

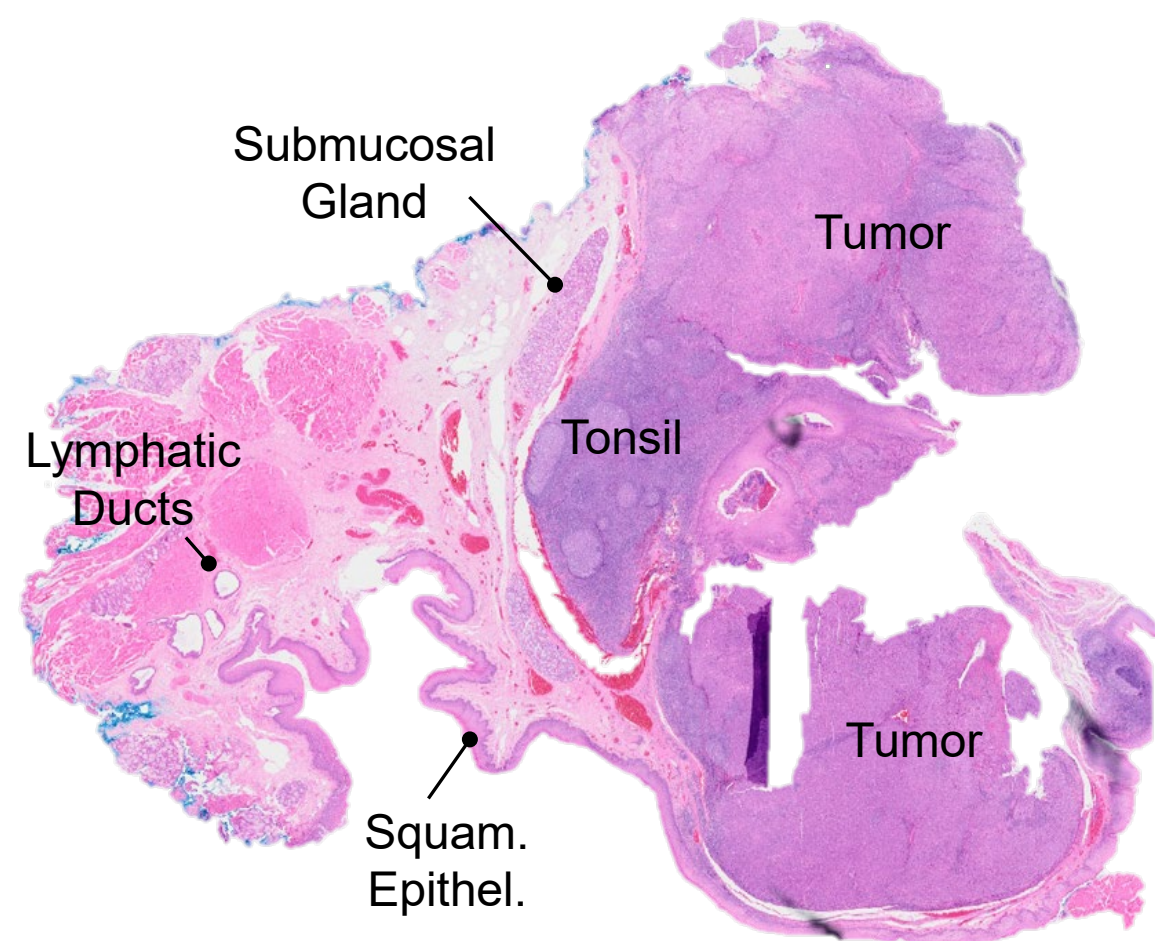
Multiomic Spatial Phenotyping of Immunotherapy Responses in Head and Neck Cancer

Oliver Braubach¹, James Monkman², Ken O'Byrne³, Brett Hughes⁴, Niyati Jhaveri¹, HaYeun Ji¹, Aditya Pratapa¹, Will Agnew-Svoboda¹, Ning Ma¹, Bassem Ben Cheikh¹, Arutha Kulasinghe².

¹Akoya Biosciences, Marlborough, MA, USA. ²The University of Queensland, Brisbane, Australia. ³The Princess Alexandra Hospital, Brisbane, Australia. ⁴The Royal Brisbane Women's Hospital, Brisbane, Australia.

1. Introduction

Immune checkpoint inhibitors (ICI) have proven to be game-changing treatments for mucosal head and neck squamous cell cancer (HNSCC). Early successes with anti-PD-1/PD-L1 ICIs have produced durable responses and prolonged survival in some patients but there remains a need for better biomarkers to guide patient selection. The tumor microenvironment (TME) composition, texture, and cellular architecture are key to understanding immune responsive and resistant HNSCC phenotypes. In this study, we designed a single-cell, multiomic spatial phenotyping experiment to characterize a HNSCC tissue from a patient with a partial response to Pembrolizumab/Nivolumab treatment. The image shown below is an H&E-stained section from the aforementioned patient. Path annotations indicate a large tumor mass, a regionally intact tonsil, as well as an Esophageal Submucosal Gland, Lymphatic Ducts and normal Squamous Epithelium. In this poster, we demonstrate how **Multiomic Spatial Phenotyping** was used to produce a uniquely comprehensive analysis of the patient's TME and how it may explain the patient's partial response to ICI therapy.



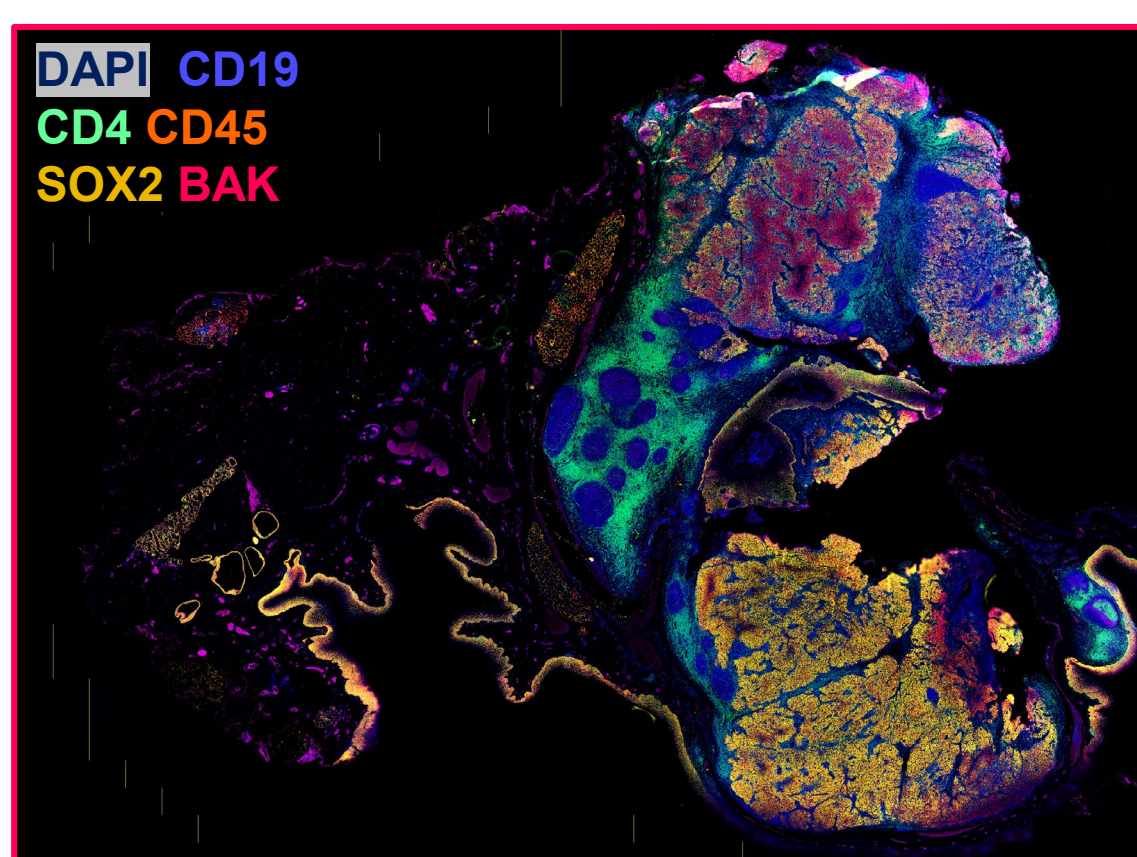
2. Multiomic (RNA & Protein) Spatial Phenotyping on PhenoCycler-Fusion



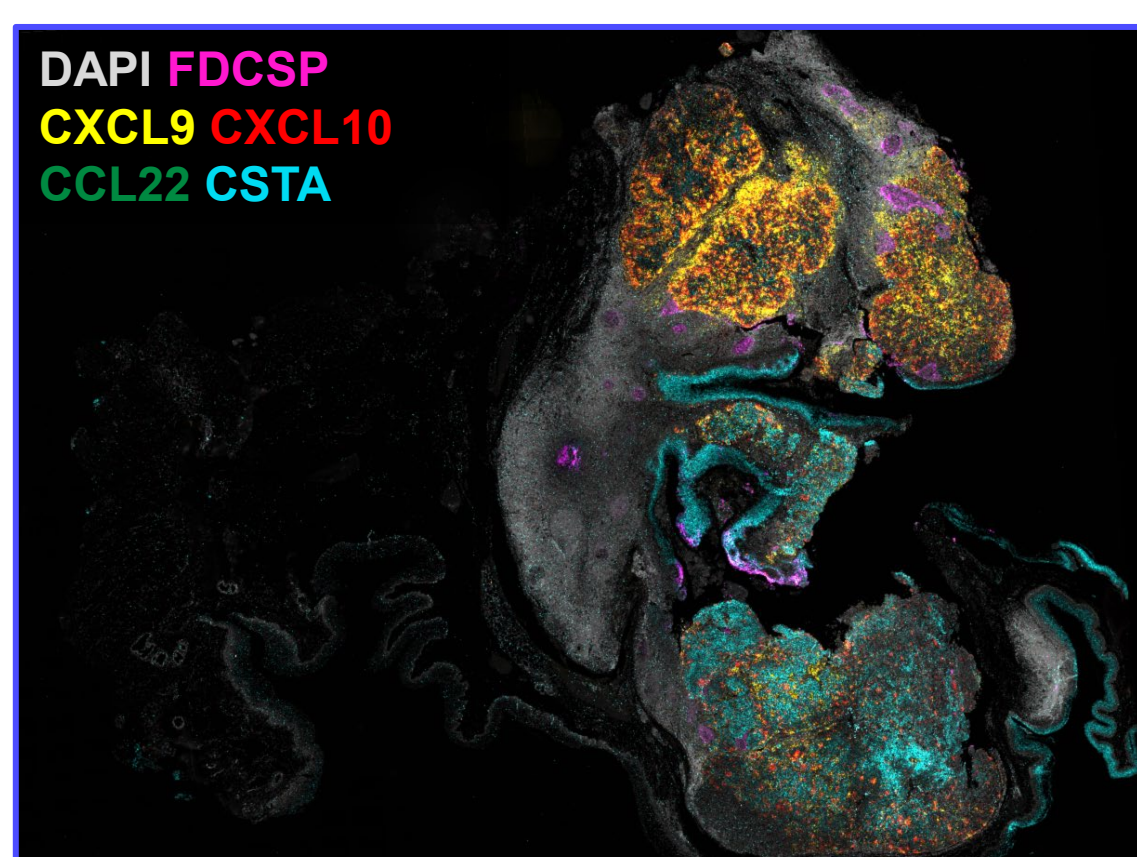
The **PhenoCycler®-Fusion** system is a spatial biology solution that enables multiomic ultrahigh-plex imaging of whole tissues at single-cell resolution. The high-throughput coupled with in-flight data processing and file compression algorithms enables routine analyses of whole-slide tissue sections, which often contain millions of cells. The unified PhenoCycler chemistry can be used to detect both RNA and protein targets, thereby allowing thorough interrogation of complex tissues. In this study, we analyzed HNSCC tissues with an ultrahigh-plex panel containing **101 antibodies** that label immune cell lineages, checkpoints, the stroma, as well as metabolic and cell stress markers. We then conducted single-cell resolution RNA imaging with **45 RNA probes** on serial sections from the same tissue blocks. RNA probes were designed to detect cytokines and chemokines and other molecules that may modulate immune cell function in the HNSCC TME.

3. Multiomic Panel Design

101-plex AB panel										45-plex RNA probes									
CD4	CD107a	HLA-E	PCNA	Na/K	CPT1A	B2M	CCL20	ACTB		CD4	CD14	CCL21	ARHGDI8						
CD68	CD21	IFNG	C1Qa	ATPase	GLUT1	CD14	CCL21	ARHGDI8		CD68	CD27	CCL22	COL1A1						
CD20	CD163	CD69	CCR6	HK1	pNRF2	CD52	CCL5	COL3A1		CD20	CD11b	CCL22	COL3A1						
CD11c	CD11b	CD227	AXL	LDHA	ATPA5	CD74	CXCL10	COL4A1		CD11c	CD141	CXCL11	CSTA						
Ki67	CD141	CD138	HLA-DR	G6PD	E-cadherin	CD74	CXCL10	COL4A1		Ki67	Pax5	CXCL11	CSTA						
CD45RO	FOXP3	CD15	BAK	TP63	Histone H3	CD12	CXCL12	CSTB		CD45RO	FOXP3	CXCL12	CSTB						
	CD15	BAK	TP63	Histone H3	CD12	CXCL12	CSTB												
PanCK	Gran B	MPO	BCL-XL	S100A4	β-actin	PECAM1	CXCL16	GAPDH		PanCK	Gran B	CXCL16	GAPDH						
CD3e	CD38	iNOS	BAX	TFAM	Podoplanin	GNAS	CXCL2	KRT10		CD3e	CD38	CXCL2	KRT10						
CD44	CD39	HLA-DPB1	BAD	GP100	SMA	CLU	CXCL5	KRT13		CD44	CD39	CXCL5	KRT13						
CD45	CD79a	LEF1	Cyt.C	CK17	Vimentin	TXNIP	CXCL8	KRT4		CD45	CD79a	CXCL8	KRT4						
HLA-A	PD-1	GAL9	LC3B	CK19	Collagen IV	HIF1A	CXCL9	S100A11		HLA-A	PD-1	CXCL9	S100A11						
CD14	PD-L1	CD138	Beclin-1	GATA3	CD34	β-catenin	EGFR	TMSB4X		CD14	PD-L1	EGFR	TMSB4X						
CD56	ICOS	MC-	H2AX	SOX2	SDHA	EPCAM	TUBA1B	VIM		CD56	ICOS	TUBA1B	VIM						
CD57	LAG3	Tryptase	pRPS6	SDHA	ZEB1					CD57	LAG3								
CD19	IDO1	OX40	Cyc D1	Cit. Syn	EPCAM					CD19	IDO1								
CD2	CD40	ZAP70	ASCT2		LaminB1					CD2	CD40								
CD1a	MMP-9									CD1a	MMP-9								



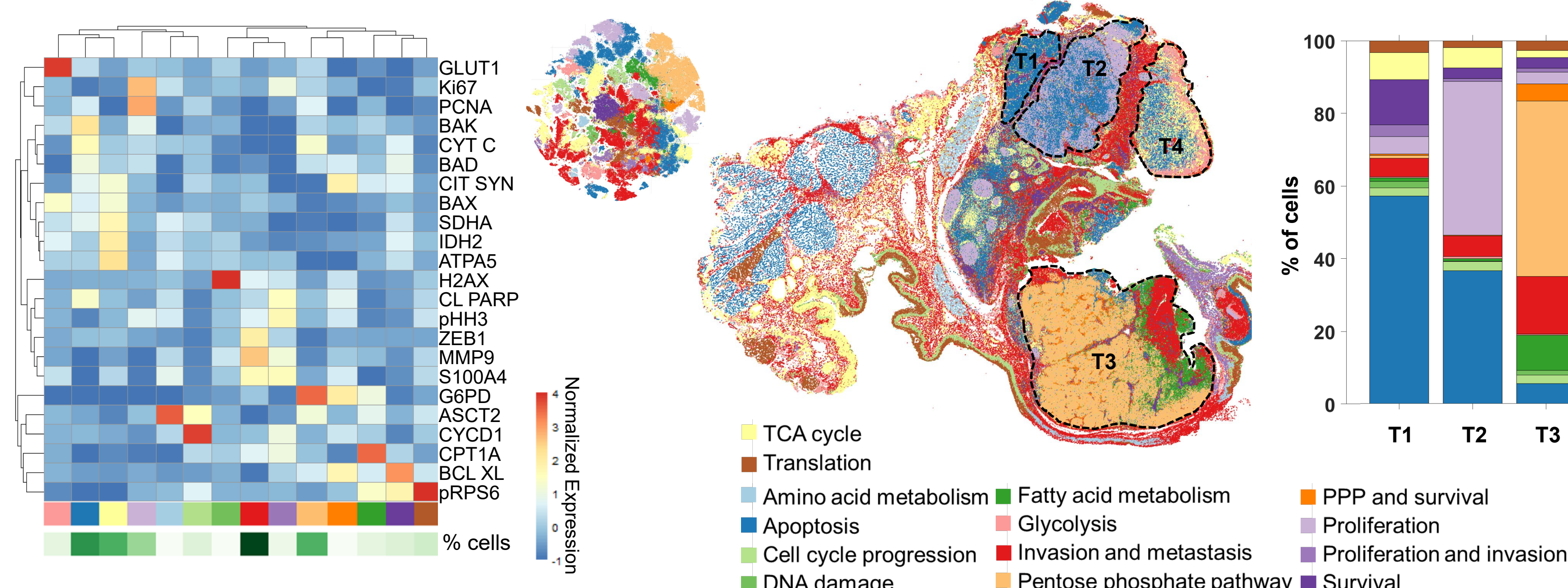
The **101-plex antibody panel** included comprehensive immune and metabolic markers, which enabled the discovery of substantial heterogeneity within the HNSCC tumor regions; this heterogeneity may be indicative of a partial ICI response (see Section 4).



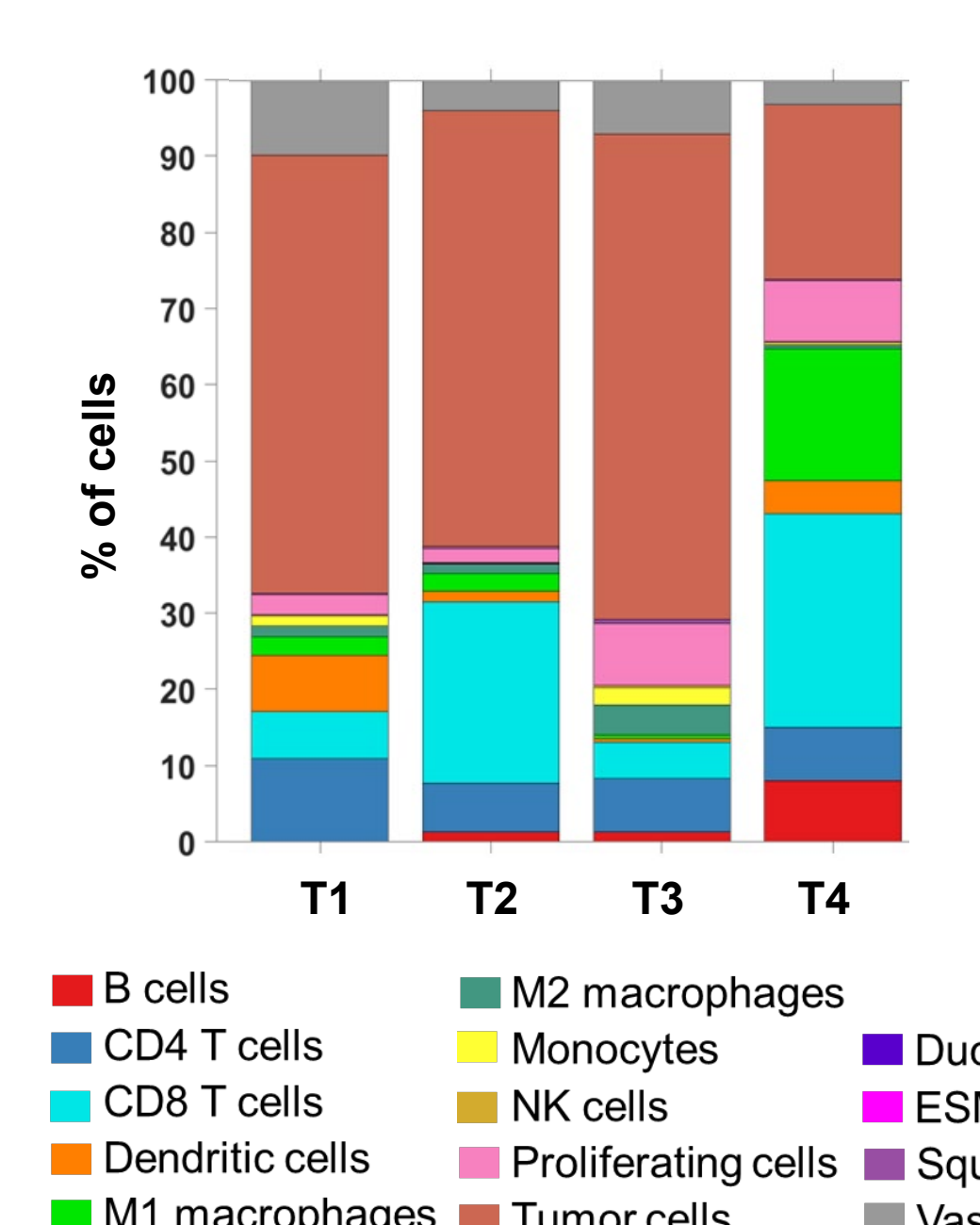
The **45-plex RNA probes** produced complementary readouts for an extensive list of TME cyto- and chemokines. Integration of RNA data with the protein spatial phenotyping revealed distinct inflammatory niches across the tumor regions (Section 5).

4. 101-Plex "Cancer Hallmark" AB Panel Reveals 4 Distinct Tumor Region with Metabolic and Immune Heterogeneity

4.1 Metabolic/Stress Spatial Phenotyping Illustrates Tumor Heterogeneity



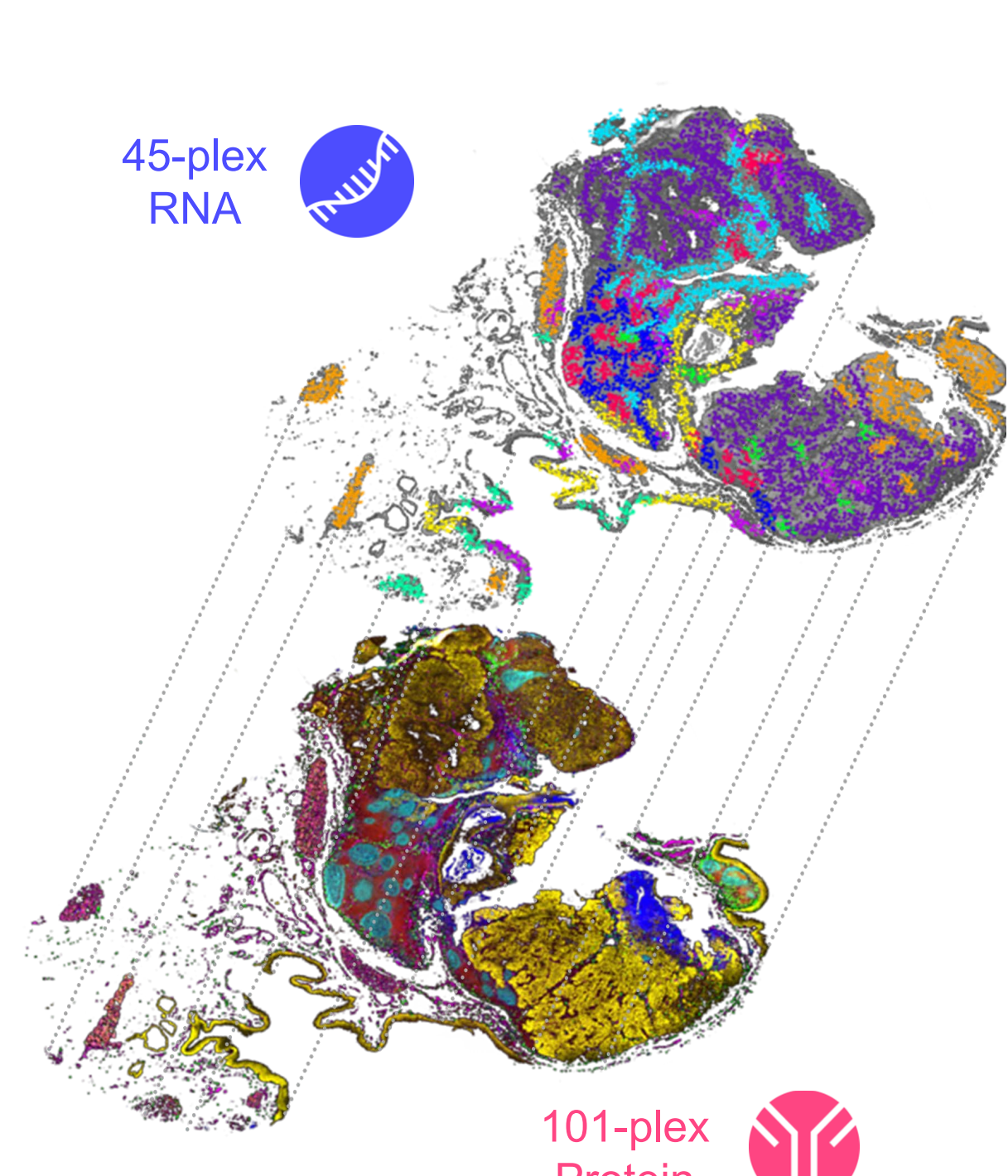
4.2 Tumor Immune Infiltration



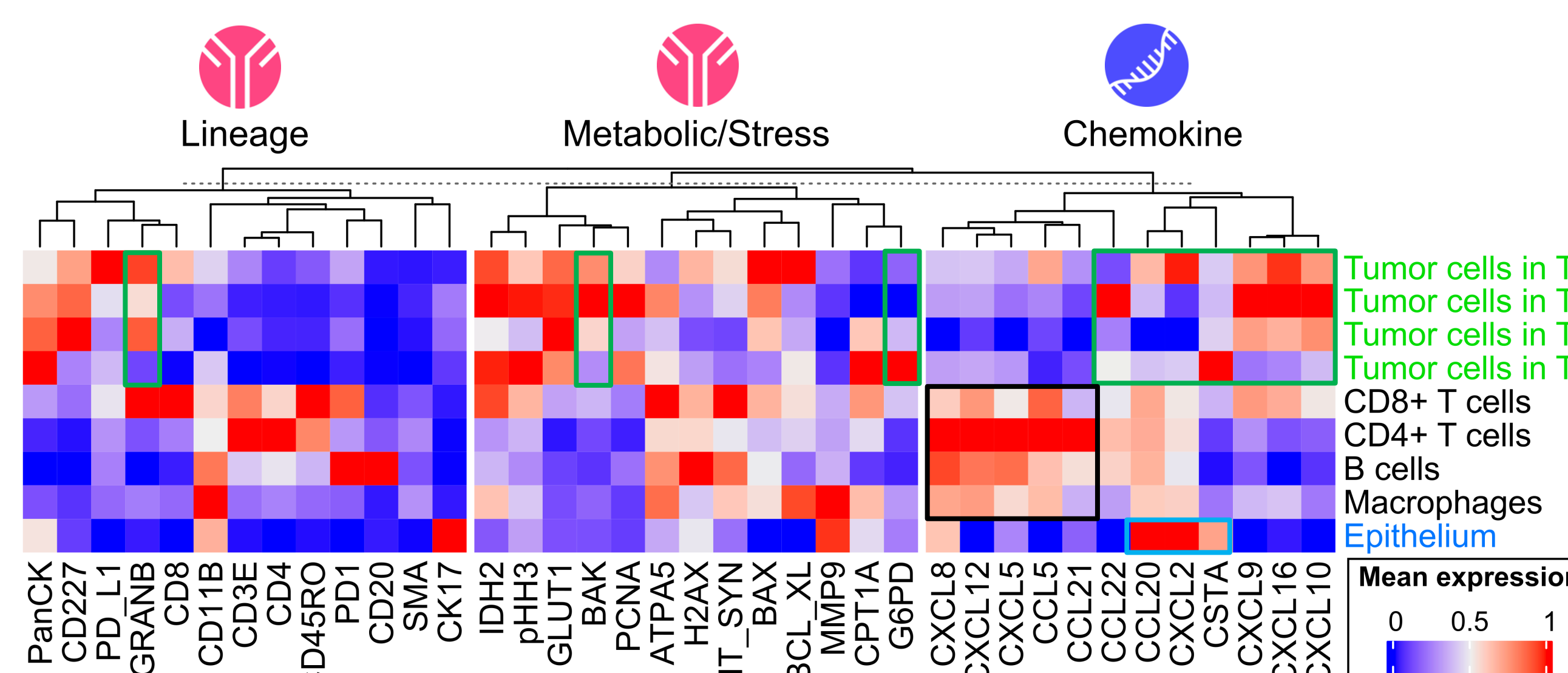
4.1 Metabolic Spatial Phenotyping of 23 antibodies identified 14 functionally specialized cell phenotypes within four distinct tumor regions: Tumor 1 (T1) had a high degree of apoptotic cells (blue); T2 had a central apoptotic core (blue) surrounded by proliferating cells (lilac); T3 was linked to the pentose phosphate pathway (peach) and apoptosis (blue). **4.2 Targeted immune-phenotyping of the four tumor regions revealed clear differences in immune cell infiltration, concordant with metabolic & stress profiles of each tumor region.**

5. Integrated Multiomic Spatial Phenotyping Uncovers Distinct Inflammatory Niches in Different Tumor Regions

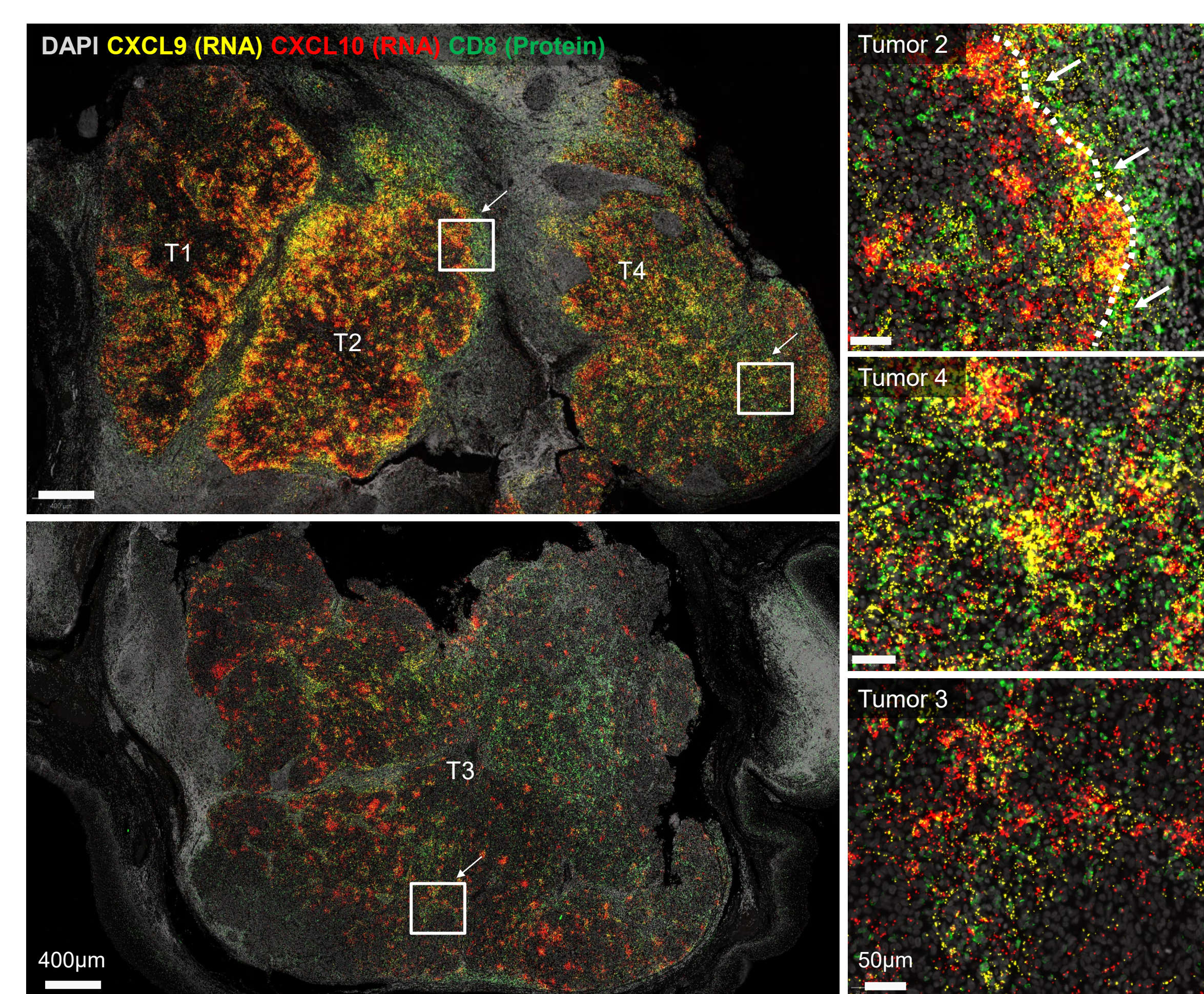
5.1. Data Integration Strategy



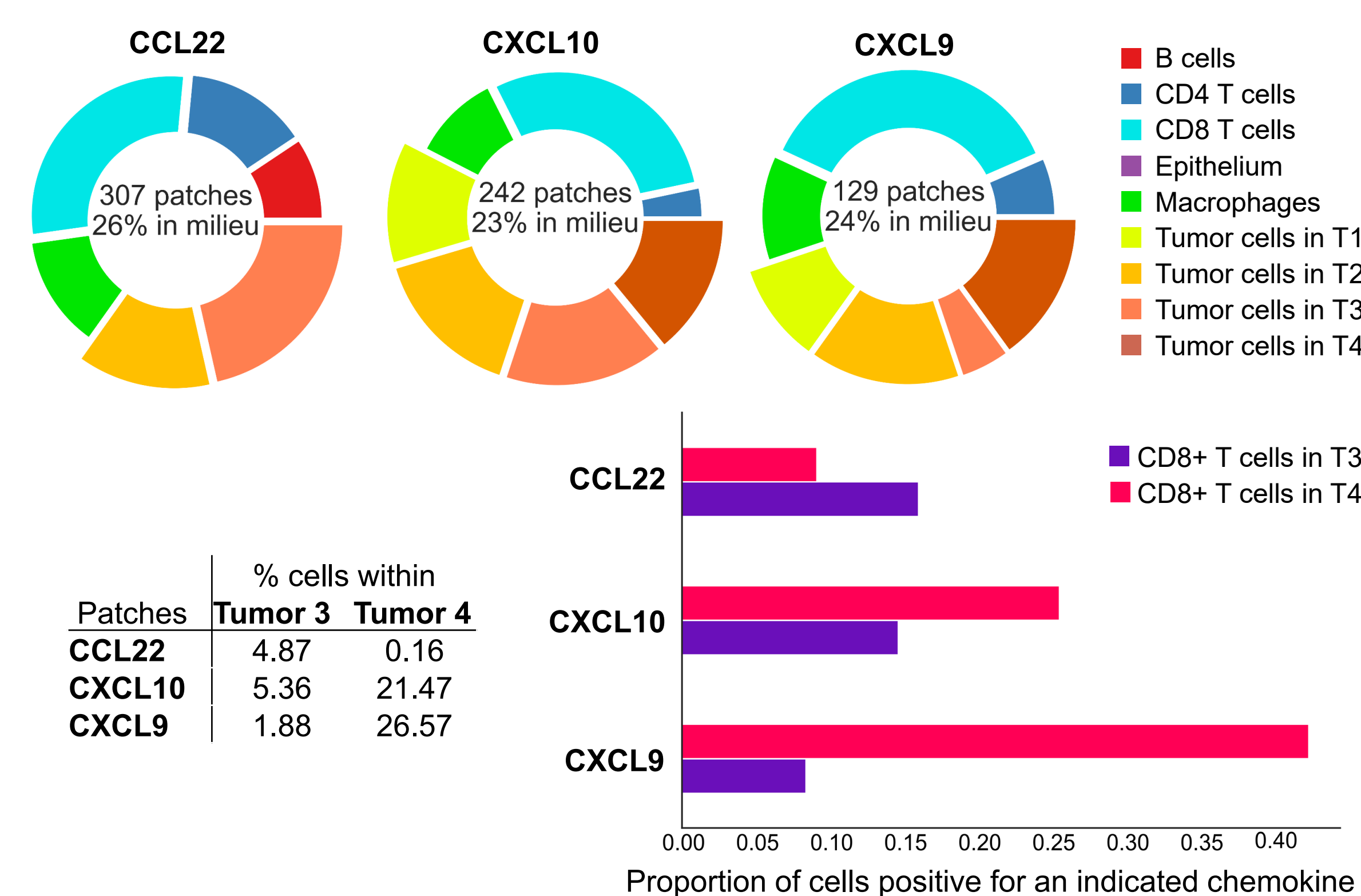
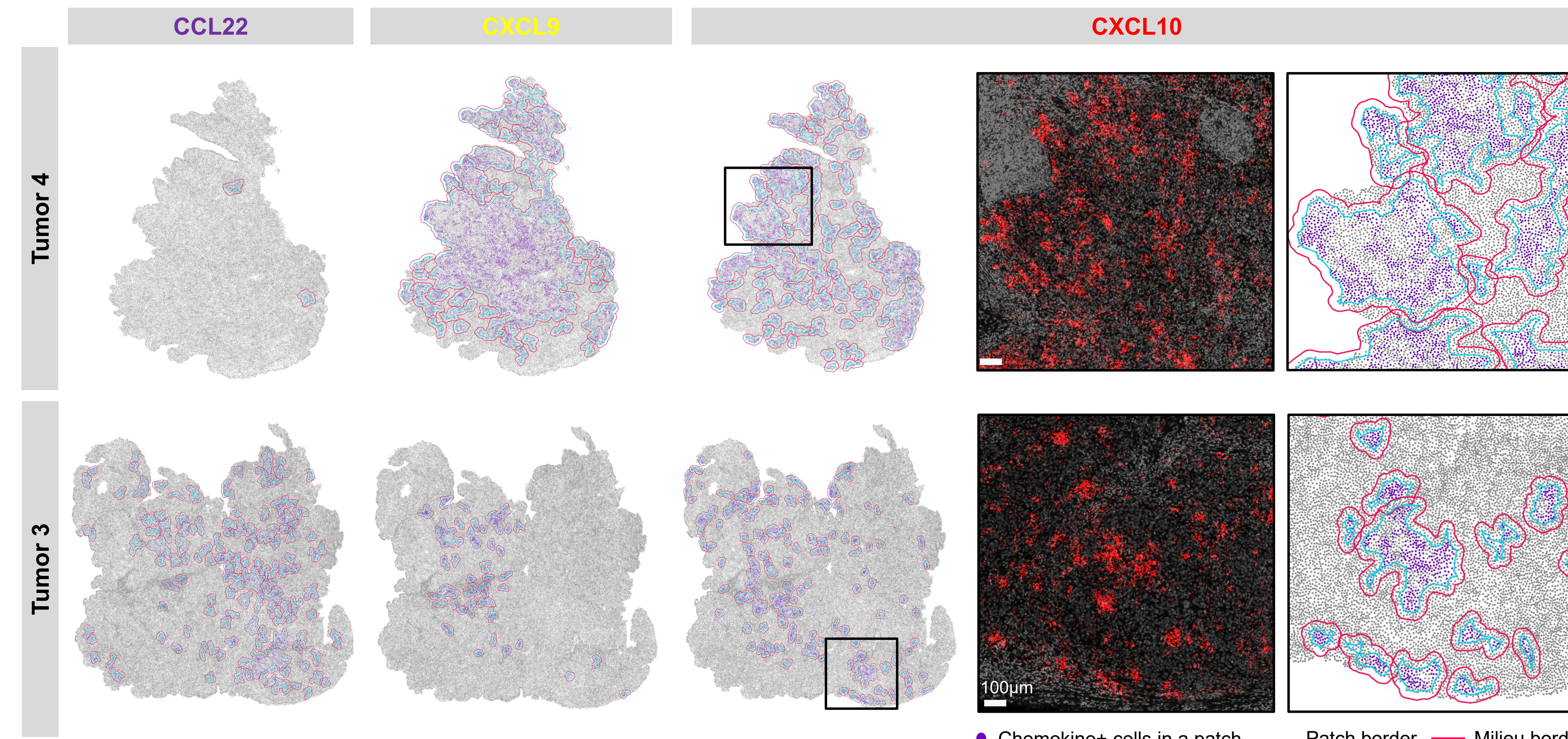
5.2. Single-cell Metabolic and Chemokine Signatures Reveal TME Heterogeneity in Tumor Regions



5.2 Chemokine expression landscape for distinct cell types, in combination with the cell lineage and metabolic profile (protein data), produced a multiomic spatial signature that distinguishes the four tumor regions identified in Section 4. The heatmap (top) shows chemokines with differential mean expression over tumor regions. Combined with the immune cell infiltration profile (protein data), we identified a correlation between CD8 T-cells and CXCL9 & 10. Integrated multiomic images (right) show **CXCL9 & CXCL10** overlaid on **CD8 T-cell** positions in the four tumor regions. Boxed regions, magnified on the right, illustrate distinct marker expression per tumor region. White dotted lines and arrows in the magnified Tumor 2 region depict putative CD8 T-cell recruitment at the T2 tumor margin. **Tumor regions 4 & 3 show clear differences in the amount of CD8 T-cell infiltration, which appears to be recruited by CXCL9 and CXCL10.**



5.3. Localized Patterns of Chemokine Expression Leads to Distinct Chemokine-derived CD8+ T Cell Recruitment in Different Tumor Regions

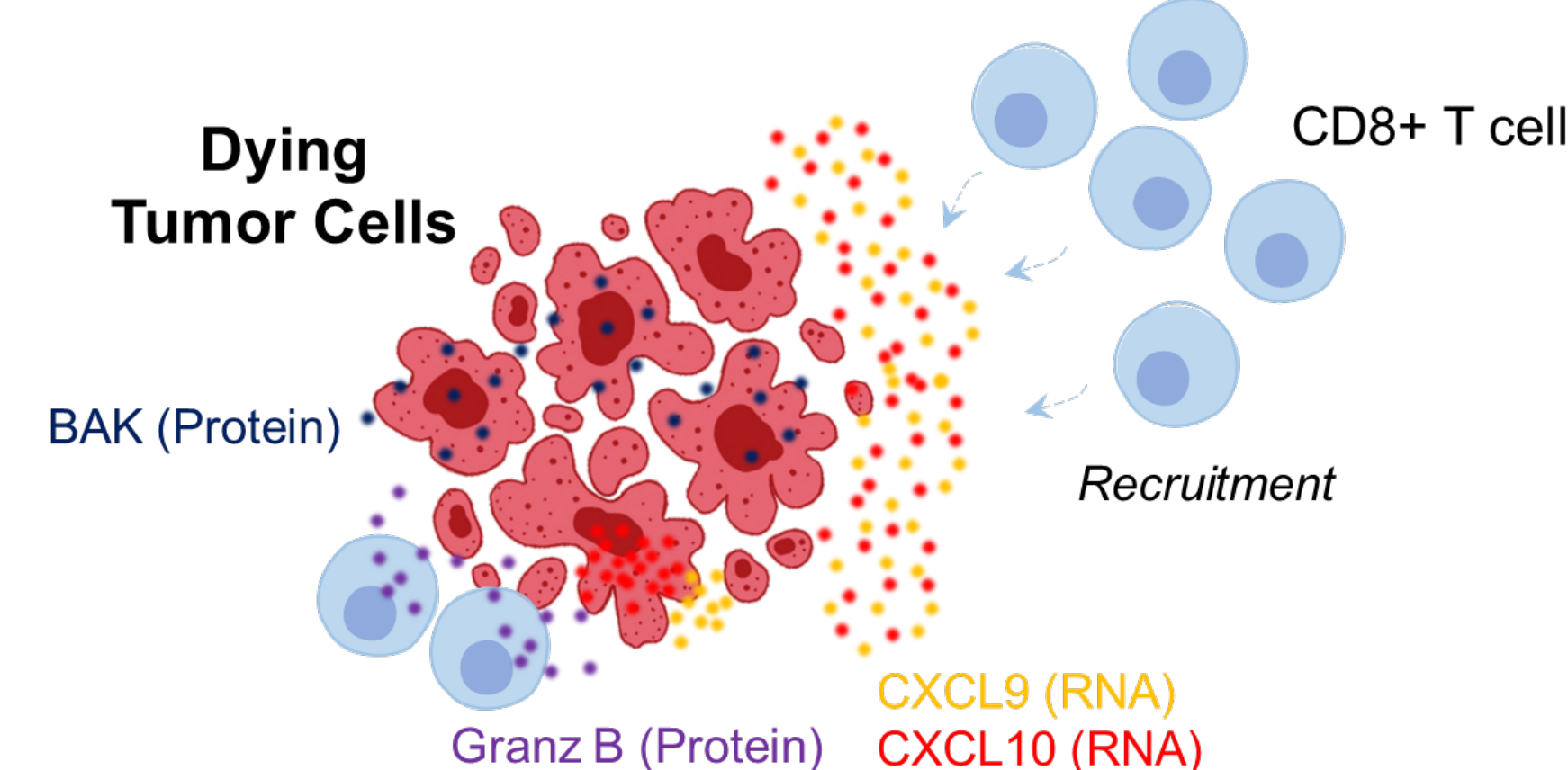


The sunburst plots (top) summarize the quantification of distinct chemokine milieus across the entire tissue, where each circle shows the fraction of cell types (as shown in the legend) associated with a certain chemokine milieu. **The differences in the proportions of CD8+ T cells that lie within the local enrichments of CCL22 or CXCL9 & 10 correlate with the TME heterogeneity in Tumor 3 & 4 regions.**

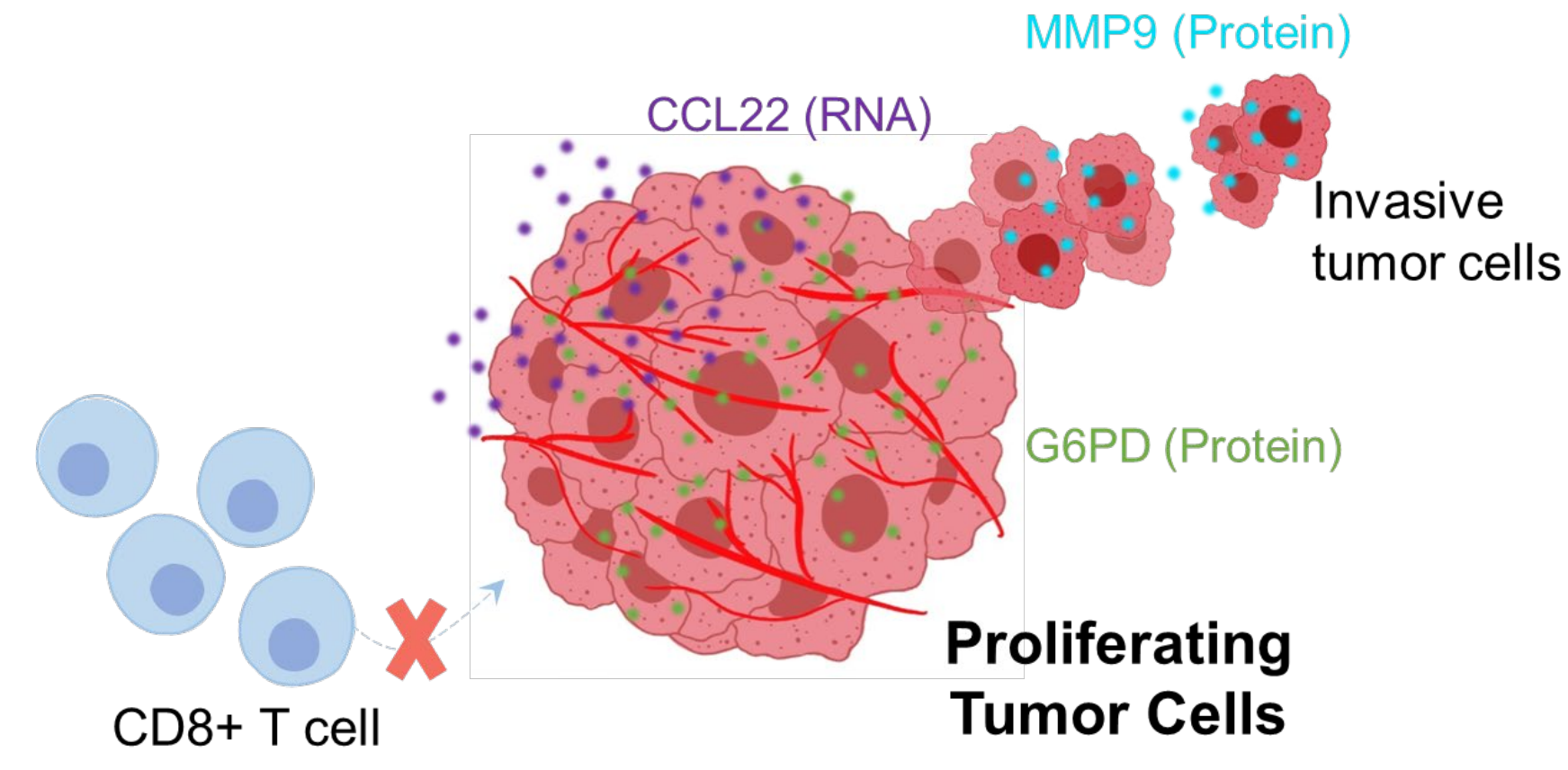
¹Hoch et al. Sci Immunol 2022. PMID: 35363540

6. Multiomic Spatial Phenotyping Provides Mechanistic Insights into Partial Immune Response of HNSCC Patient

Anti-tumoral activity (Immune-responsive)



Pro-tumoral activity (Immune-suppressive)



We used **multiomic single-cell spatial phenotyping** on the PhenoCycler-Fusion to provide a uniquely comprehensive anato-functional HNSCC tumor analysis.

- Deep spatial protein phenotyping uncovered tumor regions with heterogeneous metabolic, stress and immune-infiltration profiles. Distinctively, we saw tumor regions that were highly apoptotic (T4) versus regions that were proliferating and invasive (T3).
- Subsequent RNA-based spatial phenotyping of chemokine landscapes revealed potential regulatory roles, where (1) localized patterns of CXCL9 and 10 in T4 promoted anti-tumoral activity by recruiting CD8 T+ cells that release Granzyme B to induce apoptotic function, while (2) the expression of CCL22 produced the opposite effect in T3.

Collectively, these data demonstrate the power of multiomic spatial phenotyping and may, ultimately, produce information about new Spatial Biomarkers that can be used to stratify patients for ICI therapy.