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Introduction

Resolving cell phenotypes and spatial relationships between cell populations within the tumor microenvironment will have a significant impact on our understanding of tumor progression and response to immunotherapies.

Using Miltenyi Biotec's MACSima™ Imaging System and portfolio of pre-tested antibodies, hundreds of protein markers can be visualized on a single tissue section in a

non-destructive manner. Here we report on a novel combined spatial transcriptomics and proteomics approach on the MACSima Imaging System. We combined the MACSima Imaging Cyclic Staining technology (MICS) and Miltenyi Biotec's new gene expression technology RNAsky™ (for details on the RNAsky technology please refer to poster 5643) to characterize human FFPE tumor samples.

Methods

1 Spatial multiomics using the MACSima™ platform

In brief, human FFPE tissue samples were prepared for MICS (Figure 1). H&E staining was performed on an adjacent serial section for ROI selection. RNA transcript data was collected by using specifically amplified oligonucleotide detection probes conjugated to fluorochromes and using a proprietary signal removal system. Subse-

quently, antibody staining was performed on the same sample, using the newly established REAscreen Immuno-oncology Antibody Panel for human FFPE samples (Figure 2). Image processing, segmentation and single-cell analysis was performed in Miltenyi Biotec's MACS*i*Q View software suite.



Figure 1. The MICS technology enables the simultaneous analysis of hundreds of markers on a single sample. The cyclic process comprises three main steps that are conducted repeatedly by the MACSima Imaging System in a

fully automated manner: Staining with fluorochrome-conjugated antibodies (01), image acquisition of the stained sample (02), and erasure of the fluorescent signal (3).

2 REAscreen Immuno-oncology Antibody Panel

Cell State		Structure		Immune Cell Profiling		Tumor Characterization	
Proliferation	PCNA	Stroma and Vasculature	β-Actin	Lymphoid	CD45	Vimentin	Cytokeratin 19
	Ki-67		CD44		CD45RO		Cytokeratin 14
	Death				CD45RB		Cytokeratin 20
Cleaved PARP-1	Bcl-2	aSMA	CD31	Myeloid	CD279 (PD-1)	pan-Cytokeratin	CD66e (CEA)
			CD8a		CD274 (PD-L1)		β-Catenin
			CD8a		CD223 (LAG-3)		MART-1
		Podoplanin	CD20	Immune Activation/Function	CD107a	Cytokeratin HMW	PMEL
			CD11c		CD107b		p53
			CD38		CD163		
		Collagen I	CD79a	HLA-DR	CD204	Cytokeratin 5	
			CD138		CD209 (DC-SIGN)		
			CD56		CD1c		
		Calponin	CD57	CD134 (OX40)	Mast Cell Tryptase	Cytokeratin 8	
					HLA-ABC		

Figure 2. The REAscreen Immuno-oncology Antibody Panel for human FFPE samples contains 61 markers to identify immune cells, tumor stroma including blood and lymphatic vessels, and malignant epithelial cell pop-

ulations, as well as their proliferative or apoptotic state. It enables the analysis of at least 12 potential immune cell subsets within the tumor microenvironment, as well as the activation/checkpoint status of those populations.

Results

1 Spatial proteomics using MICS reveals distinct cell populations within the tumor microenvironment

FFPE tissue samples of colorectal adenocarcinoma (CRC), pancreatic ductal adenocarcinoma (PDAC), head and neck squamous cell carcinoma (HNSCC), cervical squamous cell carcinoma and melanoma were reviewed by a

pathologist in order to select ROIs. 2.6 x 1.7 mm ROIs were placed in tumor regions showing high immune infiltrate. FFPE tonsil tissue was used as internal control for each MICS experiment.

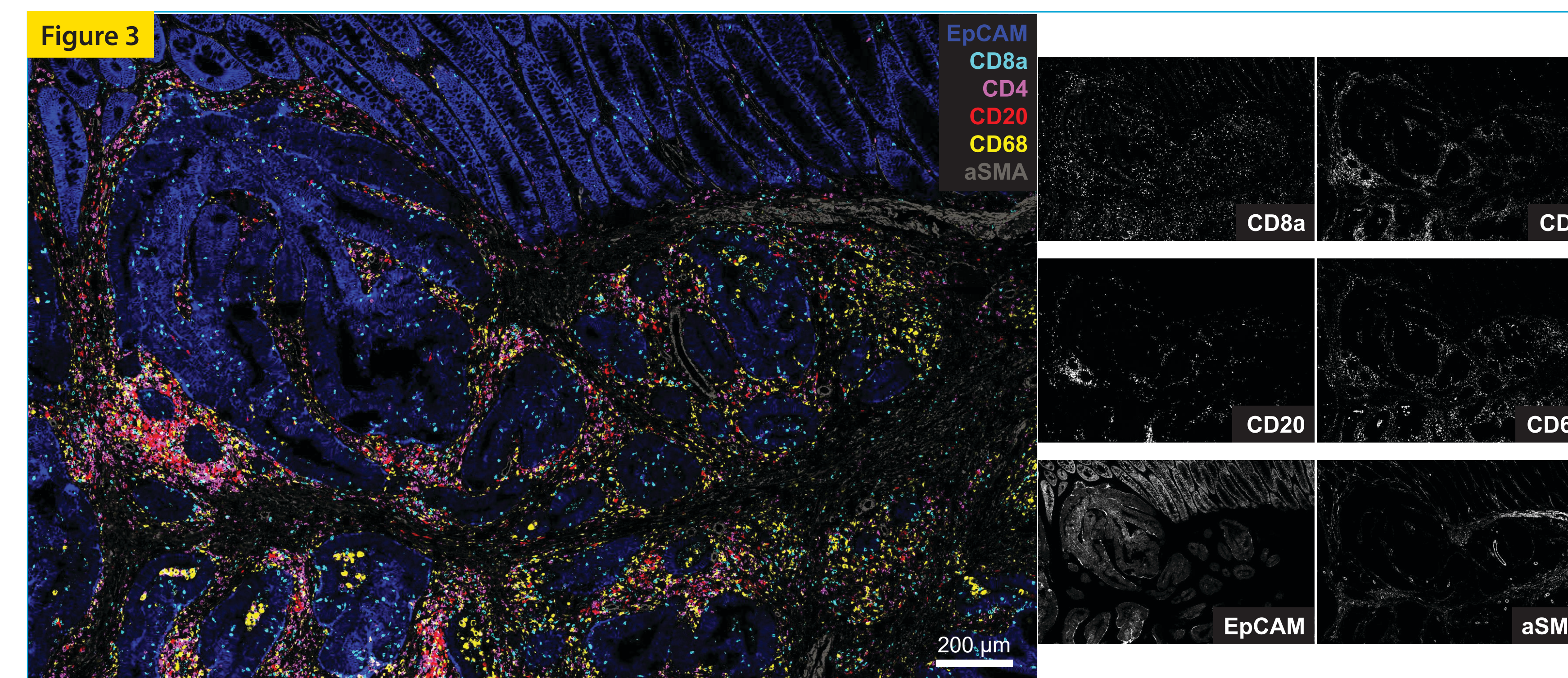


Figure 3. Whole ROI overview of a multiplex immunofluorescence staining of human colorectal adenocarcinoma tissue. The sample shows a low-grade infiltrating adenocarcinoma with colon mucosa and submucosa (upper

right) and a prominent lymphocyte infiltrate. A tertiary lymphoid structure is visible towards the left of the ROI. Biomarkers as indicated; single stains of each marker are displayed to the right.

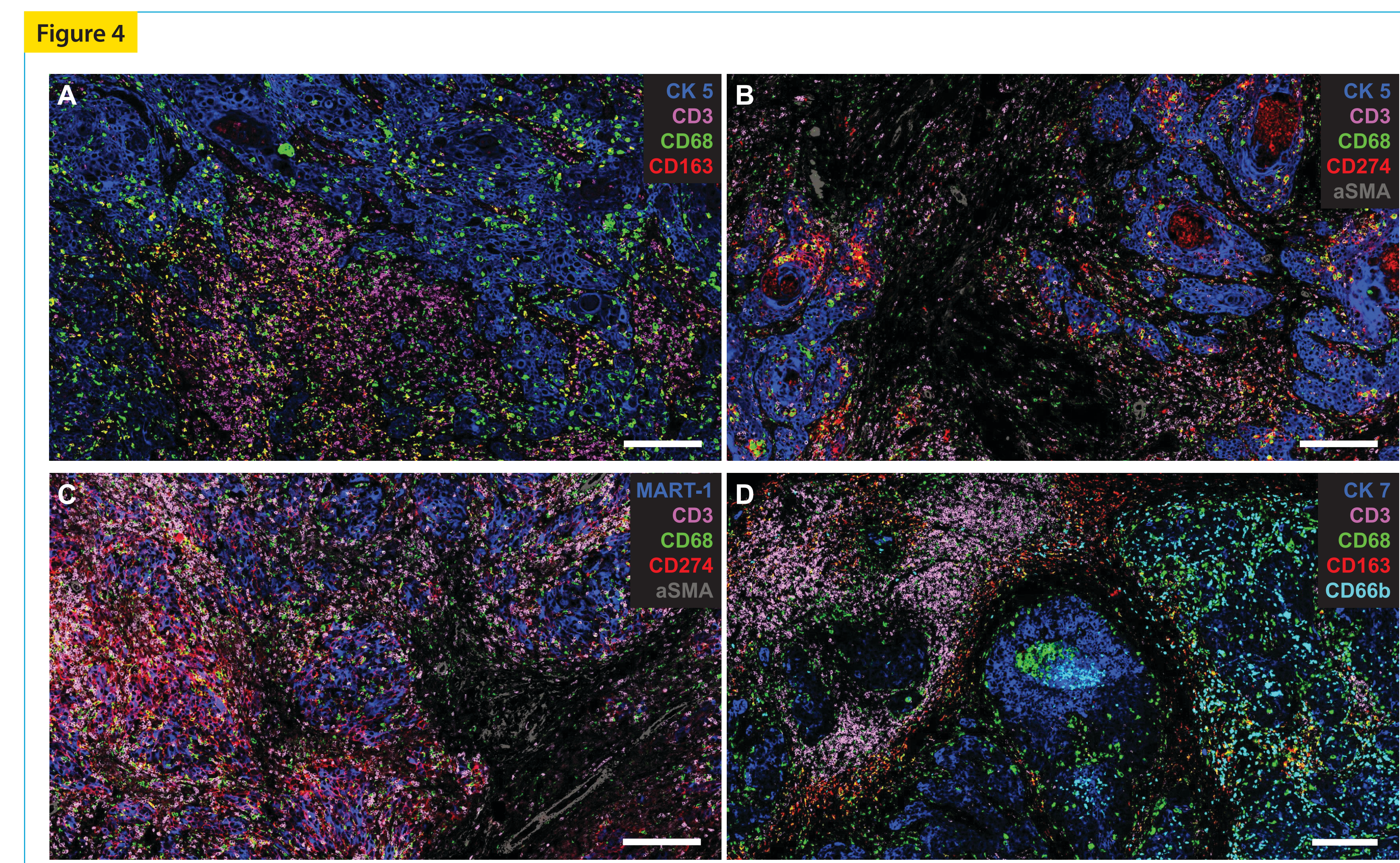


Figure 4. Magnified overviews of multiplex immunofluorescence stainings of human cancer tissues: A) cervical squamous cell carcinoma, B) head and neck squamous cell carcinoma, C) melanoma, D) pancreatic ductal adenocarcinoma. The different tissues show distinct distri-

bution of several immune cell populations within the tumor microenvironment. Biomarkers as indicated, overlap of red and green staining results in yellow color. Scale bars: 200 µm.

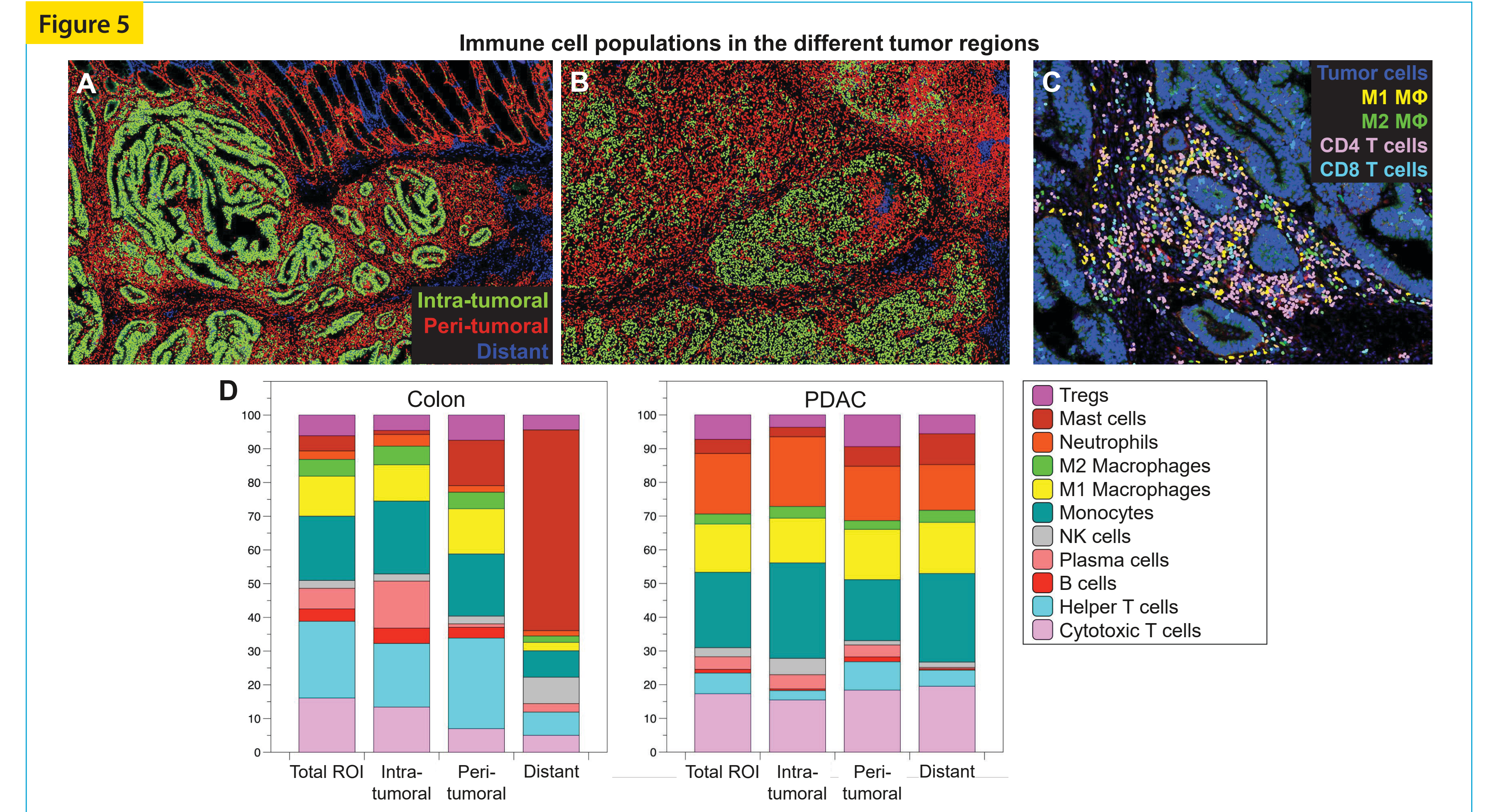


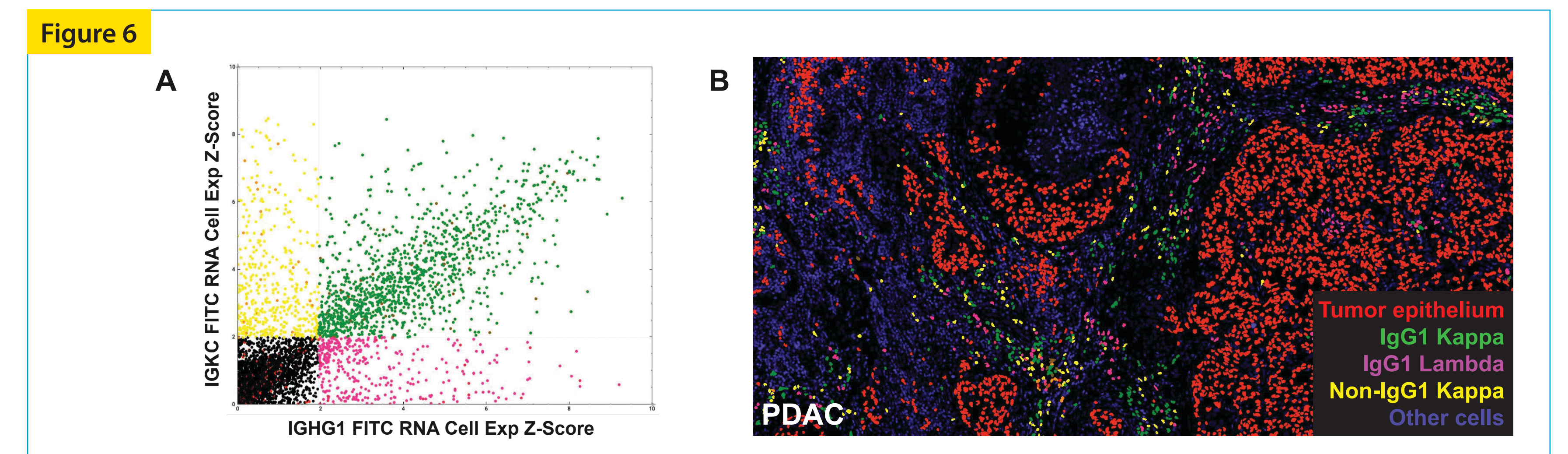
Figure 5. Immune cell populations were identified by cell gating, and distance maps were created. The intra-tumoral region was defined by the tumor cells and a surrounding distance of 5 µm, the peri-tumoral region included the adjacent 300 µm. Distance maps of the human CRC (A) and PDAC (B) samples. C) Magnification of the CRC sample, showing the identified immune cell populations. D) Percent of the indicated immune populations

per total number of immune cells within the respective tumor region. Cell populations were gated as follows: Tregs - CD4⁺ FoxP3⁺, mast cells - mast cell tryptase⁺, neutrophils - CD11b⁺ CD66b⁺, M1 macrophages - CD14⁺ CD68⁺ CD163⁺, M2 macrophages - CD14⁺ CD68⁺ CD163⁺, Monocytes - CD11b⁺, NK cells - CD56⁺, Plasma cells - CD79a⁺, B cells - CD20⁺, helper T cells - CD4⁺, cytotoxic T cells - CD8⁺.

2 The combination of proteomics and transcriptomics allows for unprecedented insight into cell phenotypes in a spatial context

Within a PDAC sample, cells were segmented and plasma cells identified by the marker CD79a. Subsequently, fluorescence signals for IGKC and IGHG1, generated by the RNAsky technology, were gated (Figure 6A) to determine plasma cell relationship based on immunoglobulin kappa or lambda light chain usage. 45% percent of IgG1-

expressing plasma cells expressed a lambda light chain, 55% a kappa light chain, indicating a normal kappa/lambda ratio of the tumor infiltrating plasma cells. Distribution of plasma cell clones within the tumor microenvironment is shown in Figure 6B.



Conclusion

With the introduction of the RNAsky™ technology, the MACSima™ has evolved to a true multiomics platform enabling protein and RNA detection on a single section. The shown characterization dataset demonstrates the ability of our platform to perform standardized in-depth

phenotyping of sample cohorts. This will enable discovery and further development of predictive and prognostic biomarkers critical to patient stratification, clinical management and response to immunotherapy.

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