APPLICATION NOTE

SPATIAL PROFILING OF IMMUNE CELL GENE SIGNATURES IN THE TUMOR MICROENVIRONMENT

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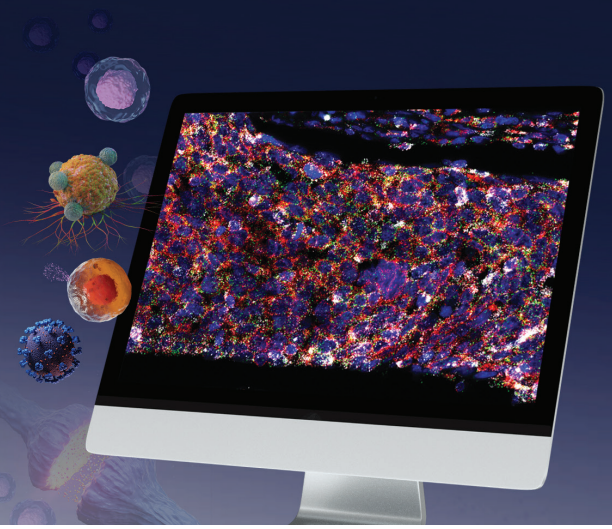


INTRODUCTION

Complex tissues with high cellular heterogeneity require single cell technologies both at the transcriptomic and spatial level to fully interrogate the cell types within them. Simultaneous detection of multiple markers is critical to obtain meaningful information from patient samples using single cell platforms. Single cell technologies enable precise analysis of heterogenous tissues to elucidate key therapeutic targets and biomarkers. In addition, studying target gene expression with spatial context is vital for understanding the cellular organization and function within the tissue. Recognizing the significance of spatial biology, *Nature Methods* declared spatially resolved transcriptomics as the method of the year in 2020[1].

RNAscope™ *in situ* hybridization (ISH) technology has been used for single molecule detection of RNA transcripts at single cell and sub-cellular resolution in the tissue. The proprietary "double Z" probe design in combination with the advanced signal amplification enables highly specific and sensitive detection of the target RNA[2]. Over the years, the RNAscope technology has evolved to encompass the detection of short RNA targets and splice variants, miRNAs and more recently, DNA targets.

This document demonstrates the capabilities of a multiplexed *in situ* transcriptomic approach for the spatial mapping of target genes in highly complex and heterogenous FFPE tumor tissues using the RNAscope HiPlex v2 assay.

PROFILING THE TUMOR MICROENVIRONMENT

The tumor microenvironment (TME) is a network of complex interactions between the tumor cells, immune cells, endothelial cells, fibroblasts, and the surrounding extracellular matrix. Immunotherapies including immune checkpoint blockade have demonstrated therapeutic efficacy and durable responses for several tumor types, however most patients are either nonresponsive or develop resistance to immunotherapies[3]. For developing next generation immunotherapies, it is important to identify reliable biomarkers for predicting treatment response. Correlating immune cell population and their activation states in treated tumors to the therapeutic response and clinical outcome can be one strategy[4]. Evaluating gene signatures of the tumor cells and infiltrating immune cells can provide prognostic and predictive information. The RNAscope ISH assays have demonstrated successful visualization and spatial mapping of immune cell markers , secreted cytokines[5,6] and tumor markers[7] within the TME.

Comprehensive spatial analysis is best accomplished by multiplexing solutions that allow simultaneous visualization of the target genes in the same sample.

THE RNAscope™ HiPlex v2 ASSAY FOR SINGLE CELL AND SPATIAL TRANSCRIPTOMIC PROFILING IN FFPE TUMOR TISSUES

The RNAscope HiPlex v2 assay is capable of multiplex fluorescent detection of up to 12 targets in fresh/fixed frozen and FFPE tissues. This assay has been especially optimized for detection of target RNA in FFPE samples. It is challenging to detect clean signal due to the autofluorescence observed in FFPE samples. By introducing the FFPE reagent as a part of the HiPlex v2 assay, RNA signal can be clearly visualized to assess target gene expression.

After a series of highly effective and specific signal amplifications, the signal is detected iteratively, where up to four target genes are visualized in four distinct fluorescent channels. Similar to previous RNAscope assays, a single RNA transcript can be visualized as a punctate dot. The assay uniquely employs the cleavable versions of different fluorophores, and the iterative process is to be repeated until all 12 targets are detected. The fluorophore cleaving procedure is rapid with minimal to no effect on RNA and tissue morphology. Finally, the images from various rounds can be merged using RNAscope™ HiPlex Image Registration Software v2 (FIGURE 1). This software allows the user to toggle and overlay expression of only select targets thereby making data interpretation very simple.

KEY FEATURES OF THE HIPLEX v2 ASSAY:

- Simultaneous detection of 12 RNA targets in FFPE tissues
- Spatial mapping and validation of novel cellular subtypes in complex tissues
- Single cell and single molecule resolution
- Easy data interpretation using an exclusive image registration software

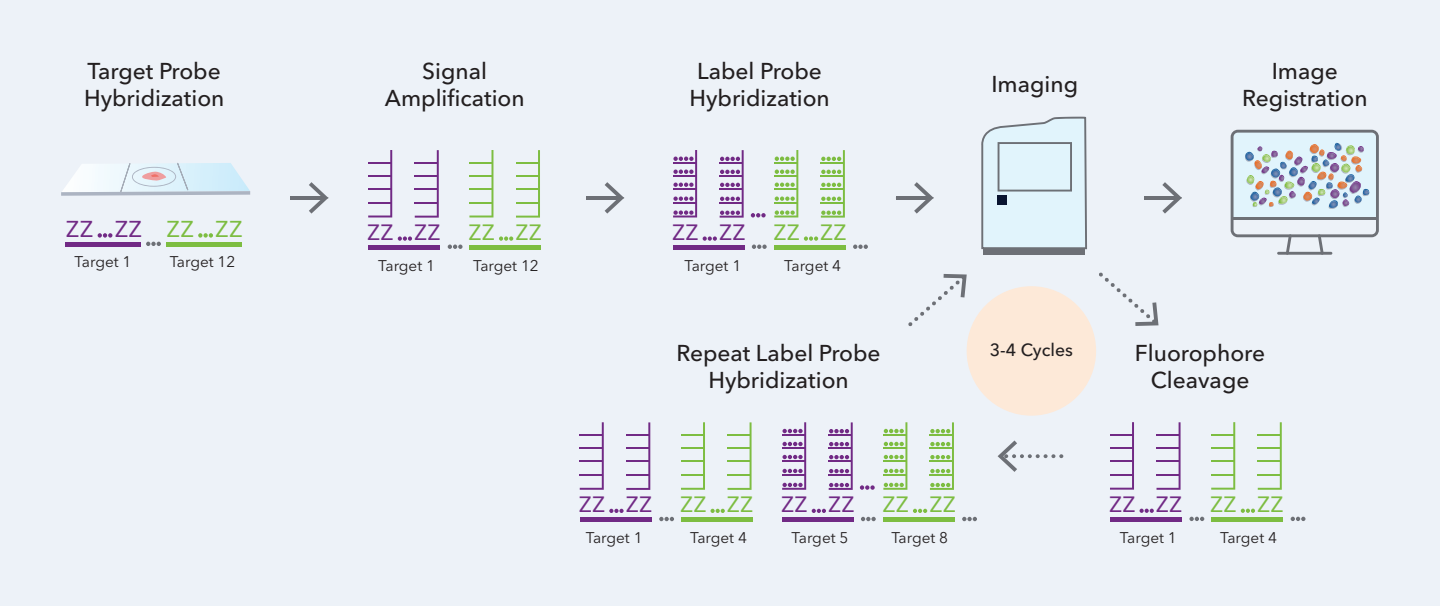


FIGURE 1. RNAscope HiPlex assay provides signal amplification for simultaneous visualization of up to 12 RNA targets in FFPE samples by performing iterative fluorescent imaging rounds.

HiPlex ASSAY ENABLES VISUALIZATION OF ACTIVE IMMUNE CELLS, CYTOKINES AND CHEMOKINES WITHIN THE TME

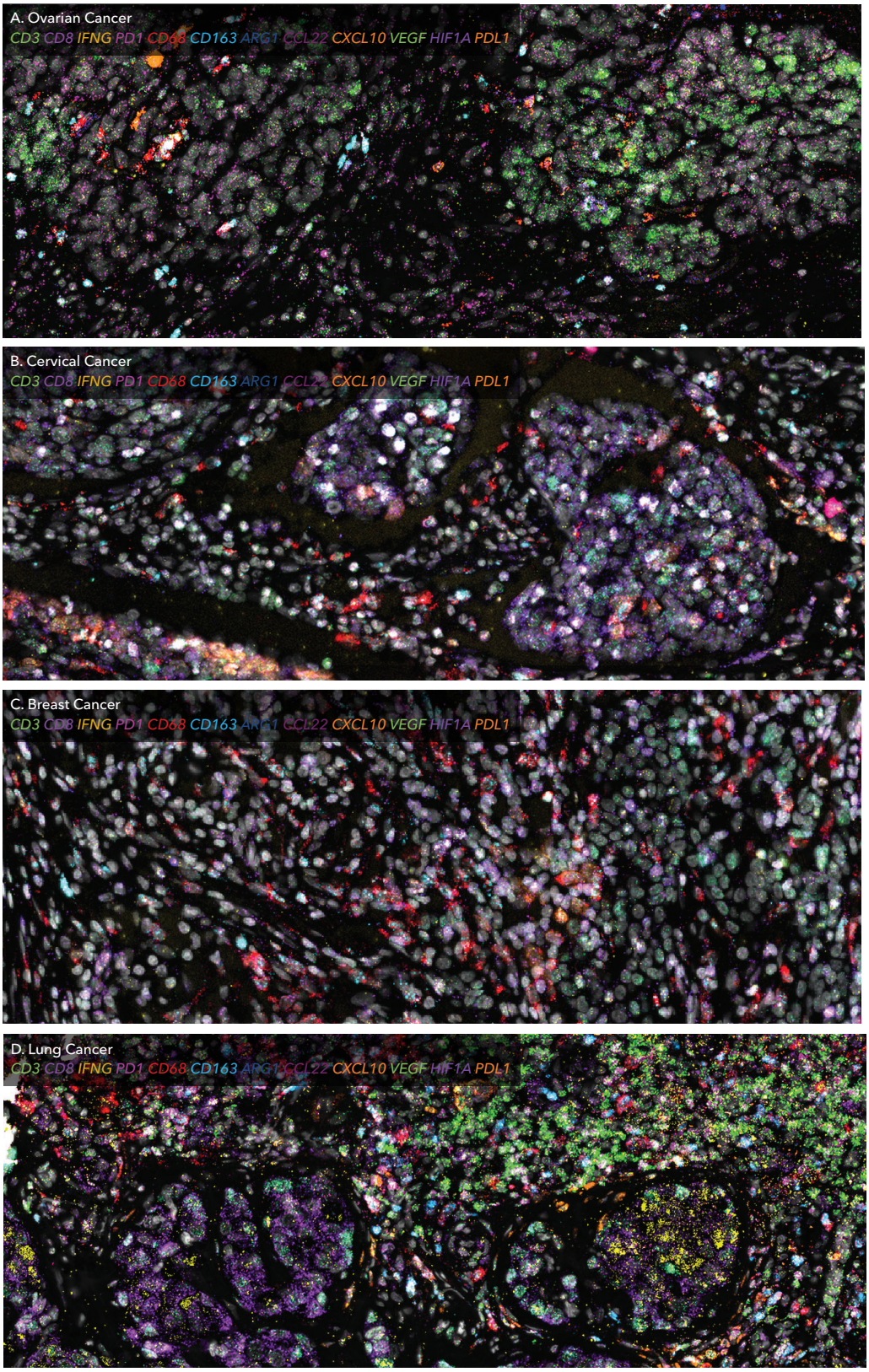
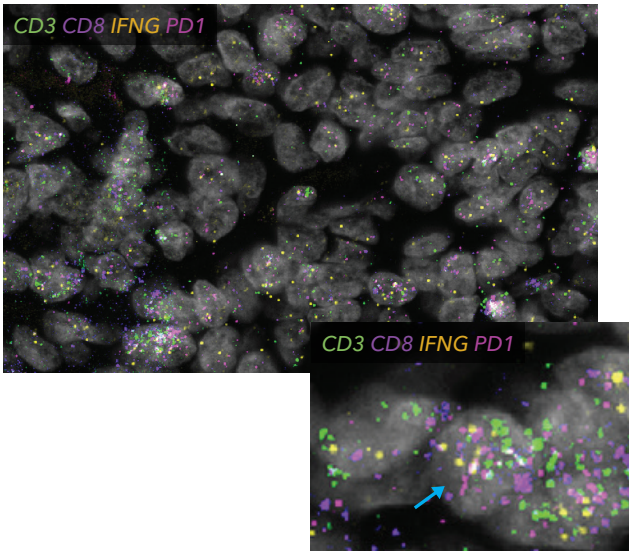
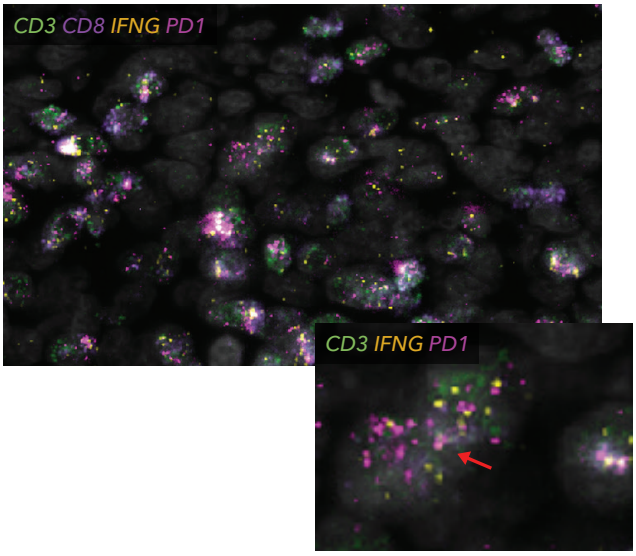


FIGURE 2. Profiling different cell types within the TME. 12 target specific marker probes were used to detect immune cells, tumor cells, chemokines and cytokines in (**A**) ovarian cancer, (**B**), cervical cancer, (**C**), breast cancer and (**D**), lung cancer. Nuclei stained with DAPI (gray).

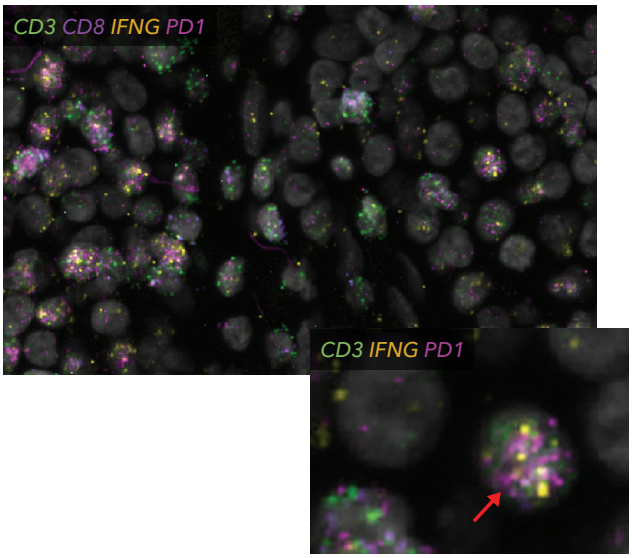
A. Ovarian Cancer



B. Cervical Cancer



C. Breast Cancer



D. Lung Cancer

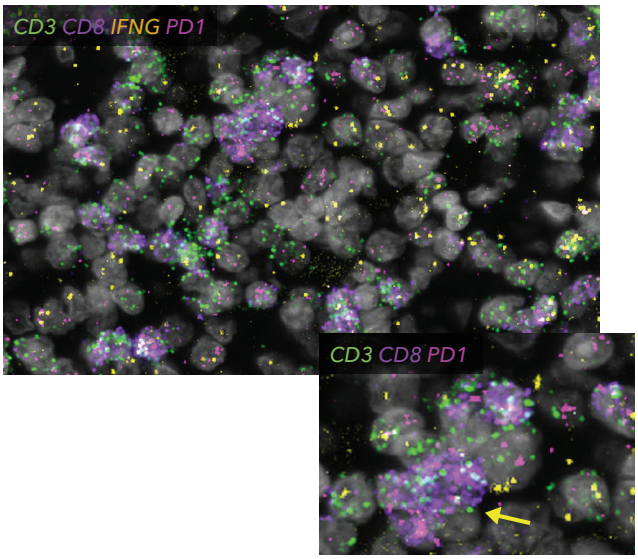


FIGURE 3. Detecting infiltrated T cells subsets within tumors. Target probes for *CD3*, *CD8*, *IFNG* and *PD1* were used to identify different subset of T cells in (**A**), ovarian cancer, (**B**), cervical cancer, (**C**), breast cancer, (**D**), lung cancer. *CD3+/CD8+/PD1+/IFNG+* cytotoxic T cells (↑) *CD3+/PD1+/IFNG+* T cells (↑) and *CD3+/CD8+/PD1+* T cells (↑). Nuclei stained with DAPI (gray).

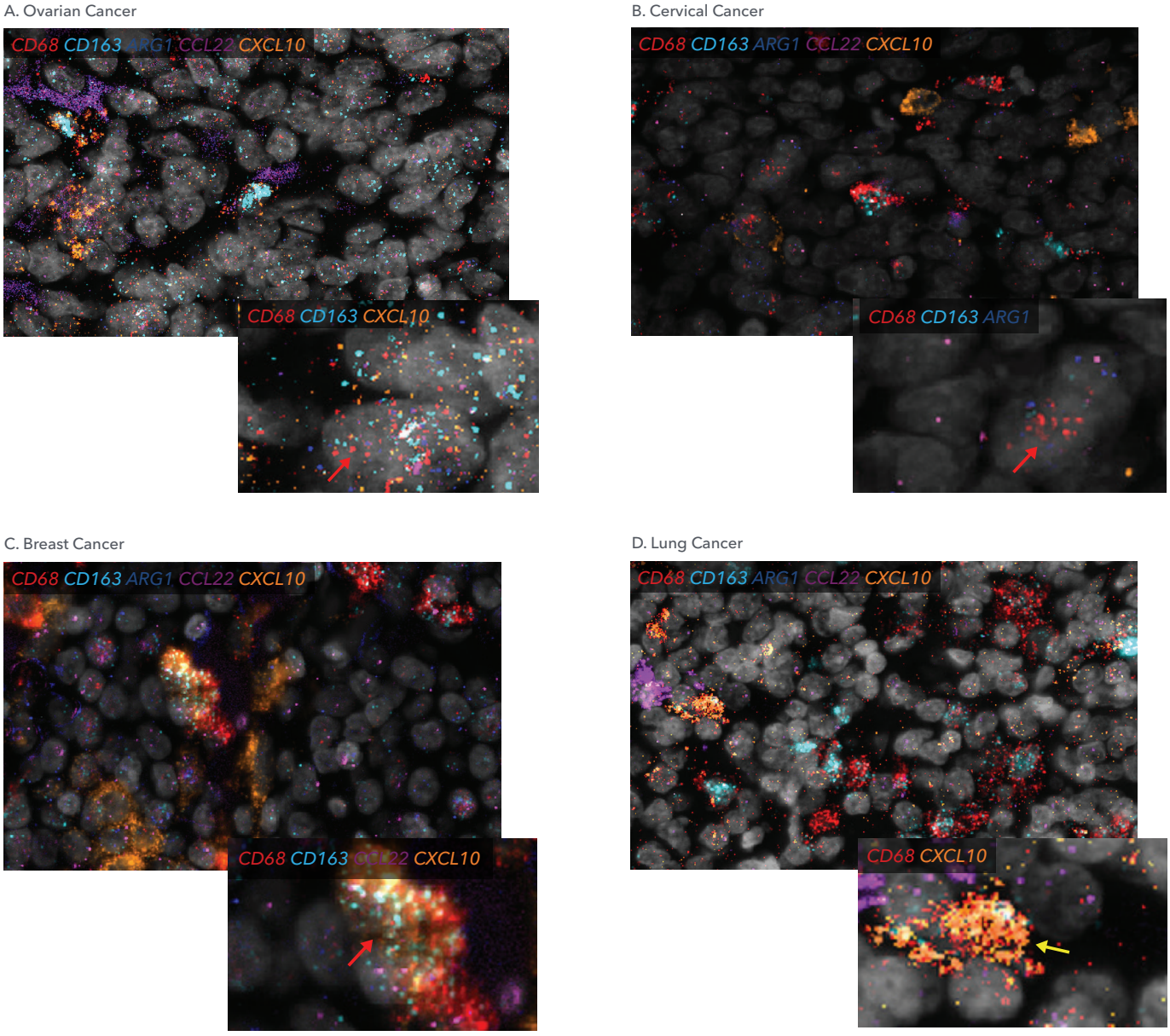
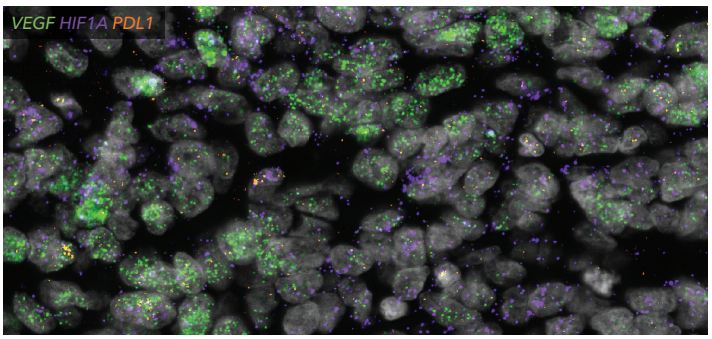


FIGURE 4. Detecting macrophages markers, chemokines and cytokines within tumors. Target probes for *CD68, CD163, ARG1 CCL22, CXCL10* were used to detect macrophages and visualize the cellular source of secreted factors in (**A**), ovarian cancer, (**B**), cervical cancer, (**C**), breast cancer, (**D**), lung cancer. *CD68+/ CD163+/ ARG1+/ CCL22+/ CXCL10+* , *CD68+/ CD163+/ CCL22+/ CXCL10+* , *CD68+/ CD163+/ ARG1+* M2 macrophages (↑) and CD68+/ CXCL10+ M1 macrophages were detected (↑). Nuclei stained with DAPI (gray).

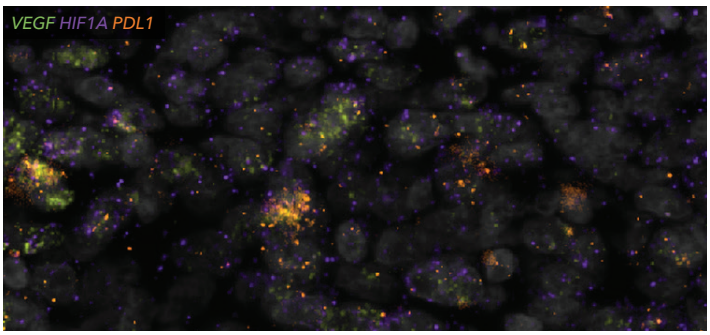
To demonstrate the utility of the HiPlex assay in profiling the TME, target probes were used to identify specific immune cell populations, secreted cytokines, immunosuppression markers etc., within breast cancer, cervical cancer, lung cancer and ovarian cancer tissues (FIGURE 2). Using T cell marker probes such as *CD3*, *CD8* and *PD1*, we were able to detect infiltrating T cells within these tumor samples. Additionally, we determined the activation states of CD8+ cytotoxic T cells by visualizing the expression of *IFNG* (FIGURE 3). Furthermore, infiltrating macrophages were detected by using a combination of markers such as *CD68*, *CD163*, *ARG1* and *CXCL10* (FIGURE 4). Intratumoral macrophages secreting CXCL10 are important in augmenting the anti-tumor immune response by increasing CD8+ T lymphocyte infiltration. Studies have also revealed that certain immune checkpoint inhibitor (ICI) therapies result in upregulation of CXCL9 /CXCL10 chemokines and these elevated levels are associated with better clinical response[8].

DETECTION OF IMMUNOSUPPRESSIVE SIGNATURES WITHIN TUMOR SAMPLES

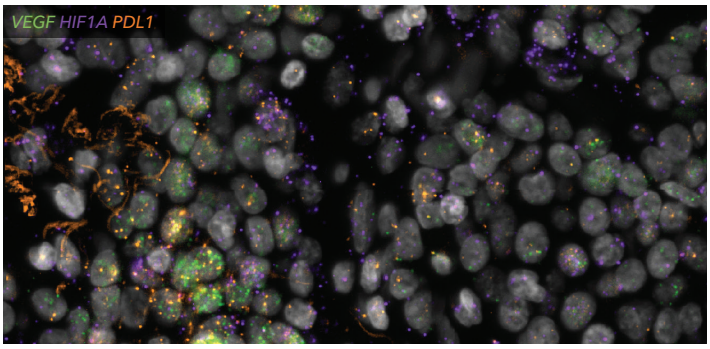
A. Ovarian Cancer



B. Cervical Cancer



C. Breast Cancer



D. Lung Cancer

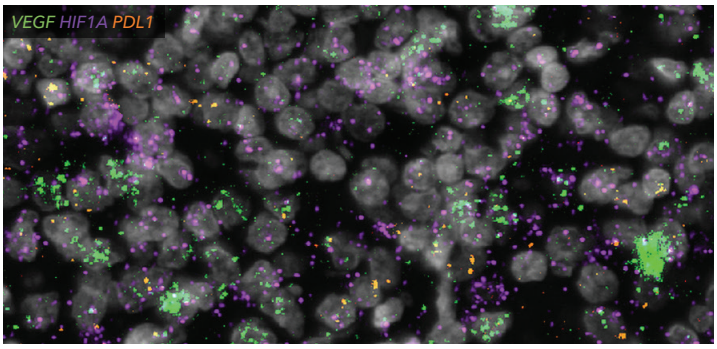


FIGURE 5. Detecting hypoxia markers in the tumor microenvironment. Level of hypoxia was assessed in different tumor samples (**A-D**), by detecting the expression of *HIF1A*, *VEGF* and *PD-L1*. Nuclei stained with DAPI (gray).

Accumulation of regulatory T cells (Tregs) in the tumor can lead to suppression of CD8+ cytotoxic T cell activity. Chemokines such as CCL22 secreted by tumor cells and tumor-associated macrophages bind to the CCR4 receptors on Treg cells and increase their recruitment into the tumor[9]. *CCL22* expression was detected in all four tumor types using a target-specific probe (**FIGURE 4**). Immunosuppressive conditions can also result from increased hypoxia within the tumors. Proliferating cancer cells can cause reduced oxygen levels in the tumor, creating an inhospitable environment for immune cells leading to reduced anti-tumor immunity. Hypoxic conditions cause upregulation of transcription factors such as HIF1-α that regulate angiogenesis by promoting VEGF expression and also cause increased expression of PD-L1 in tumor and immune cells.[10,11]. Spatial analysis of these tumors revealed that *HIF1A* and *VEGF* expression was strong in all four samples (**FIGURE 5**).

Visualizing and spatially mapping different cellular subtypes within the TME is easily enabled by the RNAscope HiPlex v2 assay. Using the image registration software, we were able to overlay the expression of all 12 targets or simultaneously visualize only a select few genes at a time to identify sub-populations of immune cells such as T cells and macrophages. To ensure good staining and quality images, we recommend using our positive and negative control probes to confirm the RNA quality is preserved and determine if the standard assay conditions are the best for target detection. The positive control probe cocktail for the RNAscope HiPlex assay uses a set of target genes that are ubiquitously expressed in most mammalian tissues (**FIGURE 6**). Researchers interested in cell segmentation can use our pre-labeled 18s rRNA probe to assist in signal quantification.

SUMMARY

In this document we have demonstrated detection of 12 target RNA markers on same sections of four different tumor tissues to characterize specific regions of interest. Using the RNAscope HiPlex v2 assay we were able to successfully profile the TME to identify immune cell sub-types and assess the inflammatory signature within each tumor sample. This assay is also a powerful tool for single cell sequencing validation to reveal spatial organization of cells and provide deeper insight into the function and phenotype of novel cell populations. By expanding the capability to FFPE tissues, the RNAscope HiPlex v2 assay provides a highly resolved spatial multiplexing solution to interrogate complex tissues.

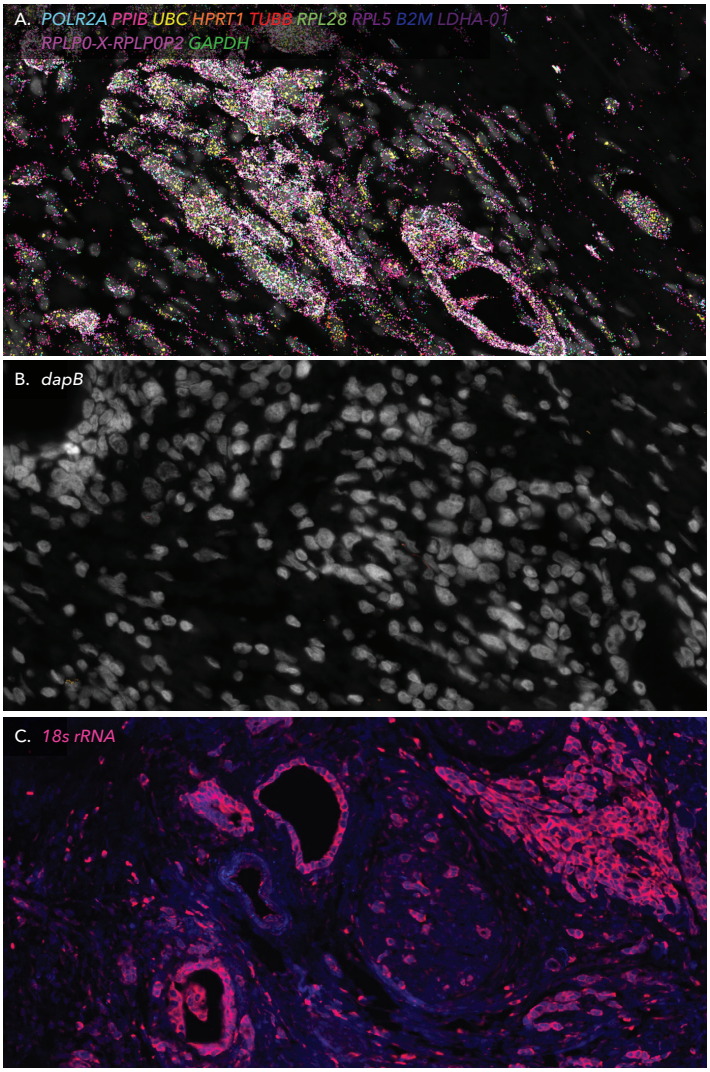


FIGURE 6. Researchers interested in cell segmentation can use our pre-labeled 18s rRNA probe to assist in signal quantification . (**A**), RNAscope HiPlex 12-plex positive control target detection, (**B**) No signal detected in any channel with the RNAscope HiPlex negative control probe, (**C**) Detection of 18s rRNA as a cell segmentation marker. Nuclei stained with DAPI (gray).

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