 6.6µL
345 LHS Human WT probes, and 6.6µL RHS Human WT probes, 2.6µL LHS viral probes, and 2.6µL RHS viral probes,
346 per sample. For control patient samples, two consecutive sections (technical replicates) were processed as described
347 for the COVID-19 samples, with adding Human WT and SARS-CoV-2 spike-in probes to all sections. For step 4.1.d,
348 qPCR (Bio-Rad) step 4 was run for a total of 30 cycles. For step 4.2.d, the Sample Index PCR was performed with 15
349 cycles for 1C, 15-16 cycles for 3C, 18-19 cycles for 2C, 16 cycles for 4nC, and 18 cycles for 5nC. After step 4.4, the
350 concentration of sequence libraries were determined with 2µL of each sample run with the dsDNA HS Qubit assay
351 (Thermo Fisher Scientific).

352 **Spatial Transcriptomics Hematoxylin & Eosin Imaging**

353 Hematoxylin & Eosin brightfield images were acquired with a Zeiss Axiolmager.Z2 VSlide Microscope using the
354 Metasystems VSlide scanning system with Metafer 5 v3.14.179 and VSlide software. The microscope has an upright

355 architecture, uses a widefield system, and a 20X air objective with the numerical aperture (NA) 0.80 was used. The
356 camera was a CoolCube 4m with a Scientific CMOS (complementary metal-oxide-semiconductor) architecture and
357 monochrome with a 3.45 x 3.45 µm pixel size. All brightfield images were taken with a Camera Gain of 1.0 and an
358 Integration Time/Exposure time of 0.00011 seconds.

359 **Spatial Transcriptomics Sequencing**

360 Sequencing libraries were pooled and diluted with Elution Buffer (EB) to a final concentration of 10nM, using a target
361 sequencing depth of 50,000 mean read pairs/spot to determine the dilution for each sample[20]. After sample pooling,
362 pooled library concentrations were checked with qPCR (Bio-Rad) before loading into the sequencer. Libraries were
363 sequenced on an Illumina NovaSeq 6000 with paired-end, dual indexed sequencing run type and parameters following
364 those specified in the Visium Spatial Gene Expression Reagent Kits for FFPE User Guide sequencing instructions[20]
365 [R1: 28 cycles, R2S: 50-52 cycles], with a spike-in of PhiX at 1% concentration, except one sample, 3C, was run with
366 R2S: 75 cycles.

367 ***In situ* sequencing (ISS)**

368 Optimal RNA integrity and assay conditions were assessed using *MALAT1* and *RPLP0* housekeeping genes only using
369 the HS Library Preparation kit for CARTANA technology (part of 10x Genomics) and following manufacturer's
370 instructions on 5µm tissue sections from representative sample 1C. Since the control probes test showed positive and
371 expected results, *in situ* sequencing was then performed on two 5µm consecutive sections from sample 1C and one
372 consecutive section from each control sample (4nC and 5nC). Superfrost Plus microscope slides (#631-9483, VWR)
373 containing 5µm tissue sections were stored at 4°C until processing. FFPE sections were baked for 1 hour at 60°C to
374 partially melt paraffin and increase tissue adherence. Next sections were deparaffinized using xylene for 2x7 minutes
375 followed by an EtOH gradient to remove xylene and rehydrate the sections. Sections were then permeabilized using
376 citrate buffer pH 6.0 (C9999 Sigma Aldrich) for 45 minutes at 95°C. For library preparation, chimeric padlock probes
377 (targeting directly RNA and containing an anchor sequence as well as a gene-specific barcode) for a custom panel of
378 SARS-CoV-2 S and E genes were hybridized overnight at 37°C, then ligated before the rolling circle amplification
379 was performed overnight at 30°C using the HS Library Preparation kit from CARTANA technology and following
380 manufacturer's instructions. All incubations were performed in SecureSeal™ chambers (Grace Biolabs). For tissue
381 section mounting, Slow Fade Antifade Mountant (Thermo Fisher) was used for optimal handling and imaging. Quality
382 control of the library preparation was performed by applying anchor probes to simultaneously detect all rolling circle
383 amplification products from all genes in all panels. Anchor probes are labeled probes with Cy5 fluorophore (excitation
384 at 650 nm and emission at 670 nm). All samples passed the quality control l and were sent to CARTANA (part of
385 10x Genomics), Sweden, for a single cycle *in situ* barcode sequencing, imaging and data processing. Briefly, adapter
386 probes and a sequencing pool (containing 4 different fluorescent labels: Alexa Fluor® 488, Cy3, Cy5 and Alexa
387 Fluor® 750) were hybridized to the *in situ* libraries to detect SARS-CoV-2 gene-specific barcodes. This was followed
388 by multicolor epifluorence microscopy, scanning the whole area and thickness of the tissues. Raw data consisting of

389 20x magnification images from 5 fluorescent channels (DAPI, Alexa Fluor® 488, Cy3, Cy5 and Alexa Fluor® 750)
390 and individual z-stacks, were flattened to 2D using maximum intensity projection with a Nikon Ti2 Nikon Ti2
391 (software NIS elements) utilizing Zyla 4.2 camera. After image processing, which includes image stitching,
392 background filtering and a sub-pixel object registration algorithm, true signals were scored based on signal intensities
393 from individual multicolor images. The results were summarized in a csv file and gene plots were generated using
394 MATLAB.

395 **RNAscope assay and imaging**

396 RNAscope assay was performed on lung 5 µm FFPE sections on Superfrost Plus microscope slides (#631-9483, VWR)
397 cut from depths consecutive to the sections mounted on Visium slides. The slides were baked in a dry oven for 1 h at
398 60 °C and then deparaffinized in xylene (2x 5 min) and absolute ethanol (2x 1 min) at room temperature. After drying,
399 the sections were incubated in RNAscope Hydrogen Peroxide for 10 minutes at room temperature, followed by
400 washing steps (2x) in distilled water. Target retrieval was performed using a 1x RNAscope Target Retrieval Reagent
401 for 15 minutes, at a temperature constantly kept above 99 °C in a hot steamer. The slides were then rinsed in distilled
402 water, incubated in absolute ethanol for 3 minutes and dried at 60 °C. After creating a hydrophobic barrier, the slides
403 were left to dry overnight. The second day, the sections were incubated in RNAscope Protease Plus solution for 30
404 min at 40 °C, followed by washing in distilled water. RNAscope V-nCov2019-S probe, RNAscope Positive Control
405 probe (Hs-PPIB) and RNAscope Negative Control Probe (DapB) were hybridized to separate sections for 2h at 40 °C,
406 then the slides were washed twice for 2 minutes in 1x Wash Buffer. The probe-specific signal was developed with an
407 RNAscope 2.5 HD Detection Reagent – RED kit. Sequential hybridization of amplification reagents AMP1-4
408 happened at 40 °C for 30-15-30-15 minutes, while AMP5 and AMP6 were applied at room temperature for 1 hour
409 and 15 minutes, respectively, with two washing steps in 1x Wash Buffer after each incubation period. For signal
410 detection, each section was incubated for 10 min at room temperature in 120 ul RED Working Solution, consisting of
411 Fast RED-B and Fast RED-A reagents in a 1:60 ratio. All the protease digestion, probe hybridization, signal
412 amplification and signal detection steps were performed in a HybEZ Humidity Control Tray, which was either placed
413 into a HybEZ Oven for the 40 °C incubation steps or kept at room temperature. Following two washing steps in tap
414 water, the slides were counterstained with 50% Gill's Hematoxylin staining solution for 2 min at room temperature,
415 thoroughly rinsed with tap water, then soaked in 0.02% Ammonia water bluing solution and finally washed again in
416 tap water. The slides were then dried completely at 60 °C and then quickly dipped into xylene before mounting them
417 with VectaMount Permanent Mounting Medium. The RNAscope signal was imaged and evaluated with a Leica
418 DM5500B microscope with a HC PL APO 20x/0.70 DRY objective, using Extended Depth of Field (EDoF) imaging
419 in the Leica Application Suite X (LAS X) software platform.

420 **Spatial Transcriptomics - Data Processing**

421 **Count matrices generation**

422 The gene expression matrices were generated by spaceranger (version 1.3.0) 'count' (standard settings set except --
423 *no-bam*). The transcriptome reference was custom made from spaceranger 'mkref' using Human reference dataset

424 (GRCh38 Reference - 2020-A), and SARS-CoV-2 genome assembly (ASM985889v3). The Human Probe Set from
425 10x Genomics (Visium Human Transcriptome Probe Set v1.0) with 10x Genomics custom probes for SARS-CoV-2
426 probes appended to it, was used as the probe set reference in spaceranger 'count'.
427
428 **Quality Control**
429 The filtered count matrices (filtered_feature_bc_matrix.h5), and tissue images from spaceranger output were analyzed
430 in R using the Load10X_Spatial function available in Seurat (version 4.0.4)[58]. The filtered count matrices were
431 separated into human count data, and SARS-CoV-2 count data matrices. Spot level filtering was performed on the
432 human count matrices to keep spots with at least 400 genes, 500 UMIs, and a novelty score of 0.87. Gene level filtering
433 was applied to omit genes that did not appear in at least 1 spot. These count matrices were also filtered for Hemoglobin
434 gene counts (Supplementary Table 1). SARS-CoV-2 count matrices were normalized by dividing the SARS-CoV-2
435 gene UMI counts by the number of probes used to target the respective gene. 1 SARS-CoV-2 UMI was detected from
436 two different sections, one control and one COVID-19, that did not have SARS-CoV-2 probes added and was
437 considered as background signal.
438
439 **Clustering Analysis**
440 The Seurat SCTransform function was applied to normalize the individual filtered count matrices, and integrated in
441 Seurat using SelectIntegrationFeatures, and IntegrateData. Principal Component Analysis (PCA), and UMAP was
442 applied using 50 principal components, and 35 were further used in downstream analysis, and clustering. Batch effects
443 were addressed, and removed using RunHarmony (group.by.vars as slide ID, and 25 iterations) applied on the PCA-
444 computed matrix [59]. Clustering was applied at a resolution of 0.4.
445
446 **Differential Gene Expression**
447 Differentially expressed (DE) genes were found using 'FindMarkers' in Seurat, with default settings on the SCT
448 normalized matrix, except min.cells.group set to 2 to include at least 2 spots from each group. Both upregulated and
449 downregulated DE genes were identified, with an adjusted p-value of 0.005**.** Cell-type specific annotation of the DE
450 genes was performed manually, by using the Human Single Cell Atlas[60], PanglaoDB[61], and recently published single
451 cell transcriptomic data of the human lung[3,49] as main resources.

452 **Colocalization analysis**

453 For the colocalization analysis, a direct spot-level comparison within the COVID-19 sections was performed. The DE
454 genes distinguishing SARS-CoV-2^{+} spots from SARS-CoV-2^{-} spots were obtained as described in the Methods section
455 "Differential Gene Expression" with an additional filter of average logFC +/- 1.0.

456 **Validation by RNAScope**

457 RNAScope and ST images were manually aligned with Adobe Photoshop 2022. The RNAScope chromogenic
458 detection of the S gene with FastRed was used to distinguish RNAScope signal from lung pigmentation and tar

459 deposits. All dots of chromogenic red signal were considered as positive SARS-CoV-2 S gene signal, since the
460 majority of signal was above 1 dot per 10 nuclei area, in line with how others assessed RNAScope signal in SARS-
461 CoV-2 viral low samples[3,4,62]. RNAScope was considered as the gold standard for comparison to the ST signal. The
462 number of ST spots where the SARS-CoV-2 S gene was detected, and where the RNAScope S gene signal was also
463 obtained, was calculated. To adjust for the use of consecutive sections for ST and RNAScope experiments, the
464 agreement of ST and RNAScope in 200x200 μm^2 block areas were evaluated. Since a manual annotation of sample
465 1C was in close agreement with the computational approach, the computational approach to calculate the specificity
466 of the SARS-CoV-2 S gene detection by ST was used.

467 The computational validation was performed as follows: the RNAScope signal was detected with an ad hoc Matlab
468 (version R2021b) algorithm, which is specified in the next section "Automatic detection of RNAScope signal"; then
469 both the binary ST and RNAScope signal images were aligned and binned into 200x200 μm^2 blocks (Supplementary
470 Figures 3-4). Each block in an RNAScope/ST signal image was regarded as an observation (those blocks that contain
471 no tissue area were regarded as no observation and were excluded from any further analysis and counting). The
472 specificity of our method to capture the SARS-CoV-2 expression was calculated by considering the RNAScope
473 approach as the groundtruth and as follows:

474 $$Specificity = \frac{\#TN}{\#TN + \#FP}$$

475 Where the number (#) of True Negatives (TN) was defined as the number of blocks containing neither RNAScope nor
476 ST signals and the number of False Positives (FP) as the number of blocks containing only ST but no RNAScope
477 signals.
478

479 **Automatic detection of RNAScope signal**

480 RNAScope signals were detected with a chromatical analytic method. First, the original RGB image was transformed
481 into the Hue-Saturation-Value (HSV) format, where the bright regions in the hue channel correspond to the RNAScope
482 signals in the original histological image. The brightest regions became the foreground by thresholding the hue value
483 of the image. Morphological post processing steps were performed to refine the shape of the signal regions, the details
484 of which are available in the code (see "Code availability"). The pixels whose hue was over 0.85, saturation over 0.25,
485 and value over 0.40 were recognized as signal candidates. After performing a morphological opening operation, the
486 collection of signal candidates were output as final RNAScope signals. Supplementary Figure 5 displays the original
487 tissue subimage, the hue channel, and the RNAScope signal subimage after the thresholding.

488 **Validation by ISS**

489 ISS consecutive section images and ST images for sample 1C were manually aligned with Adobe Photoshop 2022.
490 Due to the use of non-consecutive sections, there was ~300 µm in between the ST and ISS sections. The agreement
491 between E and S gene signals for ST and ISS in block areas of 200µm was evaluated using the same computational
492 approach as used for the RNAScope validation.

493 **Data availability**

494 Raw sample sequences will be available with controlled access on the European Genome-Phenome Archive (EGA).
495 Processed gene count matrices, related metadata, corresponding ST tissue microscopy images, and images used in
496 the Validation analysis are available on Mendeley dataset under the Reserved DOI: 10.17632/xb2w7xvs2b.1. ISS
497 images for SARS-CoV-2 S and E genes can also be downloaded from Mendeley dataset with Reserved DOIs
498 10.17632/gwjk2cxsf4.1. RNAscope images for SARS-CoV-2 S gene are available on FigShare project ID 134597
499 which is currently private. RNAScope images for positive and negative control probes are available upon request.

500 **Code availability**

501 Scripts to generate the count matrices and all related R scripts used in the clustering, differential expression,
502 colocalization analysis, and the program for the computational validation can be accessed from our github repository,
503 DualST_Study.

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