WHITEPAPER

Molecularly Guided Highly Multiplexed Spatial Profiling

with the RNAscope® and GeoMx™ Assays

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Introduction

The immune response to tumors is a complex, multifactorial interaction that is shaped by the host, the tumor, and the tumor microenvironment (TME). Interactions between tumor cells and surrounding immune cells in the TME play a key role in tumor progression and treatment response, with accumulating evidence indicating a crucial role for tumor infiltrating immune cells. Immune cells can inhibit tumor growth and progression by recognizing and attacking malignant cells, but immune cells can also promote tumor cell growth, survival, and angiogenesis. Immunotherapies have demonstrated therapeutic efficacy and durable response for several tumor types, however the majority of patients are resistant or relapse after initial response. More precise characterization of the tumor and its microenvironment with a highly multiplexed, spatial approach can provide critical insight into new immunotherapeutic strategies and identify new predictive biomarkers for stratifying patients most likely to benefit from immunotherapy. In this white paper we describe a new automated workflow that uses the RNAscope *in situ* hybridization (ISH) technology to molecularly guide the GeoMx Digital Spatial Profiler (DSP) in detecting highly multiplexed gene expression with spatial resolution.

Current Methodologies to Detect Gene Expression in the Tumor Microenvironment

Classic molecular biology methods to assess gene expression include techniques such as sequencing, PCR, and Northern blotting which, while highly sensitive, specific, and/or quantitative, lack any information on spatial positioning[1]. Assessment of the spatial distribution of proteins and RNAs in intact tissue has traditionally been done by immunohistochemistry (IHC) and ISH, respectively, however each technique has its own limitations[2,3]. IHC can be challenging due to antibody availability and does not provide information on the cellular source of molecules secreted by immune cells such as cytokines and chemokines. Traditional ISH is plagued by lack of sensitivity and specificity and can be quite time consuming to perform. Furthermore, both techniques are limited in their multiplexing and quantitation of markers by the optical nature of chromogenic and fluorescent detection chemistries.

Molecularly Guided High Plex Spatial Analysis with the RNAscope ISH & DSP Technologies

Comprehensive spatial analysis requires tools that enable higher multiplex capabilities, particularly with limited FFPE samples, without sacrificing critical positional information. The GeoMx DSP combines standard immunofluorescence techniques with digital optical barcoding technology to perform highly multiplexed, spatially resolved profiling experiments[4]. The RNAscope technology is an advanced *in situ* hybridization assay that allows for the visualization of single-molecule gene expression directly in intact tissues with single cell resolution [5,6]. To precisely characterize the cells in a complex, heterogeneous tissue, a new workflow has been developed to combine GeoMx DSP with the RNAscope technology (**FIGURE 1**).

Combining the single molecule and single cell capabilities of the RNAscope ISH assay with the highly multiplexed spatial profiling capabilities of the GeoMx assay allows researchers to molecularly guide their high plex spatial analyses with single cell resolution to discrete, focused regions of interest. Samples are incubated with up to three RNAscope probes, a nuclear dye, and a cocktail of 96+ GeoMx ISH probes conjugated to photocleavable indexing oligonucleotides for high plex RNA analysis (**FIGURE 2**). Alternatively, serial sections can be incubated with up to 96 oligonucleotide-conjugated antibodies for protein analysis. GeoMx DSP performs whole slide imaging of the RNAscope probes to capture high resolution tissue morphology and enable selection of regions of interest (ROI) for high plex profiling. The RNAscope ISH assay employs a unique signal amplification strategy that allows for the visualization of target RNAs as punctate dots, where each dot represents an individual RNA molecule, with single cell resolution. The key benefits of the RNAscope assay are high sensitivity due to its signal amplification strategy, high specificity as a result of the RNAscope probe design minimizing nonspecific off-target signals, and detection and quantification of RNA with spatial and morphological context at the single cell level. Once ROI have been selected, oligonucleotide tags are released from discrete regions of the tissue via UV exposure on GeoMx DSP. Released tags are collected and quantitated in a standard nCounter® assay via hybridization to digital barcodes and counts are mapped back to tissue location, yielding a spatially-resolved digital profile of analyte abundance. Specific GeoMx RNA results can also be confirmed using the RNAscope assay at single cell resolution post-processing. With a streamlined, automated workflow performed on Leica

FIGURE 1: Workflow summary.

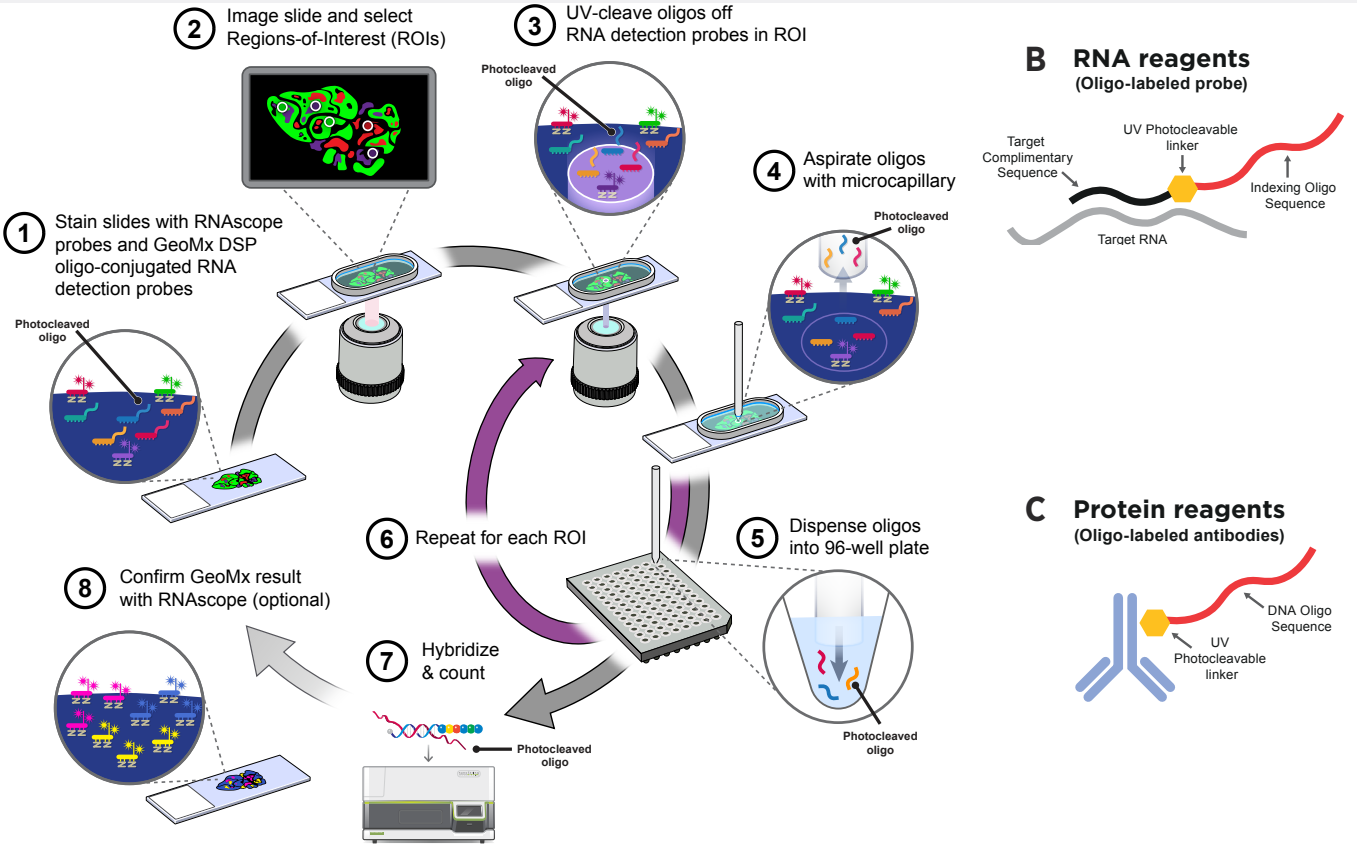

FIGURE 2: Schematic depicting the detailed workflow for molecularly guided high plex spatial analysis. (A) RNAscope molecularly guides GeoMx DSP ROI selection for RNA analysis and confirms GeoMx data on the same slide. (B) For high plex GeoMx DSP profiling of (B) RNA or (C) Protein, ISH probes or antibodies, respectively, are conjugated to indexing oligos via a photocleavable linker to enable *ex situ* quantification.

FIGURE 3: Automated workflow overview.

Biosystems' automated stainers, the GeoMx DSP, and nCounter, one can perform high plex spatial analyses in a standardized, quantitative manner with minimized inter-user variability and hands-on time, allowing for consistently reproducible results (**FIGURE 3**).

The ability to perform molecularly guided spatial profiling experiments increases the likelihood of capturing rare events often missed by typical grind and bind assays. The GeoMx assays combined with the RNAscope technology provides spatially resolved, high plex profiling of RNA targets in a single FFPE section, for deep characterization of precious samples with robust imaging and profiling data.

RNAscope ISH & GeoMx DSP Performance

Before slides are loaded onto the GeoMx DSP instrument, samples must be labeled with fluorescent markers for the identification of ROI. To show that a workflow combining RNAscope molecularly guided visualization and GeoMx DSP profiling would be feasible, we confirmed that both probe types and corresponding staining protocols are compatible (**FIGURE 4**). We first tested if the addition of GeoMx RNA probes after RNAscope visualization impacted RNAscope signal. For this, we stained RAJI FFPE cell pellets with a set of housekeeper control probes or a set of negative control probes across three channels. On control FFPE cell pellets, we found that the addition of GeoMx RNA probes after RNAscope ISH had no impact on RNAscope signal. Similarly, we found that RNAscope ISH had no significant impact on data obtained from GeoMx DSP profiling. In all, these data suggest that the two staining protocols can be used simultaneously, on the same section, with no apparent negative impact on performance of either assay.

Next, concordance between GeoMx DSP and RNAscope ISH data collected from the same tissue sections was tested. Tonsil (**FIGURE 5A**) and non-small cell lung cancer (NSCLC), **FIGURE 5B**) tissues were utilized for this study. The RNAscope LS Multiplex Fluorescent Kit (Cat. No. 322800) using probes targeting *CD19* (B cells) and *CD3E* (T cells) was visualized using either TSA Plus Cyanine 3 or TSA Plus Cyanine 5 System (Perkin Elmer). After visualization of probes with RNAscope reagents, slides were incubated with a cocktail GeoMx RNA detection reagents for analysis on the GeoMx DSP system. Twelve ROI were analyzed on both tissue types. GeoMx DSP counts were quantified with the nCounter system and RNAscope signal intensities for these ROIs was quantified using ImageJ. Biological correlation was tested between 1) targets that

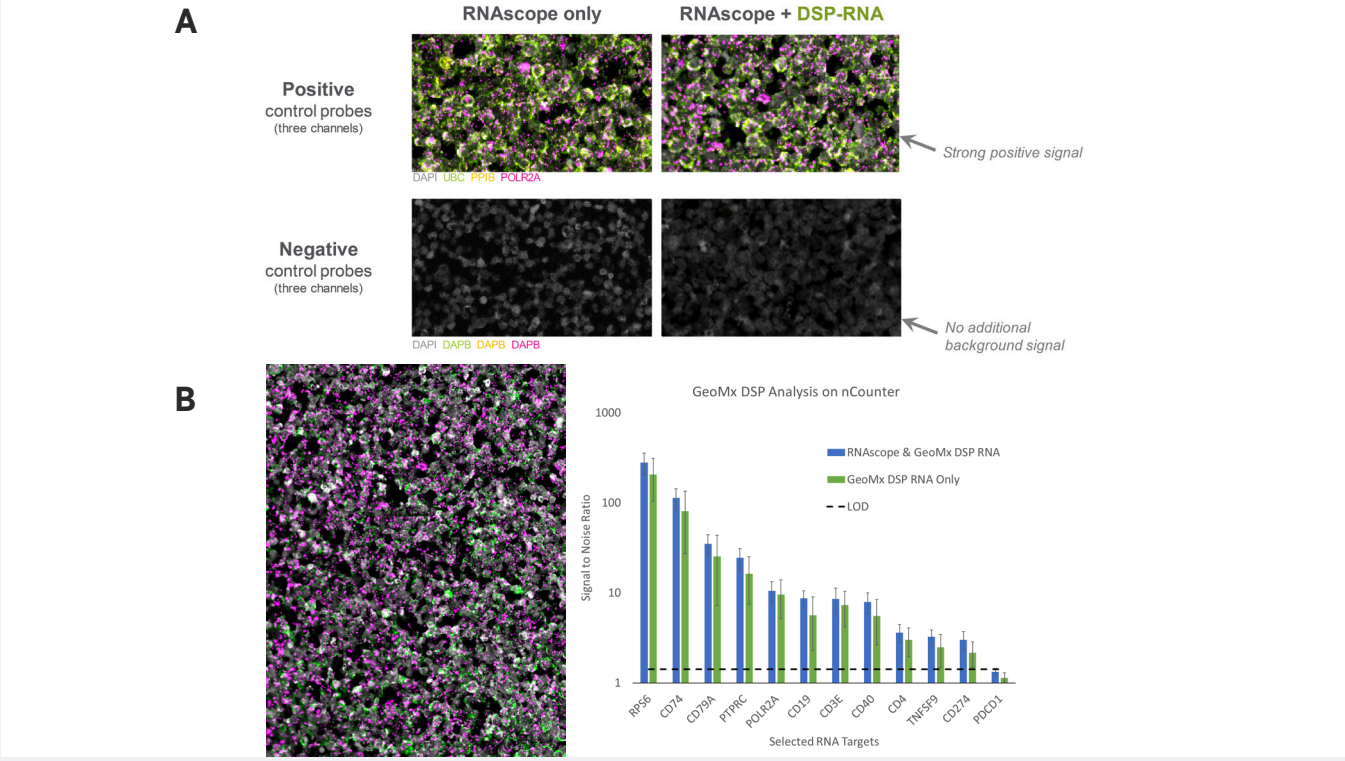

FIGURE 4: Compatibility of RNAscope ISH assay and GeoMx DSP profiling. (A) RNAscope signal is unaltered with the addition of the GeoMx RNA assay and (B) GeoMx RNA signal is unaltered with the addition of the RNAscope assay..

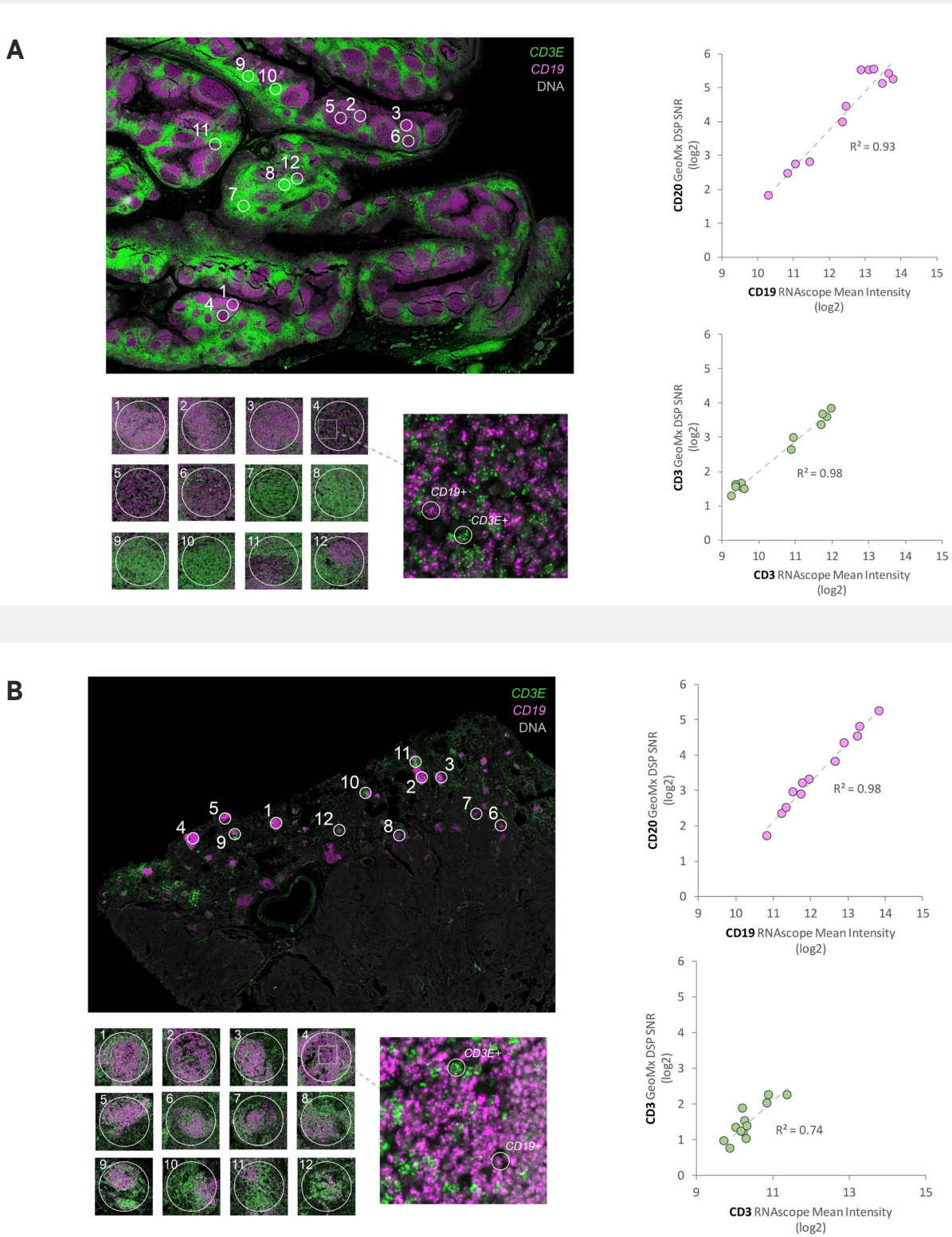

FIGURE 5: Correlation between RNAscope ISH assay and GeoMx DSP results. (A) Tonsil. (B) NSCLC.

are expected to have identical profiles but have probe sequences that would have little chance interacting because of distinct RNA sequences (*CD19* for RNAscope and *CD20* for GeoMx DSP) and 2) identical targets with probes sequence that target overlapping sequences (*CD3E* for both RNAscope and GeoMx DSP). We found that both probe configurations were compatible with the GeoMx DSP protocol with robust signal from both RNAscope and GeoMx DSP even when probe targeting sequences overlapped (*CD3E*). Concordance was generally high for both *CD19/CD20* and *CD3E* for both tissue types. Concordance was lower for *CD3E* in NSCLC, likely due to the relatively low expression levels seen for *CD3E* across the ROI analyzed. In all, these data suggest that high-quality RNAscope and GeoMX DSP signal quantification can be obtained on the same section with this protocol.

For our final experiment, the full automated workflow cycle was tested using the RNAscope assay for ROI selection, GeoMx DSP results obtained from RNAscope guided ROI, and the RNAscope assay for confirmation, at the single cell level, of the GeoMx DSP results on serial sections in NSCLC (**FIGURE 6**). Two ROI from NSCLC tissue are shown to highlight these results (**FIGURE 6A**). One ROI was enriched for *CD19* (B cells), while the other ROI was enriched for *CD3E* (T cells). As expected, GeoMx detected higher *CD20* counts for the *CD19*-enriched ROI, along with counts for other markers preferentially expressed in B cells (**FIGURE 6B**). Similarly, for the *CD3E*-enriched ROI, enrichment of *CD3E* and other transcripts preferentially expressed in T cells were observed via GeoMx DSP analysis (**FIGURE 6B**).

We confirmed the differentially expressed genes identified by GeoMx DSP in the *CD3E*-enriched ROI using the RNAscope Multiplex Fluorescent assay on serial sections. We confirmed the expression of immunoregulatory molecules such as *CTLA4*, *PD-L1, PD-1* and *ICOSLG* that are important therapeutic targets for NSCLC and other malignancies (**FIGURE 7A**). While these targets were detected in both ROI, the T cell-enriched ROI demonstrated significantly higher expression of these immune checkpoint markers. To determine the activation state of these immune cells we compared the two ROI and observed a significantly increased inflammatory signature in the T cell-enriched ROI compared to the B cell-enriched ROI, as demonstrated by elevated levels of cytokines and chemokines such as *CCL5*, *CXCL9*, and *IFNG* (**FIGURE 7B**).

Summary

In this white paper we present a robust workflow that overcomes the historical limitations of ISH and IHC by combining high-resolution imaging with high plex profiling. The work described here demonstrates compatibility of the RNAscope ISH and GeoMx

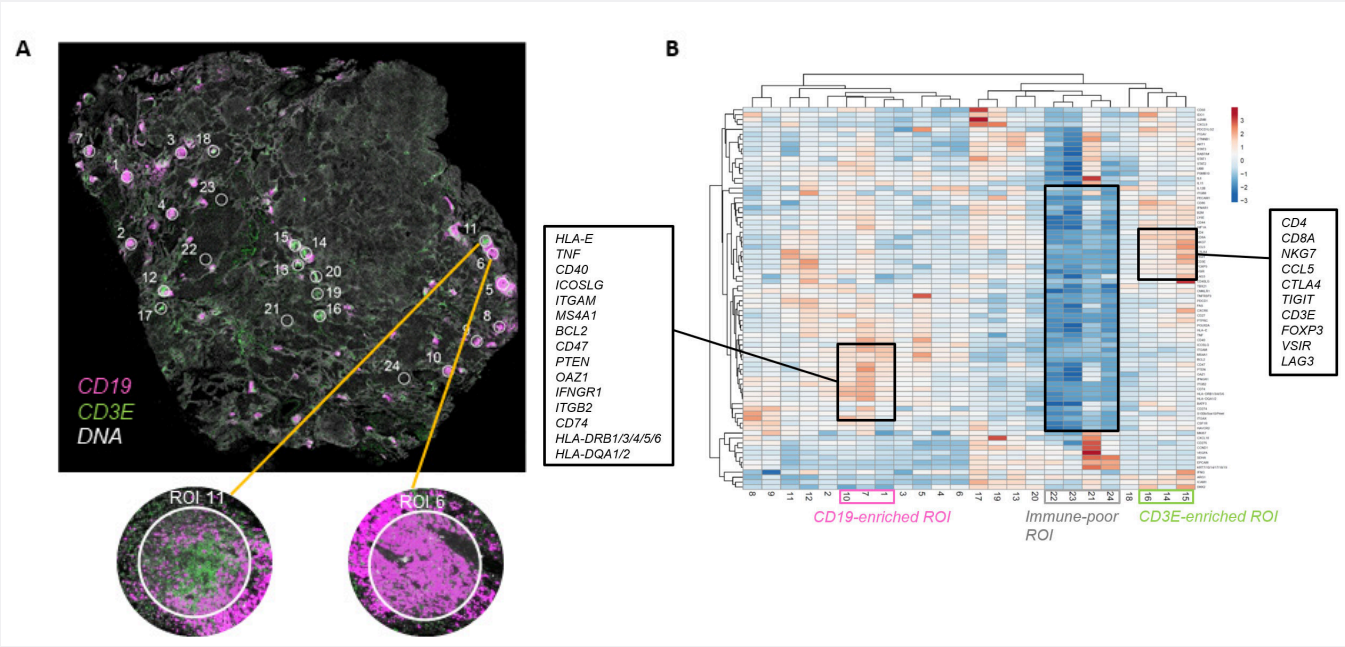

FIGURE 6 High plex spatial analysis with the RNAscope ISH and GeoMx DSP assays in NSCLC tumor sample. (A) *CD19* and *CD3E* positive ROI were profiled by GeoMx DSP for high plex RNA analysis. ROI11 is an example of a *CD3E*-enriched ROI and ROI6 is an example of a *CD19*-enriched ROI. (B) Unsupervised hierarchically clustered heatmap of all ROI and targets analyzed in this section. Specific clusters are highlighted for *CD19*-enriched, *CD3*-enriched, and immune-poor ROI. *CD19* and *CD3*-enriched ROI show enrichment of expected target, while immune-poor ROI show wide-ranging under-expression of this immune content-focused panel.

DSP workflows for molecularly guided high plex RNA profiling with spatial resolution.

The combined RNAscope ISH and GeoMx DSP workflow is now available via NanoString's DSP Technology Access Program (TAP) (www.nanostring.com/DSPTAP) prior to commercialization of the GeoMx DSP instrument (**FIGURE 8**). FFPE samples of interest are sent to NanoString for processing. RNAscope probes are selected (https://acdbio.com/all-about-probes) for ROI selection and optional post-processing confirmation of RNA results. Molecular guides for ROI selection may include cell type markers such as *CD3* and *CD4*, and immune checkpoint markers such as *PD-L1* and *CTLA4*. ROI modalities available in TAP include geometric profiling for assessment of tissue heterogeneity, segment profiling to maximize cellularity and profile distinct biological compartments based on tissue morphology, or contour profiling for analysis of the impact of distance on the expression profile. For high plex profiling, currently available content includes up to 96 RNA targets to characterize the global immune response, investigate microenvironment immune activity, quantify tumor reactivity, and evaluate the tumor inflammation signature[7]. Protein content covering key immuno-oncology or neuroinflammation and neurodegeneration targets is also available. Samples are processed by NanoString scientists and data is delivered in a publication ready format.

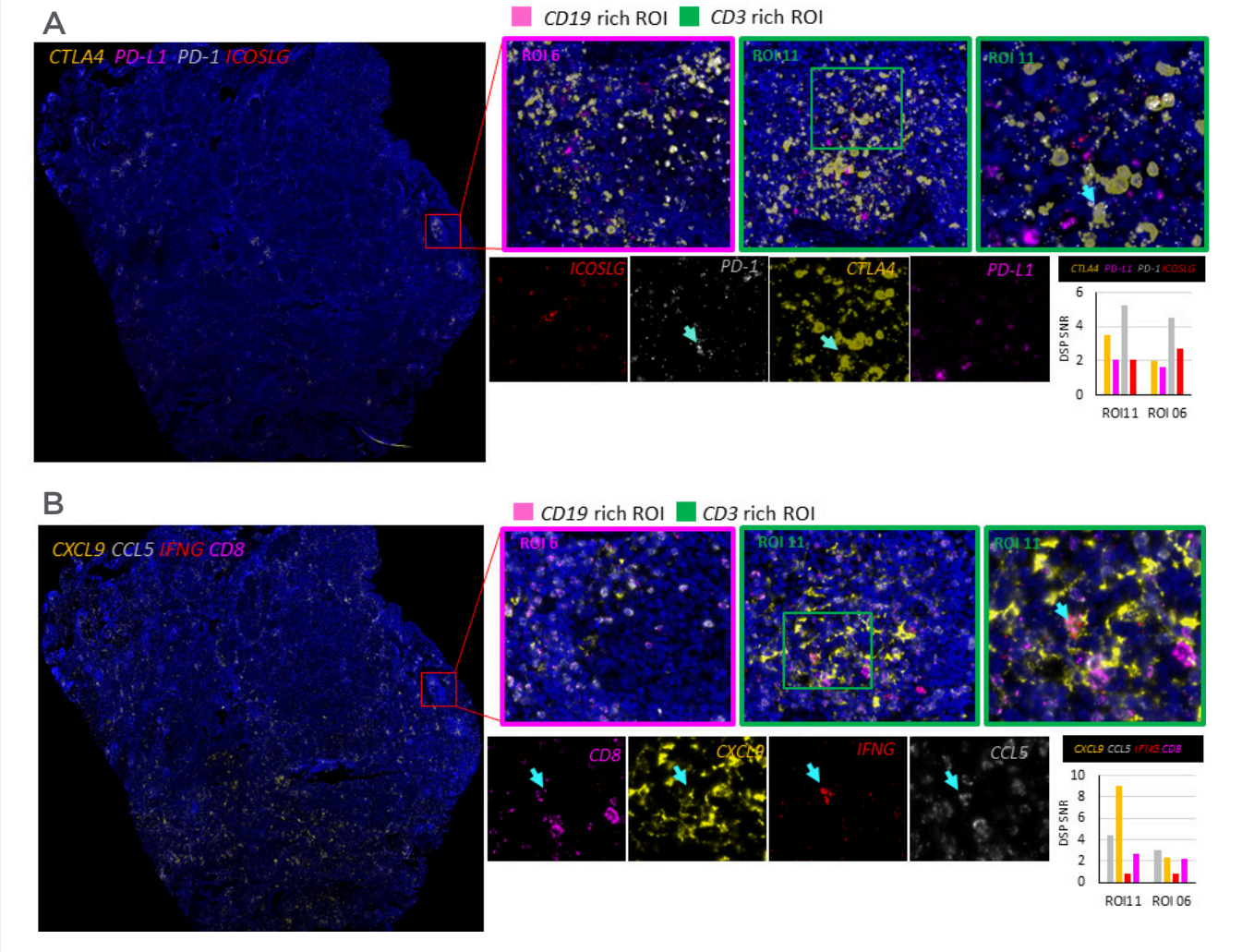

FIGURE 7: Confirmation of high plex GeoMx DSP analysis using the RNAscope ISH assay in NSCLC tumor sample. (A) Checkpoint markers such as *CTLA4* , *PD-L1, PD-1* and *ICOSLG* were expressed at a higher level in the *CD3*-enriched ROI compared to the *CD19*-enriched ROI. Blue arrow indicates a *CTLA4+/PD1+* cell. Graph indicates GeoMx DSP expression data for *CTLA4, PD-L1, PD1* and *ICOSLG*. (B) Inflammatory chemokines such as *CXCL9* and *CCL5* and cytokine, *IFNG* were expressed at a significantly higher level in the *CD3*-enriched ROI compared to the *CD19*-enriched ROI. Blue arrow indicates a *CCL5+/CXCL9+/IFNG+/CD8+* T cell. Graph indicates GeoMx DSP expression data for *CXCL9, CCL5, IFNG* and *CD8*. SNR, Signal to noise ratio.

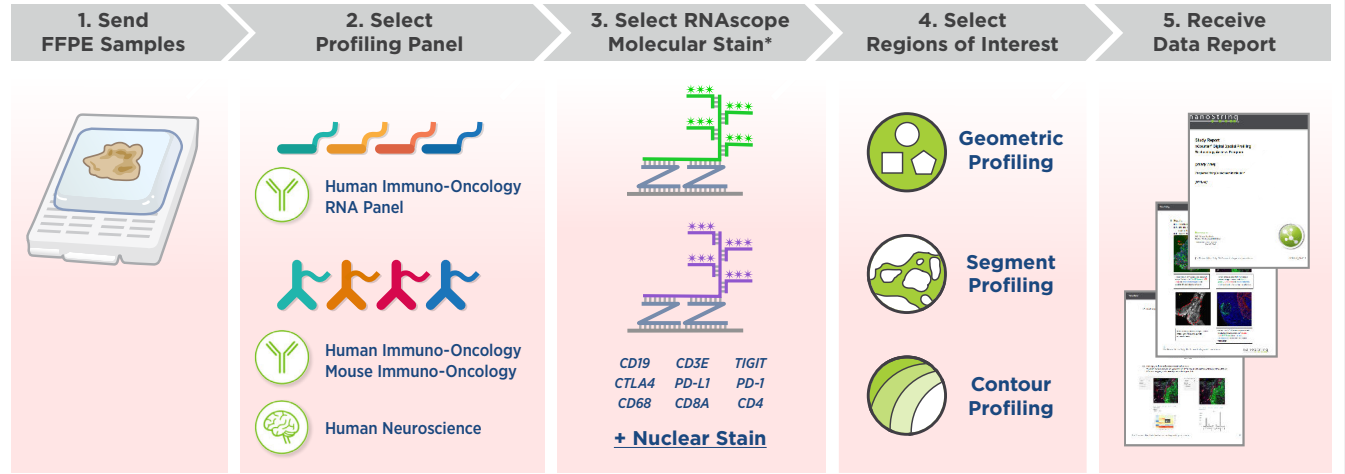

FIGURE 8: DSP Technology Access Program overview.

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