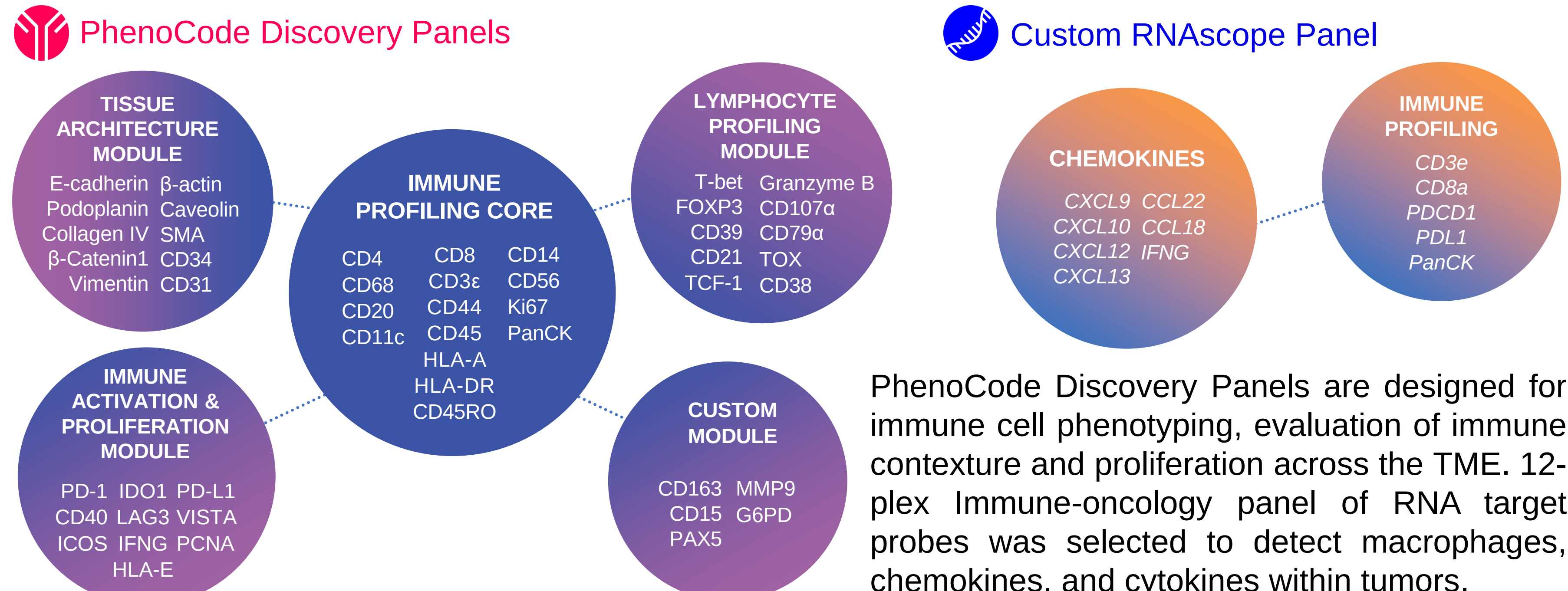


1. Introduction

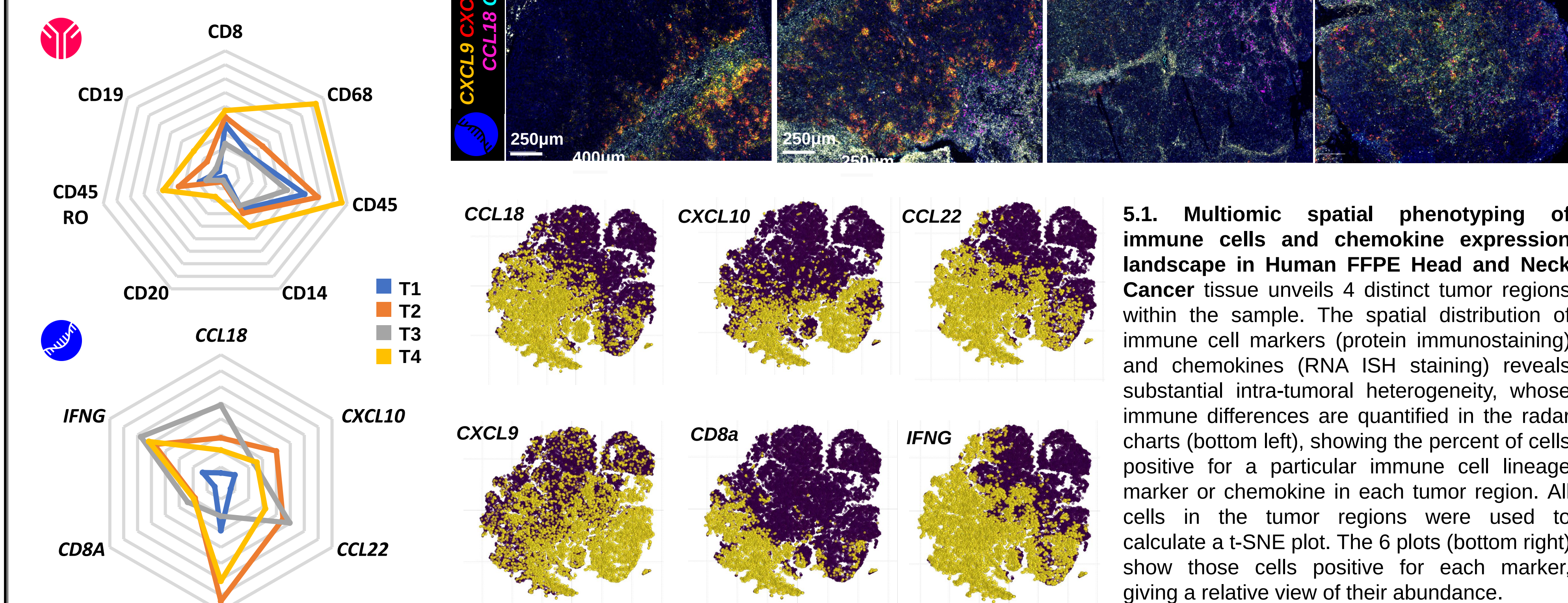
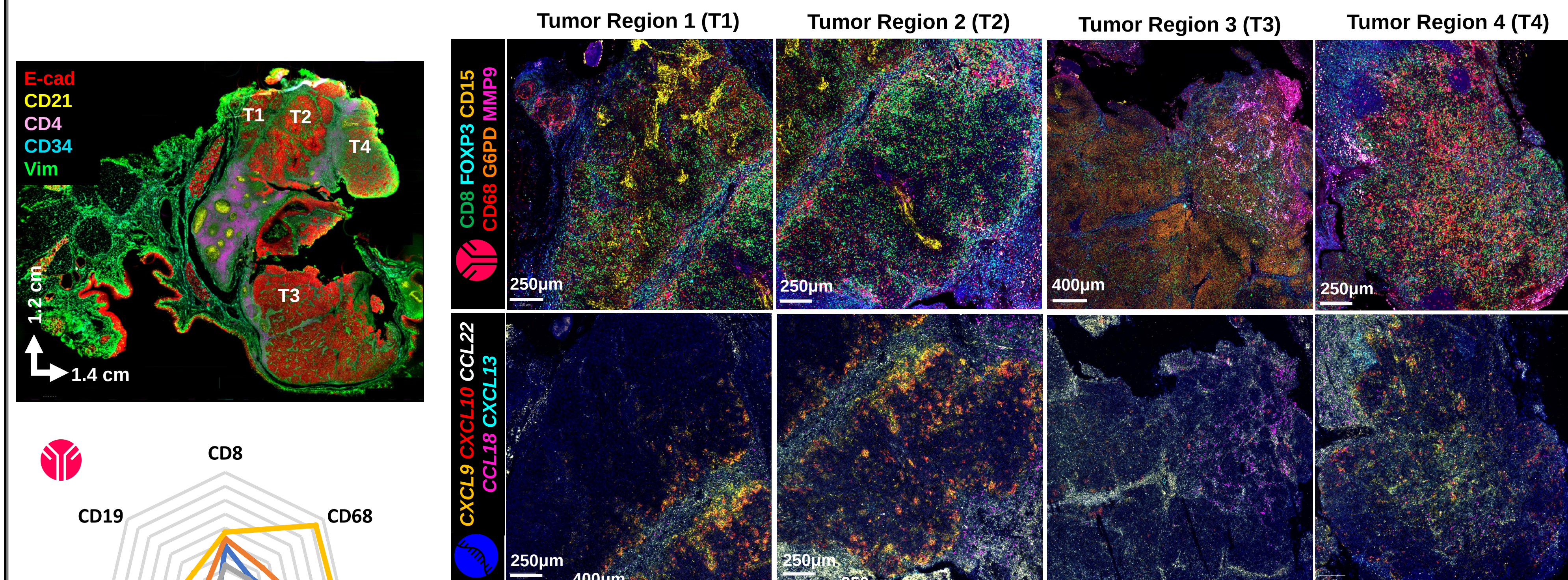
Effective strategies for cancer immunotherapies will require a deep understanding of the factors that shape both the tumor microenvironment (TME) and the immunological components within tumors, known as the tumor immune microenvironment (TiME). Here, we describe a spatial multiomics approach that utilizes RNAscope™ ISH technology paired with high-plex whole-slide spatial phenotyping on the PhenoCycler®-Fusion platform. This two-step approach is compatible with human FFPE tissues and enables researchers to characterize the spatial biology of the TiME more accurately by detecting RNA and protein markers on serial sections. Cell phenotypes and spatial associations are further analyzed through the Visiopharm Phenoplex™ software. The resulting multiomic spatial data more accurately reveals the interplay between TME and TiME by giving insight into cell lineages, surrounding structures, as well as secreted chemokines and cytokines that exist within the TME ecosystem.

3. Multiomic Panel Design



5. Multiomic Spatial Phenotyping of Human FFPE Head and Neck Cancer Tissue Reveals Distinct Chemokine and Immune Signatures within the TiME

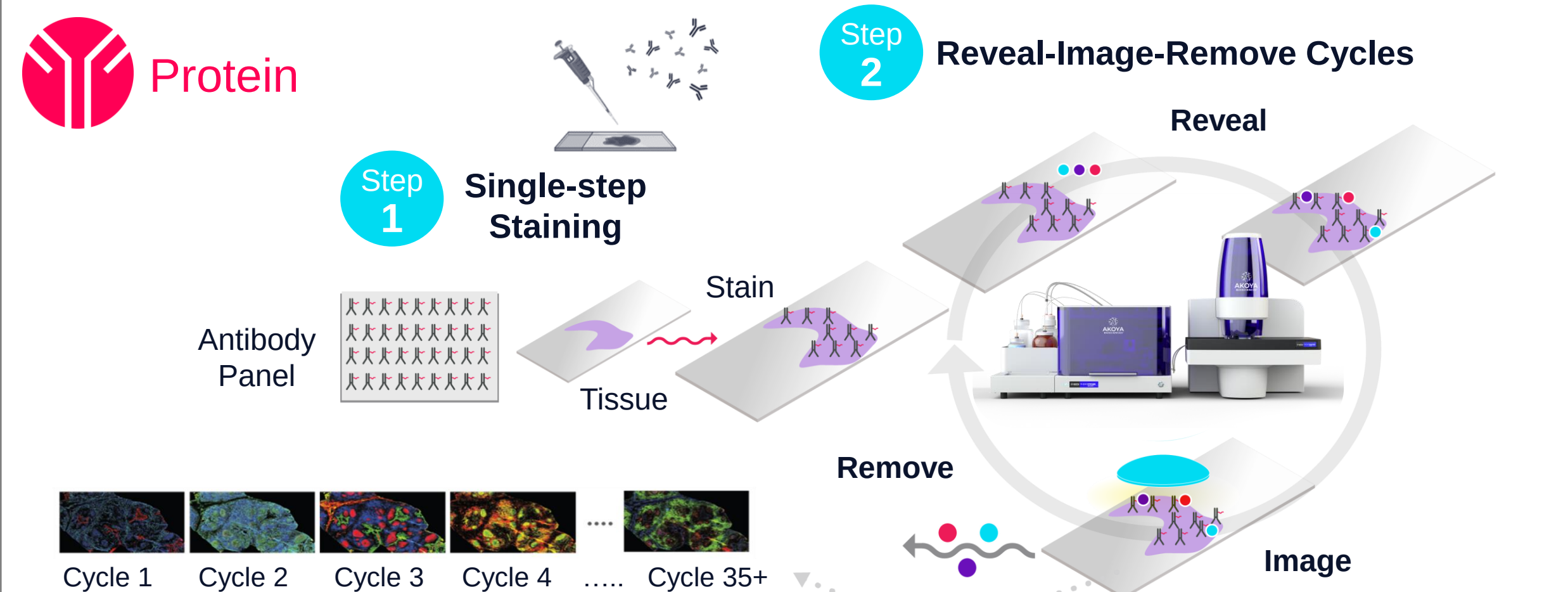
5.1. Whole-Slide Multiomic Spatial Phenotyping Reveals Intra-tumoral Heterogeneity



5.1. Multiomic spatial phenotyping of immune cells and chemokine expression landscape in Human FFPE Head and Neck Cancer tissue unveils 4 distinct tumor regions within the sample. The spatial distribution of immune cell markers (protein immunostaining) and chemokines (RNA ISH staining) reveals substantial intra-tumoral heterogeneity, whose immune differences are quantified in the radar charts (bottom left), showing the percent of cells positive for a particular immune cell lineage marker or chemokine in each tumor region. All cells in the tumor regions were used to calculate a t-SNE plot. The 6 plots (bottom right) show those cells positive for each marker, giving a relative view of their abundance.

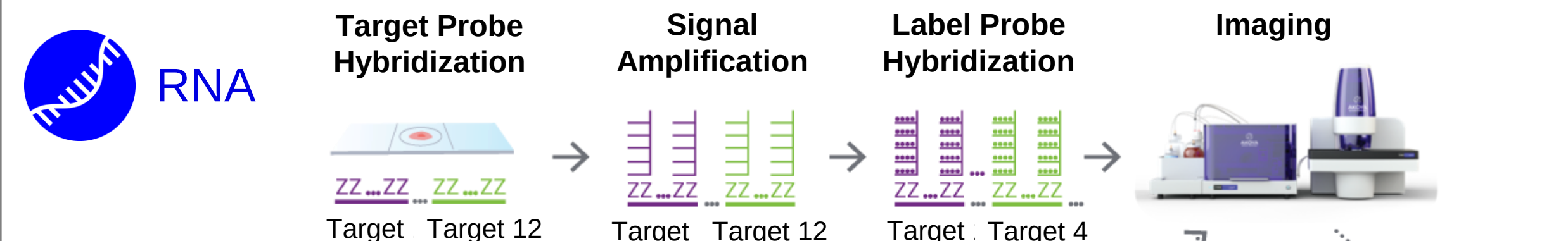
2. Multiomic Spatial Phenotyping Workflow

2.1. Spatial Protein Phenotyping on the PhenoCycler-Fusion



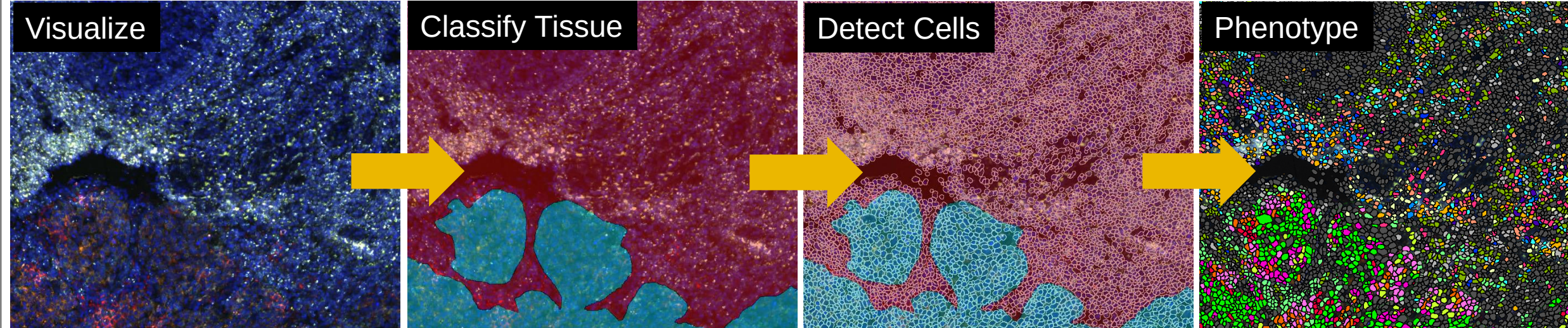
PhenoCycler-Fusion seamlessly integrates automated fluidics and imaging, creating an end-to-end solution for high-resolution spatial imaging with rapid turnaround times. Here we analyzed human FFPE tissues using the PhenoCode™ Discovery Panels that comprehensively labels both immune and metabolic markers.

2.2. RNAscope HiPlex v2 on the PhenoCycler-Fusion



RNAscope HiPlex v2 assay is capable of simultaneous detection of 12 RNA targets using the RNAscope ISH technology using the proprietary “double Z” probe design. Here we paired RNAscope HiPlex v2 assay with PhenoCycler-Fusion to perform the iterative fluorescent imaging to label a custom RNAscope chemokine panel.

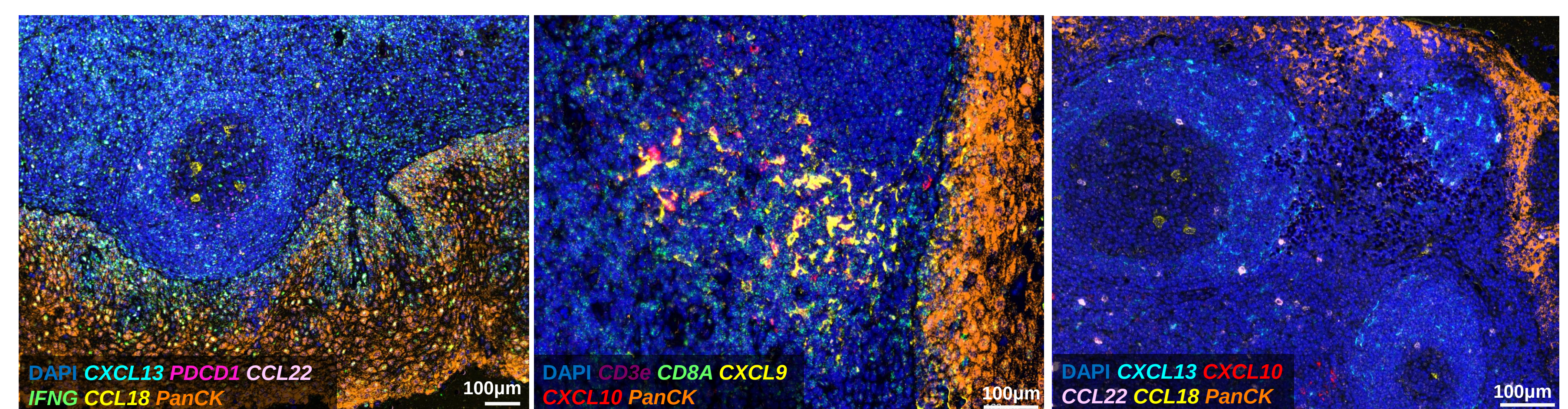
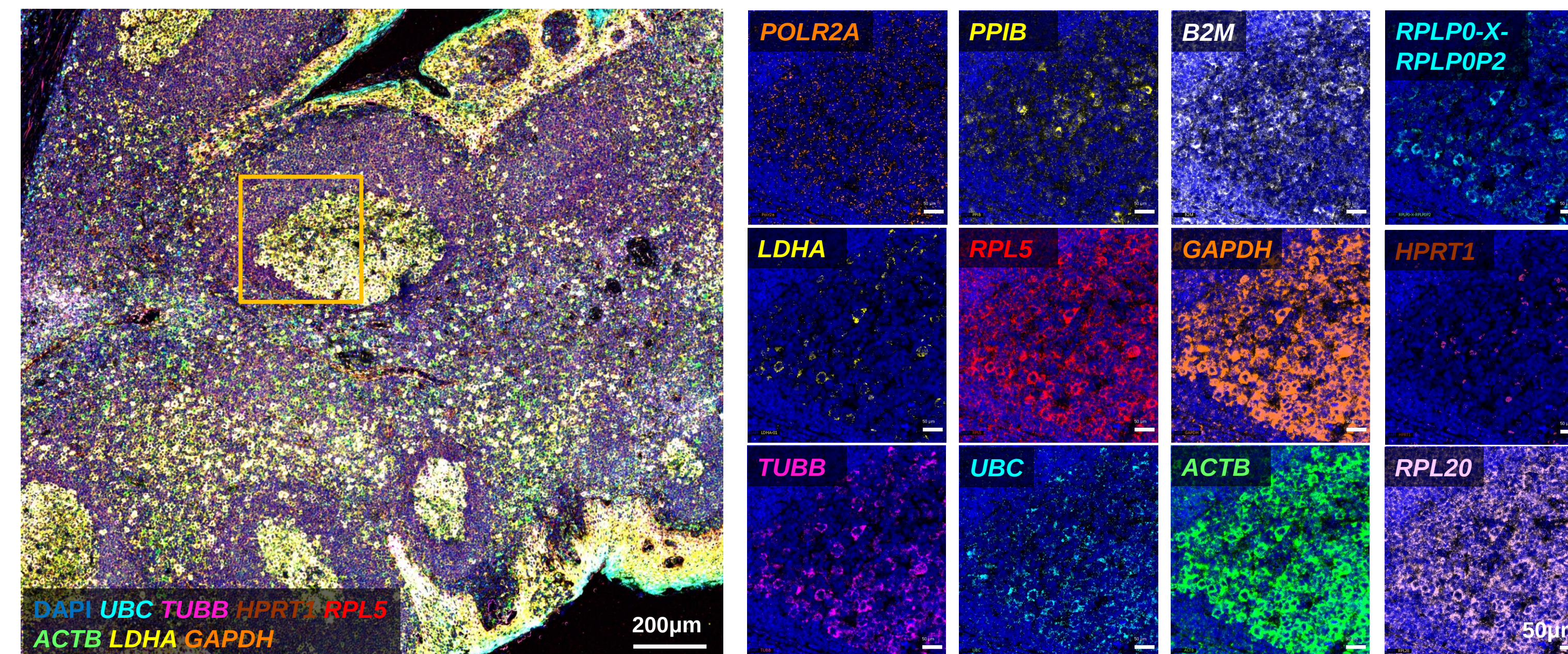
2.3. Data Analysis with Visiopharm Phenoplex software



Phenoplex software offers end-to-end workflow including importing multiplex images, Paint-to-Train AI-based tissue segmentation, deep-learning-based nuclear and cell segmentation, as well as a guided workflow for cellular phenotyping.

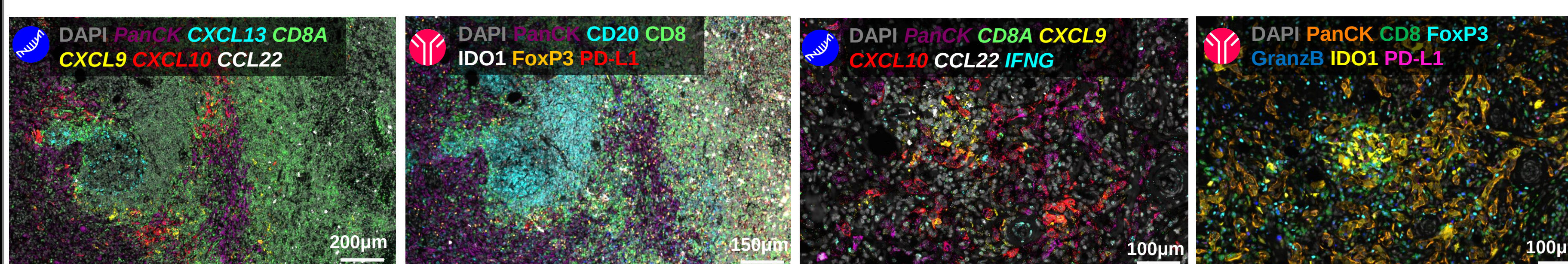
4. RNAscope ISH paired with PhenoCycler-Fusion on Human FFPE Tissues

4.1. Detection of RNAscope 12-plex probes in Human FFPE Tonsil with PhenoCycler-Fusion



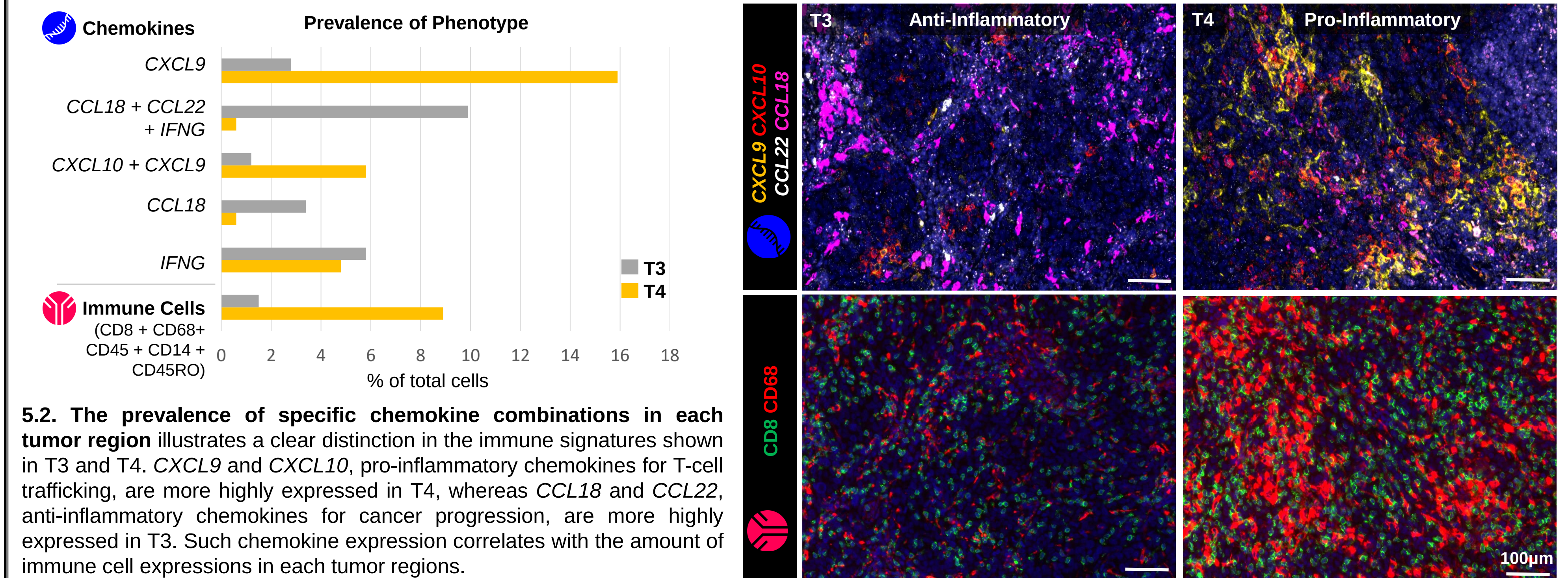
4.1. Detecting 12 target genes in a Human FFPE Tonsil tissue. To demonstrate the feasibility of automating RNAscope HiPlex v2 on PhenoCycler Fusion, 12 control probes targeting a housekeeping human gene in each of 12 separate channels were used for ISH detection in a human FFPE tonsil tissue (top images). Additionally, the custom RNAscope panel (listed in Section 3) was used to identify immune cells, tumor cells, chemokines and cytokines in a human FFPE tonsil tissue (bottom images).

4.2. Multiomic Detection of Protein & RNA Panel on Human Squamous Cell Carcinoma tissue



4.2. Multiomic detection of protein markers and RNA target genes in FFPE tumor serial sections. PhenoCode Discovery Panels were utilized to detect protein markers that can phenotype tumor cells, immune cell subtypes, and immune activation states that constitute the TME. In a serial section, RNA probes were used to detect target genes for various chemokines and cytokines within the TME.

5.2. Chemokine-driven Immune Cell Landscape Leads to Distinct TIME in Different Tumor Regions



5.2. The prevalence of specific chemokine combinations in each tumor region illustrates a clear distinction in the immune signatures shown in T3 and T4. CXCL9 and CXCL10, pro-inflammatory chemokines for T-cell trafficking, are more highly expressed in T4, whereas CCL18 and CCL22, anti-inflammatory chemokines for cancer progression, are more highly expressed in T3. Such chemokine expression correlates with the amount of immune cell expressions in each tumor regions.

6. Multiomic Spatial Analysis Uncovers Interplay of Chemokines and Immune Cells within the TiME

In this proof-of-concept study, we demonstrate the utility of multiomic spatial profiling on the PhenoCycler-Fusion platform with the RNAscope assay. Analysis of the resulting multiplex imaging data not only revealed the structural organization of cells within the TME, but also the regulatory role of chemokines within the TiME. Together, this information provides a more complete functional map of immune cells within the TME and TiME and thereby enriches our understanding of tumor biology that may be deterministic of immunotherapy responsiveness.