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Spatial Phenotyping Immune Cell Subsets in Patients with Lethal COVID-19

HIGHLIGHTS:

- **Biomarker discovery within the complex tissue microenvironment requires unrestricted, non-ROI based imaging with single cell resolution.**
- **The PhenoCycler™ platform is part of a compact, end-to-end solution for ultra-high plex, quantitative imaging of 40+ protein markers.**
- **Compatibility with FFPE tissues enabled single-cell imaging of immune cell subtypes and *in situ* detection of SARS-CoV-2 virus in post-mortem tissues taken from patients with lethal COVID-19.**
- **Highly multiplexed whole-tissue spatial phenotyping characterizes different immune cells throughout distinct phases of COVID-19 disease.**

INTRODUCTION

Spatial phenotyping facilitates the analysis of how cells organize and interact with each other to influence disease progression and treatment outcomes. This approach is invaluable for elucidating mechanisms associated with the progression of infectious diseases in the respiratory system due to the highly heterogenous immune response within the tissue microenvironment. Studies of influenza virus infection have revealed cell type-specific responses to viral infections (Fay et al., 2020; Ma et al., 2019). Recent studies of cell models of SARS-CoV-2 infection also suggest cell type-specific host-pathogen interactions (Fiege et al., 2021).

Translational research and clinical pathology of infectious disease often depend on precious human tissue samples that are available as formalin-fixed paraffin embedded (FFPE) specimens. In the context of infectious diseases, where tissue biopsies are rarely collected for diagnostic purposes, research analyses are often limited to FFPE post-mortem tissue samples. However, FFPE is incompatible with many single cell biology techniques.

In addition, single cell biology requires true single cell resolution. Some techniques, like scRNA-Seq, offer single cell resolution but lack spatial information. Other emerging spatial biology platforms have adapted to analyzing FFPE samples, but their analyses are restricted to predefined regions of interest (ROIs) that are several-fold larger than the diameters of single cells resulting in the estimation of a given effect rather than true visualization.

PhenoCycler (formerly CODEX) provides a sensitive, reproducible, and highly multiplexed method for detecting 40+ proteins in FFPE tissues.

Because of its compatibility with FFPE samples and ultra-high plex imaging capability, PhenoCycler technology represents an ideal method for analyzing the effect of SARS-CoV-2 infection on immune cell distribution and microenvironment across diverse tissue types, as described in this application note

About 95% of immune cells are not present in blood, therefore the vast majority of immunopathology related to SARS-CoV-2 infection occurs at the tissue level rather than in blood.

THE PHENOCYCLER WORKFLOW

PhenoCycler is based on multiplexed imaging and is thus deployable without the need for complicated instrumentation or operational infrastructure. The PhenoCycler chemistry is an iterative workflow that relies on a DNA-based tagging approach, where antibodies are labeled with specific oligonucleotide tags (barcodes) and dye oligonucleotides (reporters) that are sequentially hybridized and dehybridized across multiple cycles. This process is completely automated through the PhenoCycler instrument and reporter readouts are acquired using standard epifluorescence optics. Sample preparation for PhenoCycler follows a complete workflow that also includes reagents and a software suite for analysis and visualization (Figure 1, page 2).

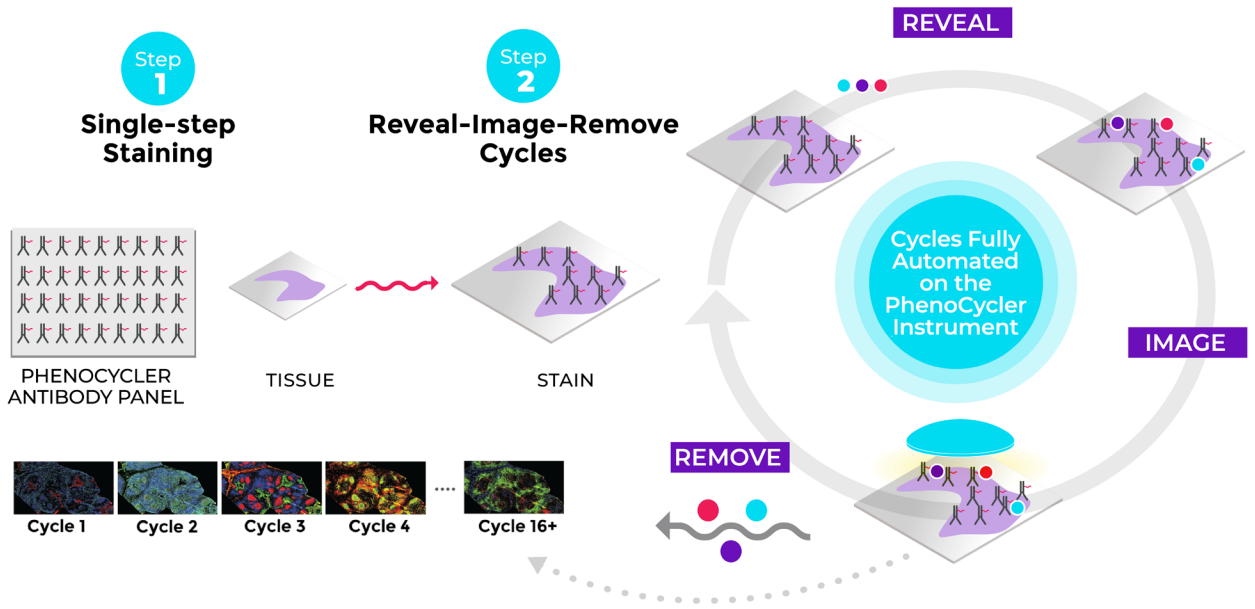


FIGURE 1. ***The PhenoCycler workflow and chemistry.*** TOP: The PhenoCycler Instrument is part of a complete workflow that also includes reagents and a software suite for analysis and visualization. The PhenoCycler system integrates with a companion fluorescence microscope through a custom stage insert. BOTTOM: The single step staining preserves sample integrity for re-analysis of specific regions. The Reveal-Image-Remove cycles are completely automated on the PhenoCycler instrument.

PHENOCYCLER USED FOR POST-MORTEM, SINGLE CELL ANALYSIS, AND *IN SITU* VIRUS DETECTION WITHIN FFPE PRESERVED TISSUE

A 35-plex PhenoCycler antibody panel was used to localize immune, stromal and functional biomarkers, as well as SARS-CoV-2 spike and nucleocapsid proteins, in post-mortem FFPE preserved tissues from 8 patients with lethal COVID-19. Post-mortem investigations were performed by Dr. Esther Youd at Cwm Taf Bro Morgannwg Health Board, South Wales, UK, and included consent post-mortems for tissue research. The study cohort included 3 males and 5 females with a median time of symptom onset to death of 13.6 days. Mortality location included 3 deaths in the community and 5 deaths in hospital. The panel of markers are shown in Table 1. Representative images are shown in Figure 2.

Immune	**T cells** CD3e CD4 CD8 FoxP3	**NK cells** CD56 CD57	**Stromal**	**Epithelium** E-cadherin	**Smooth Muscle** aSMA
	Macrophages CD14 CD68 CD163 CD11b	**Granulocytes** CD15		**Type II Pneumocytes** TTF-1	**Lymphatics** Podoplanin
	Lymphocytes CD45 CD45RO	**Dendritic Cells** CD11c CD303			
Virus	**SARS-CoV-2** Spike Nucleocapsid		**Functional**	**Multifunctional** CD34 VWF CD61 Mac2-Galectin3	**Proliferation** Ki67 **Checkpoint** TIM3 LAG3 PD1 PDL1 Ox40

TABLE 1. List of markers used to localize immune, stromal, functional, and virus proteins via multiplex PhenoCycler antibody panel single-step staining.

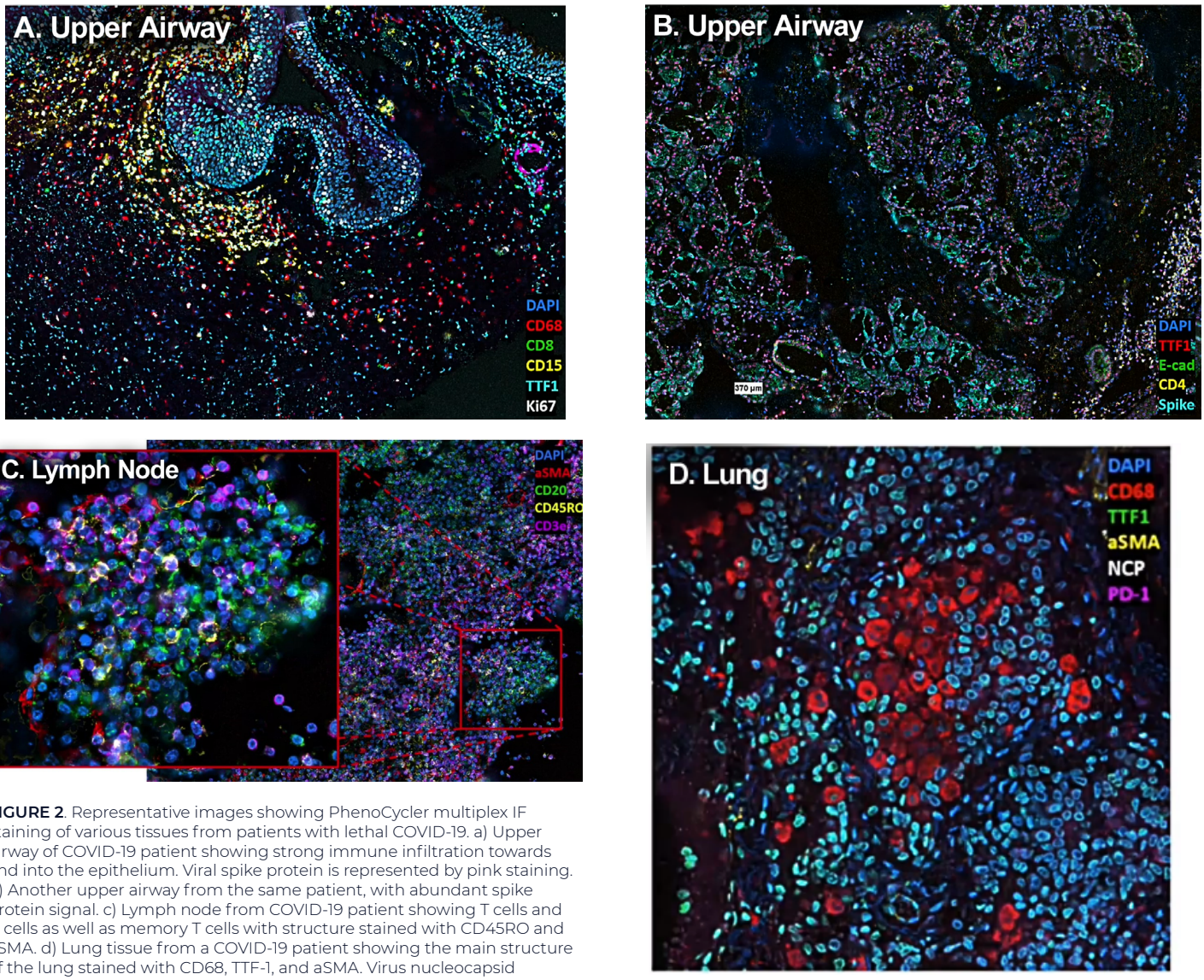


FIGURE 2. Representative images showing PhenoCycler multiplex IF staining of various tissues from patients with lethal COVID-19. a) Upper airway of COVID-19 patient showing strong immune infiltration towards and into the epithelium. Viral spike protein is represented by pink staining. b) Another upper airway from the same patient, with abundant spike protein signal. c) Lymph node from COVID-19 patient showing T cells and B cells as well as memory T cells with structure stained with CD45RO and aSMA. d) Lung tissue from a COVID-19 patient showing the main structure of the lung stained with CD68, TTF-1, and aSMA. Virus nucleocapsid staining is observed, along with blood vessels stained with aSMA.

VISUALIZATION OF SARS-COV-2 VIRAL STAINING SIGNAL ON PHENOCYCLER PLATFORM

SARS-CoV-2 spike protein and SARS-CoV-2 nucleocapsid protein antibody efficacy has been speculative due to inconsistent sensitivity, particularly in post-mortem samples. To validate expression of both proteins, images from the three patients with early-stage COVID-19 who died in the community and had higher viral loads were analyzed. Images of nucleocapsid protein expression were overlayed with SARS-CoV-2 spike protein channels (Figure 3, right), representing positive identification of both proteins. Expression patterns were additionally validated using RNA staining and transcript level measurements. Figure 4 shows positive staining for viral spike protein, nucleocapsid protein, as well as macrophages in lung tissue.

Image analysis and quantitation

In order to abstract quantitative data from image data, single cell images were segmented and the average intensity of each channel, per cell, was determined. The X and Y coordinates of the centroid of each cell were also recorded. These data not only revealed the phenotype of each cell, but also enabled investigation of the spatial relationships between phenotypes.

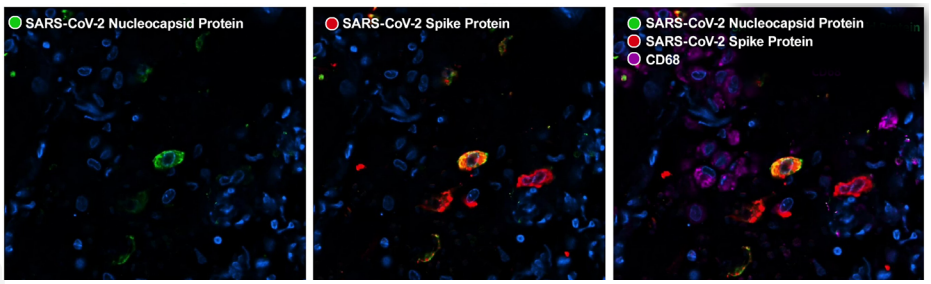


FIGURE 3. Lung from a patient known to have had high levels of viral RNA, showing positive staining for SARS-CoV-2 nucleocapsid protein, spike protein, and CD68 (macrophage signal).

Computations were performed using an R package known as the multiplex immunohistochemistry spatial interaction library ("MISSILe") built specifically for analyzing high dimensional multiplex immunohistochemistry data. Cell phenotypes were assigned using clustering algorithms to generate dimensionality reduction plots (or UMAP plot). An example of a dimensionality reduction plot shows lung tissue, where each dot represents a cell and positioning and color on the plot reflects protein expression (Figure 4, left). The heatmap (Figure 4, right) shows the average signal intensity for each channel with respect to cell phenotype.

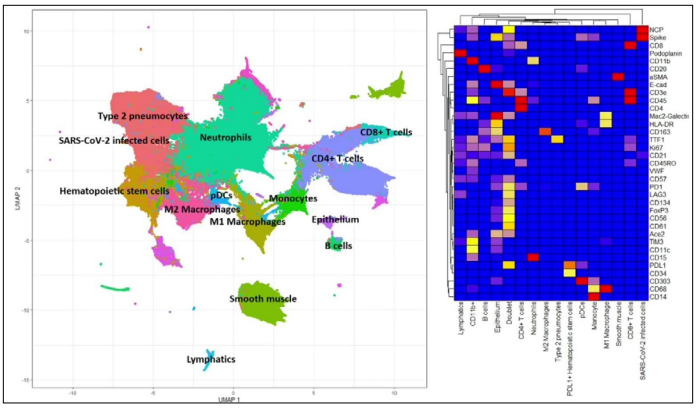
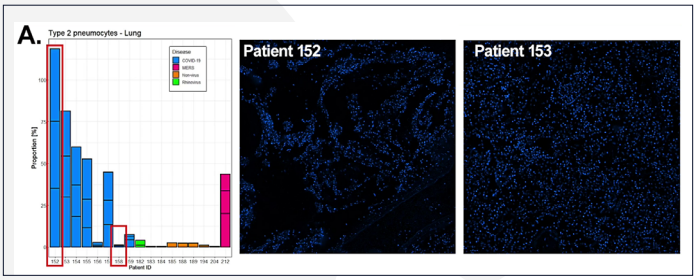
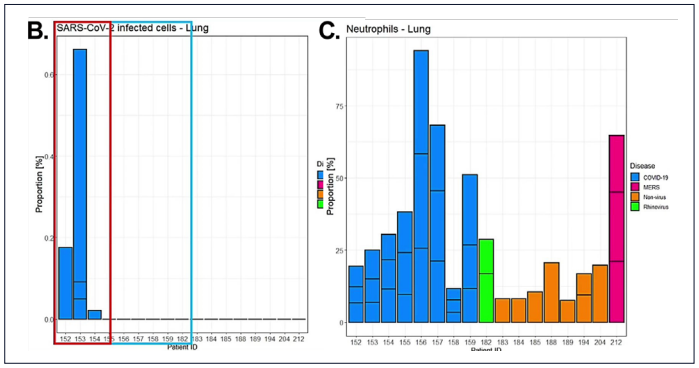


FIGURE 4. Cell phenotypes assigned using unsupervised clustering, to generate dimensionality reduction plots (left) and heatmaps showing average expression profile of each protein with respect to cell phenotype (right).

Spatial biology of lungs at various stages of COVID-19 disease progression

To compare biomarker expression and investigate spatial relationships between tissues and immune cells, the 3 patients who died with early-stage disease in the community (Patients 152, 153, and 154) were compared to the 5 patients who died with later-stage disease in the hospital (Patients 155-159) (Figure 5). All patients with COVID-19 were compared to patients who died with other infections including Middle Eastern Respiratory syndrome (MERS), rhinovirus, or non-viral infections. Patients who died in the community had increased levels of type II pneumocytes, indicating more structured lung tissue compared to those who died in hospital with later stages of disease (Figure 5a). Viral proteins were also detected in earlier stages of disease but not in later stages in which patients are hospitalized with lung damage and immune cell activation (Figure 5b). Neutrophil activation was identified in later stages of disease, but eventually reaches a threshold which may be related to lymphocyte recruitment (Figure 5c). Lymphocyte recruitment at later stage disease was denoted by increased CD4+ and CD8+ T cells in the lung (Figure 5d.)





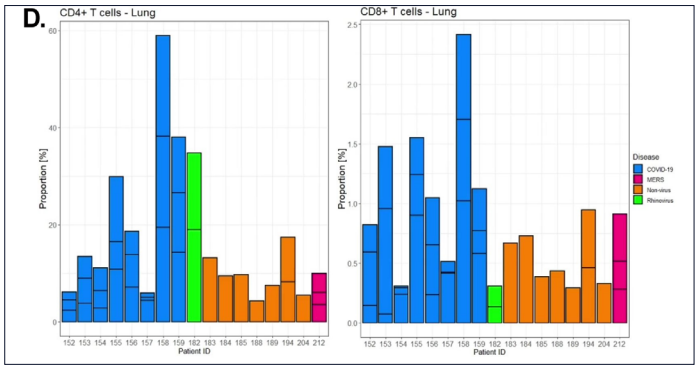


FIGURE 5. Region specific changes in lung structure, viral protein abundance, and immune cell subtypes present with respect to stage of COVID-19 disease progression. Each stack in the bar chart represents a lung region along with proportion of specific cell types (y-axis).

Interaction frequency heatmaps were used to assess spatial relationships between cell types. The heatmap shown in Figure 6 was generated using nearest-neighbor analysis of spatially resolved cell phenotype information and shows the interaction frequency between each cell type. For patient 152 (early stage disease), the infected cells seem to be interacting with each other (red box), indicating clusters of infected cells within the lungs. Infected cells are also interacting with M1 macrophages and neutrophils. Although Patient 153 died with early-stage disease, interactions between viral infected cells were slightly different. Viral infected cells appeared more isolated with fewer interactions between other infected cells, they were also not detected as existing in clusters or interacting with macrophages or neutrophils; rather, they were associated with CD4+ T cells, suggesting that these T cells may play a role in slowing viral spread within tissue.

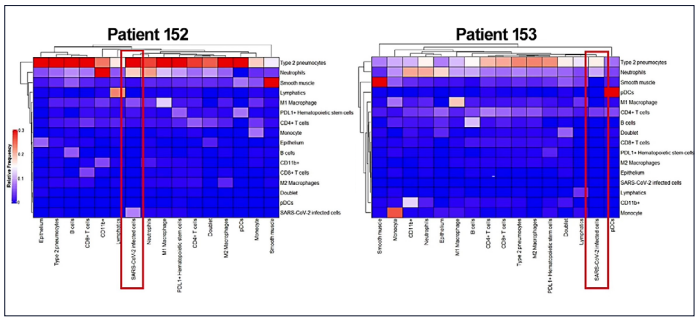


FIGURE 6. Interaction frequency heatmaps representing spatial interactions between all measured cell types. The left heatmap shows data for patient 152 (early-stage disease) and the right heatmap shows data for patient 153 (early-stage disease).

SUMMARY & CONCLUSION

PhenoCycler technology enables analysis of cell-type distribution within tissues, the complex array of cell phenotypes, and potentially their functional status. Using two antibodies against the spike protein and the nucleocapsid protein, the PhenoCycler platform provides very specific *in situ* detection of the SARS-CoV-2 virus while also validating the SARS antibodies. The current analysis revealed loss of tissue structure as well as lymphocyte-rich infiltration in late-stage disease, implicating the possibility of T cells providing a protective environment against viral spread to surrounding cells.

While the current study used relatively high-level clustering with lineage markers to group cell types, further studies should include the assessment of T cell function through deeper analysis of functional marker expression. These markers could include DNA checkpoints, proliferation markers as well as their spatial orientation within tissue.

As a follow-up to the current study, the research team has collected additional specimens from 60 patients. An analysis utilizing tissues collected from multiple anatomical sites represents an opportunity to perform high-throughput analysis of smaller, focused biomarker panels using the PhenoImager™ HT (formerly Vectra® Polaris™) platform. These experiments will provide deeper insights into the functional roles of cell subsets during disease progression.

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