Spatial Profiling of COVID-19 Samples: Experimental Designs to Capture Regional Heterogeneity with the GeoMx® DSP

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Introduction
Since the COVID-19 pandemic first began, the scientific community has responded quickly to understand the disease and potential treatments. Although there is still much to be learned, the interaction of SARS-CoV-2 with the immune system and the subsequent contribution of dysfunctional immune responses to the infection seem to be the main cause of death\(^1\). Spatial profiling of COVID-19 tissues will enable researchers to investigate heterogeneities of the host response to SARS-CoV-2 infection that may be lost with bulk or single cell profiling methods and may lead to better interventions and treatments.

The GeoMx\(^\text{®}\) Digital Spatial Profiler (DSP) allows users to generate high-plex data sets with quantitative spatial resolution of RNA and protein targets using photocleavable oligonucleotide tags called DSP barcodes (FIGURE 1). In a recent publication, differences in immunoregulatory markers were uncovered with GeoMx DSP that were not identified using traditional immunohistochemical staining and whole slide analysis, underscoring the importance of spatial analysis\(^2\). For COVID-19 applications, users can quantify over 1,800 RNA targets, including COVID-19 receptors and proteases, with the GeoMx COVID-19 Immune Response Atlas, and up to 96 protein targets using GeoMx protein assays. Both GeoMx RNA and protein assays can be customized – adding up to 30 targets to the GeoMx COVID-19 Atlas, and up to 10 targets to the GeoMx protein assays, including a COVID-19 GeoMx-formatted Custom Antibody Panel available through Abcam. The high-throughput capacity of GeoMx DSP enables collecting a large cohort of patient data sets to collate molecular information of this novel coronavirus.

The GeoMx DSP works in many tissue types, including formalin-fixed paraffin-embedded (FFPE) tissues. Organs from postmortem COVID-19 patients have been banked through rapid autopsy programs and must be preserved with FFPE due to the infectious nature of the tissue.

Experimental Design
The GeoMx\(^\text{®}\) DSP relies on antibody or RNA probes coupled to photocleavable oligonucleotide tags to spatially resolve proteins or RNA in FFPE or fresh frozen samples (FIGURE 1). After hybridization of probes to slide-mounted tissue sections, the DSP barcodes are released from discrete regions of the tissue via UV exposure. Released tags are quantitated on the nCounter or on an Illumina next-generation sequencer (NGS) and counts are mapped back to tissue location, yielding a spatially-resolved digital profile of analyte abundance.

With these samples, GeoMx DSP can easily generate large data sets comprised of tissue images and corresponding molecular data from COVID-19 decedents and compare them to patients who passed away from similar infectious disease types, such as SARS-CoV (2002) and MERS-CoV (2012), or non-viral causes, to understand the SARS-CoV-2 infections.

In this white paper we will review the experimental design and data analysis process for COVID-19 research with the GeoMx DSP.

**Experimental Design**

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Once COVID-19 patient cohorts and specific samples are selected and a whole slide image with up to 4 fluorescent markers is generated, users select regions of interest (ROIs) to deeply profile the RNA and protein targets present. Using the fluorescent images, the GeoMx software can automatically generate segments within ROIs and control the path of UV light to segment them into biological compartments (e.g., segment alveolar macrophages). Broadly speaking, GeoMx DSP COVID-19 experiments tend to address three research topics: 1) the regional immune response; 2) patient to patient immune response and variability; and, 3) viral load regional heterogeneity. These topics demonstrate the essential ability of digital spatial profiling to expose heterogeneities within and between samples, including variations that may not be identified using traditional immunohistochemical staining and whole slide analysis\(^2\). **TABLE 1** summarizes the profiling strategies most suitable for each research topic.

![FIGURE 1: GeoMx DSP Workflow. DSP indexing-oligos, or DSP barcodes, from RNA targets in the GeoMx COVID-19 Immune Response Atlas are sequenced on an Illumina next-generation sequencer (NGS). DSP barcodes from protein targets in GeoMx assays are quantified on the nCounter\(^\text{®}\) platform.](image)
TABLE 1: COVID-19 research applications of GeoMx DSP.

<table>
<thead>
<tr>
<th>Research interest</th>
<th>ROI Selection</th>
<th>Notes</th>
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<tbody>
<tr>
<td>Regional immune response</td>
<td>What do heterogeneous immune responses within regions of tissue (e.g., lung) tell us about host response to SARS-CoV-2 and disease progression?</td>
<td>Geometric or Segmented ROIs (including irregular polygon shapes) Select ROIs or segment based on morphology markers (See TABLE 2 for suggestions for lung tissue regions)</td>
</tr>
<tr>
<td>Patient to patient variability</td>
<td>What can spatial profiling tell us about differences between disease severity and other patient factors? How does the host response to SARS-CoV-2 differ from the host response to other diseases? Can population-level spatial analyses of cohorts with varied disease status or outcomes lead to a better understanding of SARS-CoV-2 infection?</td>
<td>Geometric or Segmented ROIs Select ROIs based on morphology markers, as above. We recommend collecting at least 6 ROIs per patient per tissue region type.</td>
</tr>
<tr>
<td>Viral load regional heterogeneity</td>
<td>What can profiling tissue regions with different viral loads tell us about disease progression and host response?</td>
<td>Geometric ROIs RNAscope probes and fluorescent antibodies can be used to visualize viral loads. However, in our experience viral load is generally low at time of death, so observed viral load based on RNAscope or fluorescent antibodies may be low in some samples, and we recommend screening samples with a bulk assay.</td>
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Regional Immune Response

GeoMx can compartmentalize functional tissue regions and profile protein and gene expression within sub-local regions of a tissue. This is particularly important as organ tissues are highly structured with functional regions and cell types, and each compartment may respond differently to infection. A recent GeoMx study suggests that distinct lung regions have different immune responses to the SARS-CoV-2 virus².

These functional tissue regions can be selected with either geometric or segmentation ROIs (see ROI Selection below for more details). Geometric ROI selection allows for defining large tissue regions with predefined shapes or selecting any irregular polygon shape to carve out regions or cells (FIGURE 2). This is particularly useful when tissues are highly auto fluorescent or multiple cell types show positivity to a single visualization marker and those regions aren’t desired for further analysis. Segmentation allows users to separate areas of a region of interest based on one or more fluorescent morphology markers (FIGURE 3). For example, a CD45 marker can be used to segment immune infiltrates, and a CD68 marker can be used to profile macrophages in the alveolar zone (see TABLE 2 for other suggestions for visualizing lung tissue). The number of ROIs needed for statistical power depends on the scientific question(s) and how heterogeneous the tissue is. We suggest approximately ≥6 ROIs per region type (e.g., alveolar zone vs. large airway) per patient sample for biological replicates and improved statistical power. If samples are homogeneous, fewer ROIs may suffice.

Patient to Patient Immune Response and Variability

There is wide variability in patient response to the virus²–⁵, and certain factors, including age and underlying health conditions or comorbidities tend to impact the severity of COVID-19⁶–⁷. A better understanding of this variability and how the host immune response to SARS-CoV-2 infection differs between patient groups could lead to improved interventions and outcomes. Researchers may consider inter-patient variations across select samples, population-level variations, or temporal variations in animal models. To identify population-level statistical differences in immune responses between patient cohorts requires a greater number of COVID-19 postmortem samples with accompanying clinical information. As stated in the above section, if specific tissue regions are selected for each patient sample, we suggest at least 6 ROIs per region type per patient sample, although the number of ROIs should be determined based on the scientific question and the heterogeneity of the tissue. If ROIs are selected without regards to tissue region, we suggest using ≥24 ROIs per sample.

GeoMx® DSP is compatible with tissue arrays which can be used to increase throughput and conduct population-level analyses of any patient cohort (e.g., severe vs. not severe cases, diseased vs. not diseased, SARS-CoV-2 infection vs. other infectious disease). ROIs are selected on tissue arrays, just as they are selected on single tissue samples. For population-level studies, throughput can be improved by limiting the number of ROIs per sample to 4+, which should be sufficient for statistical power with a sizable patient cohort, and employing a disciplined approach to selecting regions, such as selecting 2 alveolar ROIs and 2 large airway ROIs per sample. If tissue arrays are used, the number of ROIs per patient sample may be limited based on the tissue area, but the number of patients sampled will improve power.
One example of patient to patient or population-level analyses is addressing how the dysfunctional immune response to SARS-CoV-2 contrasts to the response to other acute respiratory distress syndromes (ARDSs). Such a study would require control ARDS patient groups in addition to a COVID-19 and normal patient group to identify unique genes and pathways for COVID-19. Although causes for ARDSs are broad, ARDSs with viral and non-viral infections could both be considered. Non-viral ARDSs and normal lung tissue groups can be used to normalize gene expression of lung tissues. Viral infection-mediated ARDSs can be compared to COVID-19 patient samples to identify differences in the host immune response.

**Viral Load Regional Heterogeneity**

Research suggests that viral loads decrease in lung tissue over time from the initial SARS-CoV-2 infection, and a good portion of postmortem COVID-19 patients’ lungs show little to no viral genes although the COVID-19 patients were positive using PCR-based nasal swab tests at the time of diagnosis. Recent research suggests that varying viral loads might represent different stages of COVID-19 progression and temporal changes of the host response to SARS-CoV-2 infection as numerous studies show viral genes are not detectable after 21 days from symptom onset.

Using the GeoMx DSP, researchers can select regions of interest with varied viral loads for profiling and subsequent comparison up front, or they can classify regions based on their viral load as measured by viral probes in the GeoMx COVID-19 Atlas after ROI collection and quantitation.

Users can also use bulk assays, including nCounter® gene expression panels (e.g., the nCounter Host Response Panel with Coronavirus Panel Plus) to assess SARS-CoV-2 viral load, and select samples for spatial analysis on GeoMx DSP based on their viral load. It’s important to note that viral load may be low at time of death for those patients who were at hospital beds for over a month, so we suggest researchers screen samples ahead of time for sufficient viral load before running on GeoMx DSP. See next section for more details on using RNAscope probes to identify regions with varying viral loads.
Morphology Markers and Region of Interest Selection

Following sample selection, each sample is imaged with up to 4 fluorescent markers, including a nuclear stain, simultaneously. The whole slide images are then used to select regions of interest (ROIs) for further profiling. GeoMx® DSP offers five ROI selection strategies, of which geometric and segmentation methods are the most relevant to COVID-19 research. Geometric ROIs can be selected with pre-defined shapes (circles and rectangles) or freeform shapes can be drawn by the user using the polygon tool. Segmentation enables ROIs to be subdivided into separate areas based on their morphology marker signal. We recommend selecting a minimum of six ROIs per sample. If different biological regions of the tissue sample are selected, we suggest a minimum of four ROIs per region type (e.g., large airway and alveolar zones), and ideally ≥6 for statistical power.

In our experience, some COVID-19 lung samples, presumably at the early stages of disease progression, still have preserved alveoli and typical low cellular density (due to the sponge-like nature of healthy lungs). In samples like these, we recommend selecting ROIs that are large enough to encompass greater than 200 cells per segmented ROI for RNA analysis. This ensures that gene expression counts are above the limit of detection. For protein analyses, as few as 10 cells can be captured in an ROI for sufficient signal. In lung samples with higher cell density (e.g., with increased immune infiltrates), smaller ROIs can be selected.

NanoString offers several morphology marker kits, each with 2 fluorescent antibodies and a nuclear stain. Users can also customize their morphology markers with commercially available fluorescent markers, including RNAscope ISH probes from Advanced Cell Diagnostics (ACD). ACD offers three probes targeting the SARS-CoV-2 virus (TABLE 2) as well as a catalog of >21,000 ISH probes. The choice of morphology markers will depend on the biological question and the tissue type.

<table>
<thead>
<tr>
<th>Pulmonary Region</th>
<th>Marker Options</th>
<th>NanoString Experience</th>
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<tbody>
<tr>
<td>Large airway</td>
<td>PanCK to visualize bronchial epithelium</td>
<td>Diseased tissues tend to lose the large airway architecture, and tissue sections may not have enough large airways to capture 4+ ROIs. To increase the ROIs of this type, users can place two or more tissue sections from one patient on the slide. To capture enough cells for a strong RNA signal, users can draw a large ROI (≥660x785 µm) encompassing two large airways, and then segment the ROI based on the PanCK+ signal.</td>
</tr>
<tr>
<td>Alveolar zone</td>
<td>CD45 and CD68</td>
<td>The CD45 marker can be used to segment immune infiltrates, and the CD68 marker can be used to profile alveolar macrophages. Once ROIs in the alveolar zone are chosen, cell types with the specific fluorescence signal can be selected with segmentation.</td>
</tr>
<tr>
<td>Vascular zone</td>
<td>Extracellular matrix around lung vasculatures are highly autofluorescent</td>
<td>As shown in Figure 2, vasculatures have a large halo at the center and are surrounded by autofluorescent extracellular matrix with endothelial cells and pericytes. Users can draw a polygon around the vasculatures, and then segment the ROI using the autofluorescent signal. Another option is selecting ROIs within the alveolar zone and then excluding PanCK+ cells with segmentation. Air sacs in the alveolar zone are lined with PanCK+ type 1 and 2 pneumocytes and capillaries. Thus, capillaries (vasculatures) in the alveolar zone can be defined with PanCK exclusion.</td>
</tr>
<tr>
<td>SARS-CoV-2 virus</td>
<td>Various SARS-CoV-2 targets</td>
<td>RNAscope — ACD offers three probes targeting the SARS-CoV-2 virus: S gene encoding the spike protein, antisense strand of the S gene, and antisense strand of the orf1ab gene. Fluorescent antibodies — Novus offers an antibody against the spike protein which we’ve used successfully in IHC imaging.</td>
</tr>
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</table>

TABLE 2 provides guidance for morphology marker use in COVID-19 lung tissue. Segmenting an ROI provides a powerful way to enrich for select compartments within a tissue or for subsets of cell types (e.g., CD3E+ T cells). While the GeoMx DSP can automatically segment ROIs based on fluorescent antibodies’ signals (FIGURE 2), the punctate nature of the RNAscope signal can often limit the ability to perform auto-segmentation. However, one researcher found a case study with an abnormally high SARS-CoV-2 viral load at time of death and was able to perform auto-segmentation on the SARS-CoV-2 viral signal using RNAscope.

Whether using fluorescent antibodies or RNAscope probes for visualization, the nuclei marker, SYTO-13, should be used. If needed, auto-segmentation can be performed on the SYTO-13 signal to separate cell-containing regions from glass regions (FIGURE 3), which is especially useful in lung tissue where cell density may be low. Removing glass regions from ROIs maximizes the signal to noise.

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TABLE 2: Morphology marker option for COVID-19 research using GeoMx DSP. Here we outline some morphology markers that we have used in COVID-19 research. Users can choose from commercial GeoMx morphology marker kits or develop their own markers suitable for one of the GeoMx DSP’s four fluorescent channels.
Data Analysis

The flexibility of selecting specific ROIs using the GeoMx® DSP allows the user to ask several biologically motivated questions. Whichever targeting approach is used, the general schema for data processing involves quality control of raw data and normalization prior to downstream analyses.

Both GeoMx RNA and protein assays include controls for QC and normalization. For GeoMx protein assays, this includes negative controls, biological controls (e.g., Ms IgG1, Ms IgG2a, Rb IgG), various housekeepers, and positive control molecules. For the GeoMx COVID-19 Atlas, this includes two positive control reference genes (SF3A3 and CC2D1B) and eight negative control probes that target alien sequences identified by the external RNA Controls Consortium (ERCC).

Quality Control of Raw Data

For the GeoMx COVID-19 Immune Response Atlas, multiple probes per gene are hybridized and sequenced using Illumina next generation sequencing platforms. Within the GeoMx DSP software, PCR duplicates that arose during the sequencing preparation stage are bioinformatically removed and the number of reads per probe are returned (FIGURE 4). The use of multiple target probes allows for precise quantification in the event of RNA degradation.

For GeoMx RNA assays outlier detection methods can then be used to identify probes within a given gene that should be removed. Once outliers are removed, the remaining probe counts are typically averaged together (geometric mean) to provide a single quantity for each gene in an AOI.

For both GeoMx RNA and protein assays, a comparison of the raw data relative to the negative control molecules can reveal genes with varying signal-to-noise (SNR). There are several methods of estimating SNR and one goal of them is to identify genes that are expressed above background expression. For GeoMx studies, however, there should be extra consideration of the ROI selection, masking, and overall experimental design when using SNR as a guide for gene-based filtering. For example, if comparing samples from vastly different tissues (lung vs liver), there’s an expectation that certain genes might not be highly expressed in these different types. In another GeoMx study, one might be interested in comparing COVID-19 patient lung samples to normal healthy lung controls. In this scenario, the expression of SARS-CoV-2 viral genes may only be elevated in the COVID-19 samples. One recommendation is to make sure that, for any given gene or protein, its expression is consistently above background in a biologically-motivated subset of samples.
Normalization
There are several methods available for normalizing or standardizing data. Broadly, counts that are generated from nCounter or NGS readout need to be standardized to account for the amount of sample input, RNA quality, assay efficiency, and other technical factors. No single normalization method works for all experimental designs and downstream analyses, but it is good practice to ensure that the normalization factors are not correlated with factors of interest. For protein-based studies, comparing concordance within and between isotype controls and housekeeping genes can identify optimum normalization strategies. For more details, please see our white paper on Introduction to GeoMx Normalization: Protein. For RNA-based studies, popular normalization methods include scaling data based on: 1) the mean of the negative probes or biological controls, 2) the mean of the housekeeping genes, 3) the upper quartile of genes. Checking that the normalization factors are not confounded or correlated with the factors of your biological question are necessary to ensure meaningful spatial insight.

Statistical Analyses
Several established methods that exist for expression analyses can be used with GeoMx data and additional analyses that make use of the spatial context are emerging.

Reduced dimensionality and clustering – For many high-plex analyses, a useful first strategy is to visualize the normalized data in a reduced dimensional space to identify clusters within or across groups of interest and to uncover any annotation errors and high-level clusters/grouping (e.g., PCA, FIGURE 5A). Another useful visualization approach is to use hierarchical clustering of any genes of interest across AOIs and groups. GeoMx heatmaps (FIGURE 5B) can also be used to identify if Covid-19 samples cluster together for a suite of genes.

Differential gene or protein expression – The expression of genes and proteins can be compared between groups and results are often summarized using volcano plots where the x-axis represents the log2 fold change between groups and the y-axis shows the significance (FIGURE 5C).

There are several methods for implementing differential expression analysis and they vary by the model assumptions and the type of normalization methods used. Wang et al. 2019 highlight different approaches for count-based data. Because multiple ROIs can be selected for a given individual tissue in GeoMx, one important statistical consideration is that samples may not be independent observations so the use of mixed effects models may be warranted. As with RNAseq data, it may be more appropriate to analyze GeoMx-based RNA data with negative binomial mixed effects models if your data consist of discrete counts.
**Gene Set Enrichment Analysis (GSEA)** – Differential expression analysis above is typically done on a per gene or per protein basis. This can lead to several significantly different features and correcting for multiple hypothesis testing is preferred before drawing inferences. Genes and proteins, however, interact within networks or pathways in which small changes across many features may be found but not picked up with differential expression. GSEA is a statistical approach that examines biological pathways or sets of genes and can be implemented with GeoMx® DSP data (FIGURE 5D). The goal of GSEA is to determine if the focal set of genes occur randomly within a ranked list of all genes or if the focal set is biased towards the top or bottom of the ranked list. In other words, GSEA is used to identify whether a given gene set or functional group is differentially expressed in different phenotypes.

**Estimating Cell Abundance with the SpatialDecon Algorithm** – NanoString has developed the SpatialDecon algorithm to quantify cell populations detected in each ROI. This algorithm integrates prespecified cell type expression profiles calculated from single-cell RNA sequencing data with the GeoMx data to estimate relative cell abundances. A recent COVID-19 study employed this algorithm to investigate the distribution of immune cell types in relation to the location of the virus and found spatial heterogeneity of the immune response across cases.

**Conclusion**

The ability to perform high-plex, spatial profiling of FFPE and fresh frozen samples with the GeoMx® DSP enables researchers to gather more information from each precious sample to better understand the immune response to the SARS-CoV-2 infection. Using the GeoMx COVID-19 Immune Response Atlas and GeoMx protein assays, including the custom COVID-19 GeoMx-formatted Antibody Panel from Abcam, researchers can investigate regional heterogeneities and intra- and inter-patient variability in the host response to the infection. Recent studies with the DSP have uncovered heterogeneity within COVID-19 patient lung samples. GeoMx DSP data from COVID-19 studies is available on NCBI GEO; visit nanostring.com/COVID19 to access.

**FIGURE 5.** Example of statistical analyses to compare Control (n=20) and Treatment (n=20) cohorts. For each individual, five AOIs were sampled and 1000 genes were analyzed. A) PCA of normalized AOIs show high-level grouping based on cohort. B) Heatmap of the top 15 differentially expressed genes in the Treatment group and the top differential expressed genes in Control groups are compared. To better compare genes—which may differ in their constitutive expression levels—more directly, hierarchical clustering was performed on the Z-scores of the log2-transformed normalized data. C) Volcano plots of individual genes show a joint effect of log2 fold change and significance. D) Top pathways between Treatment and Control are identified by GSEA. Each vertical bar in the Gene ranks column shows the location of a given pathway’s associated genes relative to their fold change across all genes in the dataset. Pathways are ranked by normalized enrichment score (NES). The data presented in this figure was simulated to demonstrate GeoMx DSP capabilities without revealing any collaborator data which is in preparation for publication.


Certain Medical Conditions and Risk for Severe COVID-19 Illness | CDC. Centers for Disease Control and Prevention.

SARS-CoV-2 infection since the exposure and post symptoms onset. Lou, B. et al. 2020, medRxiv.


Introduction to GeoMx Normalization: Protein. Available at: https://www.nanostring.com/support/product-support/support-system


Comparison of normalization approaches for gene expression studies completed with high-throughput sequencing. Abbas-Aghababazadeh, F., Li, Q. & Fridley, B. L. 2018, PLOS One.


Data from GeoMx DSP COVID-19 studies are available on NCBI GEO for continued analysis. To access the GeoMx images and data from these studies, please visit nanostring.com/COVID19.