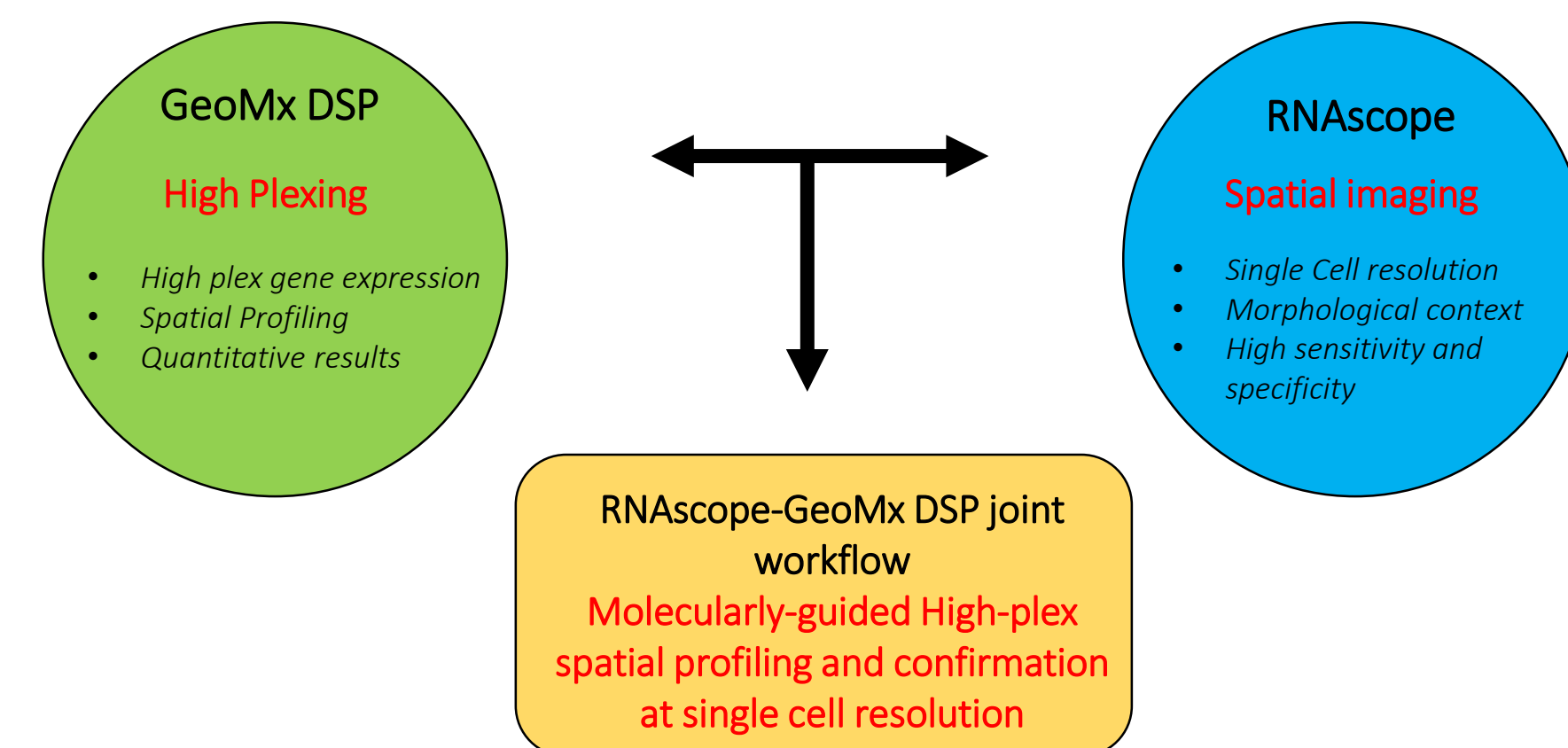


Introduction

The tumor microenvironment (TME) is a network of complex interactions between the tumor and surrounding immune and non-immune cells. Immunotherapies including immune checkpoint blockade have demonstrated therapeutic efficacy and durable responses for several tumor types, however most patients are nonresponsive or develop resistance to such immunotherapies. To identify new predictive biomarkers to better stratify patients, it is essential to comprehensively characterize the immune cells within the TME at the molecular level. Traditional methods to assess gene expression, such as sequencing, PCR, immunohistochemistry, and in situ hybridization (ISH) either lack information on spatial positioning or sensitivity/specificity. To address this, we have developed a novel workflow combining the single molecule and single cell visualization capabilities of the RNAscope™ ISH assay with the highly multiplexed spatial profiling capabilities of the GeoMx™ Digital Spatial Profiler (DSP) RNA assays.



Methods

❖ **Samples used:** Lung tumor samples

❖ **Workflow:**

Probe hybridization for both RNAscope and DSP GeoMx was performed on the Leica automated stainer. ROI selection and profiling was performed on the NanoString GeoMx DSP and the DSP quantification was performed using the nCounter.

• **ROI selection:** *CD3E* and *CD19* specific RNAscope probes were used for identifying T cell-rich and B cell-rich areas within the lung tumor using RNAscope Multiplex Fluorescent Assay V2.

• **GeoMx DSP analysis:** Selected ROIs were profiled using a panel of 78 immuno-oncology related target genes.

• **Confirmation:** Genes of interest were further interrogated and visualized with single cell resolution using the RNAscope assay.

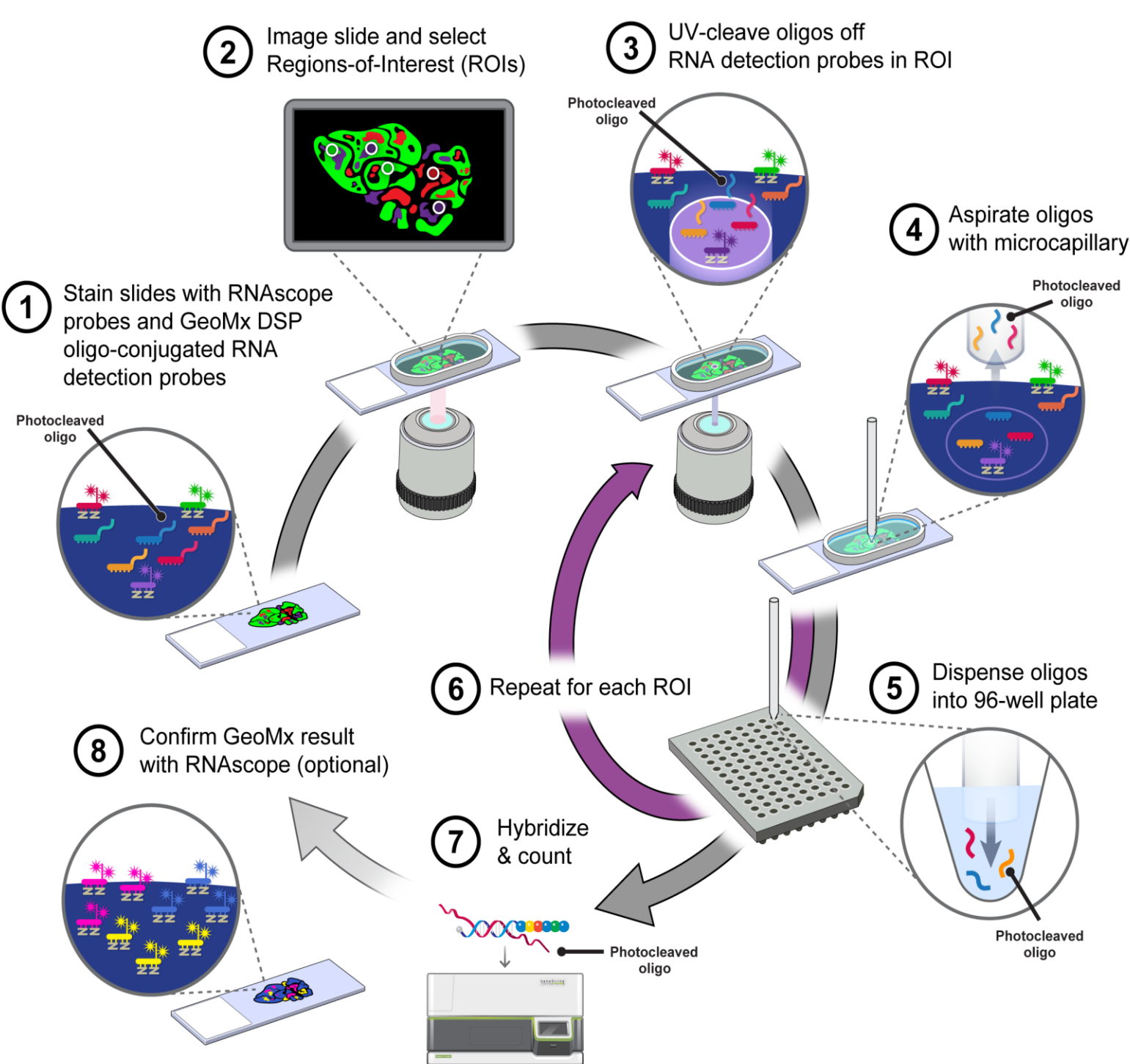


Figure 1: Step-wise depiction of the RNAscope-GeoMx DSP workflow for spatial gene expression analysis.

Results

RNAscope ISH assay and GeoMx DSP are compatible with each other

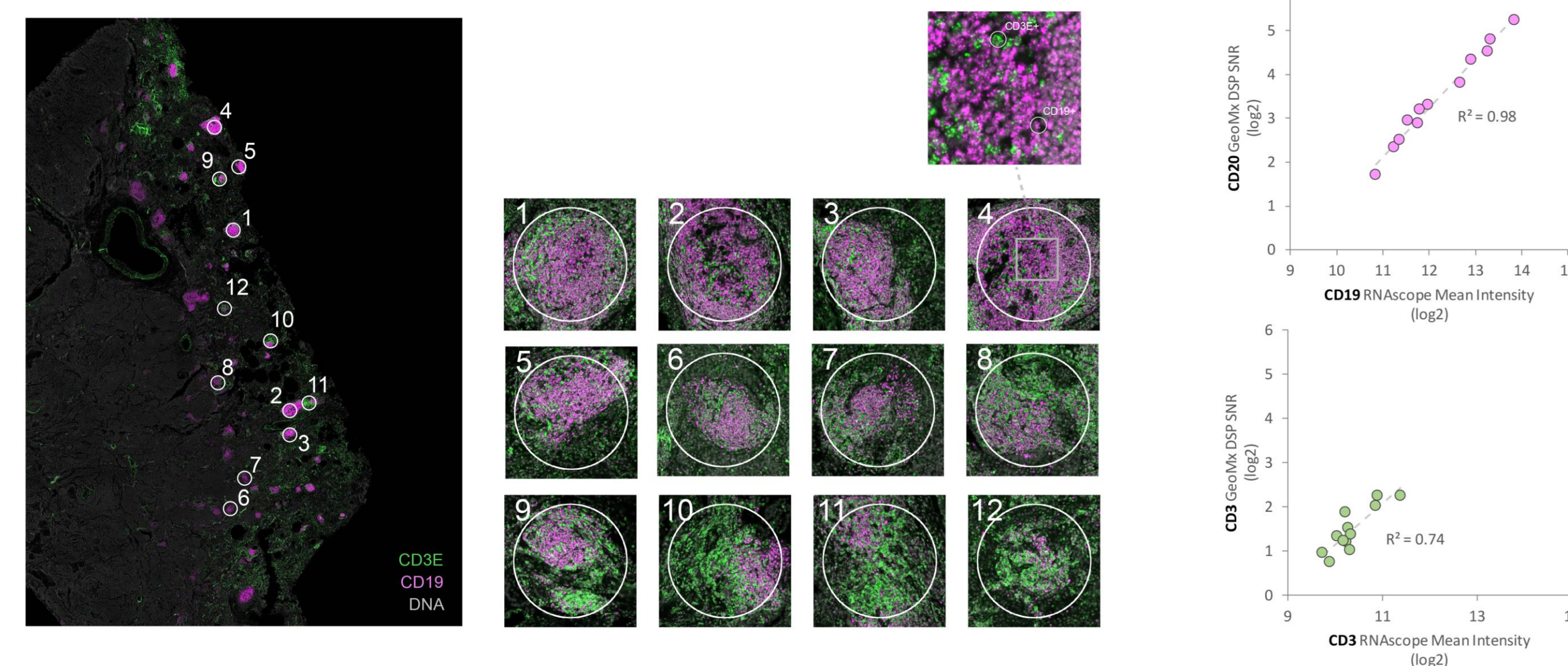


Figure 2: Concordance between GeoMx DSP and RNAscope ISH. *CD3E* & *CD19* mRNA expression was visualized and quantified using the RNAscope assay and ImageJ, respectively. *CD3E* and *CD20* mRNA expression was quantified using the DSP nCounter system. The assays showed concordance for the expression of the same gene (*CD3E*) as well as for different genes with identical expression profiles (*CD19/CD20*), indicating that the two technologies are compatible.

Molecularly guided ROI selection by RNAscope and GeoMx DSP profiling of 78 IO-related RNA targets

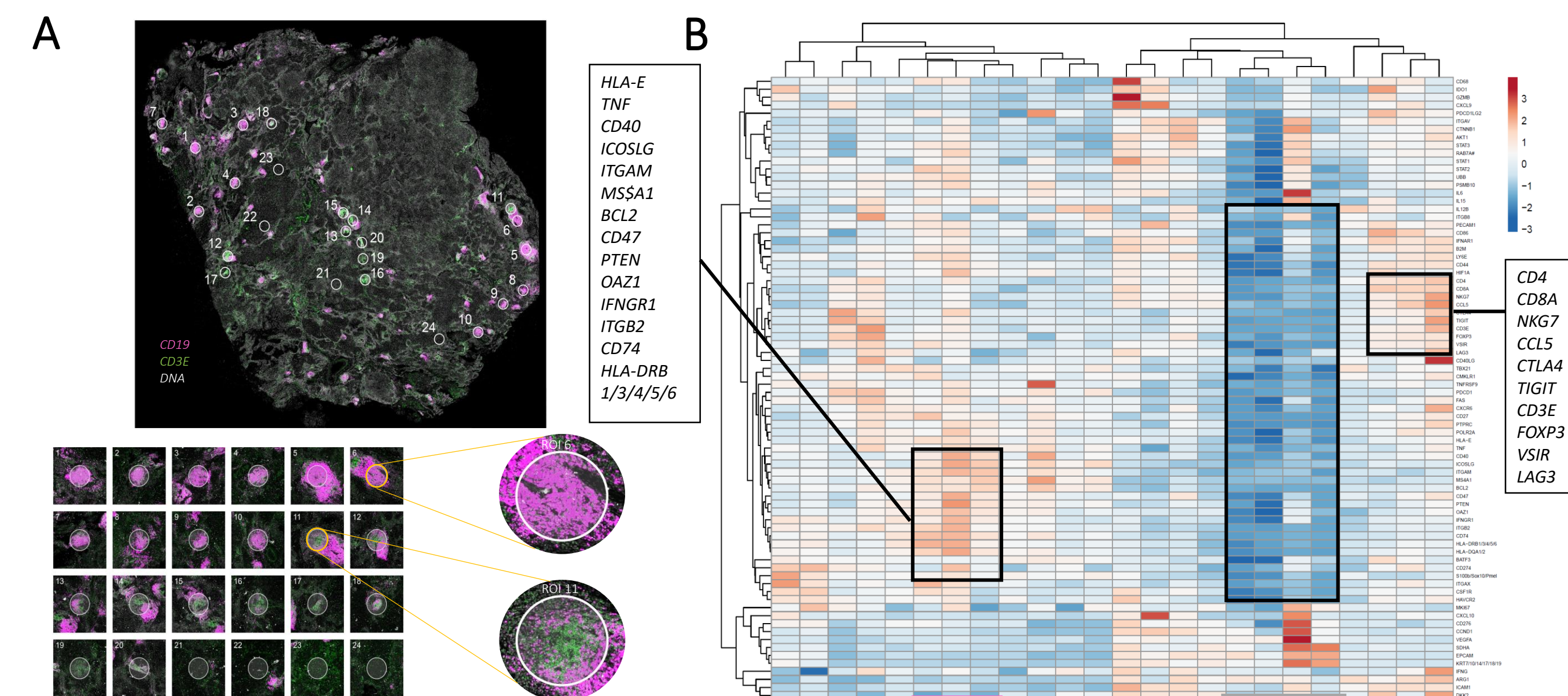


Figure 3: High plex spatial analysis with the RNAscope ISH and GeoMx DSP profiling in NSCLC. (A) *CD19* and *CD3E* positive ROI were profiled by GeoMx DSP for high plex RNA analysis in human lung cancer FFPE sample. ROI11 is an example of a *CD3E*-enriched ROI and ROI6 is an example of a *CD19*-enriched 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.

Confirmation and visualization of immunoregulatory and inflammatory marker expression at single cell resolution

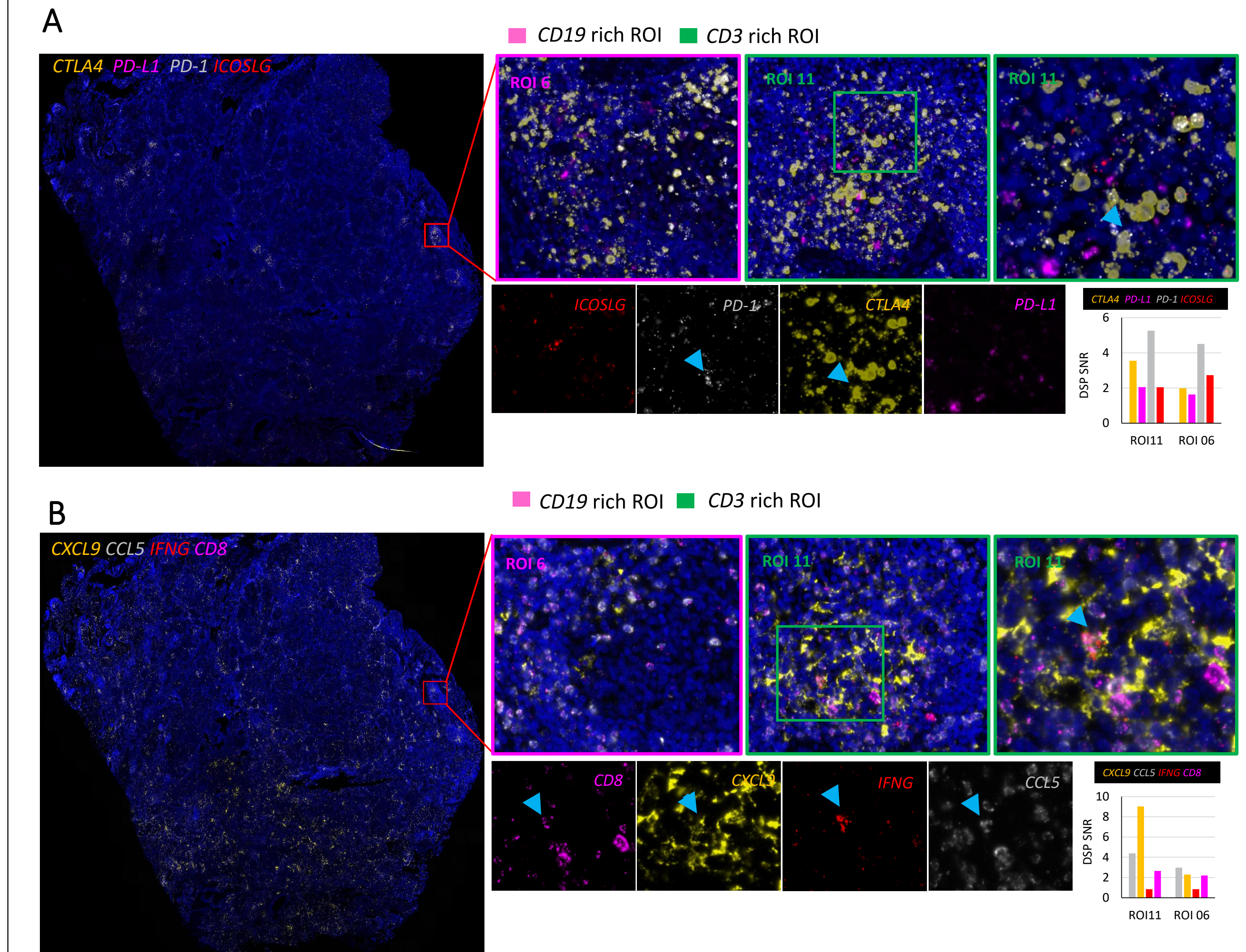


Figure 4: Confirmation of high plex GeoMx DSP analysis using the RNAscope ISH assay in NSCLC tumor sample. (A) Checkpoint markers such as *CTLA4*, *PD-L1*, *PD-1* and *ICOSLG* were expressed at a higher level in the *CD3*-enriched ROI compared to the *CD19*-enriched ROI. Blue arrow indicates a *CTLA4*+/*PD1*+ cell. (B) Inflammatory chemokines such as *CXCL9* and *CCL5* and cytokines such as *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 shows expression levels by GeoMx DSP. SNR, Signal to noise ratio.

Summary

- The RNAscope ISH assay was used to molecularly guide GeoMx DSP analysis and visualize *CD3E*-rich (T cell) and *CD19*-rich (B cell) regions in the tumor tissue.
- The GeoMx DSP analysis identified ROI-specific gene expression profiles within the heterogeneous lung cancer tumor which could be confirmed with RNAscope at single-cell resolution.
- T cell-enriched ROI demonstrated significantly increased expression of immune cell receptors and immune cell activation markers such as cytokines and chemokines in comparison to the B cell-enriched ROI.
- Taken together, this study demonstrates that the RNAscope ISH assay combined with the GeoMx DSP assay enables molecularly guided high plex RNA profiling with spatial resolution.