Mapping the cancer immune landscape in colorectal adenocarcinoma using multiplexed imaging and AI based analysis

Published Abstract # 4914

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Introduction & Aim

Colorectal cancer is a high burden cancer in the United States, leading to the second highest cause of cancer deaths. Despite chemotherapeutic intervention and surgical resection, recurrent disease may occur in some patients and typically has a poorer prognosis. Thus, there is a demand for advanced therapies such as immunotherapies for improved clinical outcomes. An in-depth understanding of the colon cancer microenvironment is necessary to improve outcome in this subset of patients. Spatial biology approaches are well positioned to comprehensively uncover the molecular and biological mechanisms of cancer cell aggression in colorectal cancer. Here, we visualized nearly 30 biomarkers in colon adenocarcinoma (CAC) tissue using the Cell DIVE multiplexed imaging workflow and validated antibodies from Cell Signaling Technology (CST®). Using this approach, we were able to probe multiple pathways of interest in cancer progression, such as vascularization of tumor tissue, immune cell responses, and cell proliferation. Together, these data allow the creation of a spatial map of tumor cell aggression and offer predictive value for the progression of the disease. Deep molecular and architectural insights on this level are possible only with a spatial biology approach and offer multiple downstream hypotheses to further understand cancer progression and interrogate the heterogeneity within the tumor microenvironment.

Results

AI-guided analysis was utilized to analyze the CAC tissue which revealed single cells characterized by expression of multiple markers for epithelial, mesenchymal, and immune cell types (**Fig.1**). Clustering analysis showed a diverse range of lymphoid cell populations with activated T-cell population and regulatory T-cells (T-regs) in clusters 16 & 4, and B-cells and NK-cells within cluster 12 (pink boxes in **Fig. 2A**). These cells also expressed immune checkpoint markers like CTLA4, PDL1, PD1, and LAG3, possibly serving as an escape mechanism from the immune system. The vasculature (clusters 13,19, & 11,5 in **Fig. 2A**) and the myeloid cell populations (2, 20, & 8 in **Fig. 2A**) showed interactions with larger groups of clusters specific to cellular metabolism (blue box in Fig. **2A**) and proliferation (cluster 7 & 9 in **Fig. 2A**). Within the myeloid cell population, tumor-associated macrophages or TAMs (cluster 20, green box **in Fig. 2A**) were closely linked with endothelial cell populations (clusters 13 and 19, red box in **Fig. 2A**). Specifically, tumor-associated macrophages (TAMs) are characterized by their ability to facilitate tumor progression by supporting angiogenesis, and preferentially accumulating around blood vessels. We noted the spatial positioning of TAMs, marked by heterogenous expression of CD68+ and CD163+ cells close to the blood vessels in CAC (Fig. **2B**). Interestingly, expression of TIM3 and vimentin was also observed in TAMs which has been associated with disease progression and poor prognosis (cluster 20, green box **in Fig. 2A**). Next, dimensionality reduction (UMAP in **Fig. 2C**) allowed us to visualize cell phenotypes (**Fig. 2D**) within the heterogeneous CAC tissue and broadly clustered them into groups associated with tumor progression, innate and adaptive immune cell populations within the stroma. Additionally, dimensionality reduction **(Fig. 2C**) revealed a distinct population expressing B-cell, T-regs, immune checkpoint and metabolic markers, potentially indicating metabolic reprogramming and autoimmune regulation within the CAC tissue. Overall, the Cell DIVE + AIVIA analysis workflow revealed key cell populations like TAMs, vascular endothelial cells, and immune regulatory cells that malignant cells may recruit to promote tumor growth (**Fig. 2**).

Table 1. Study Design: Antibodies and Tissues

Target	Clone	Conjugate	Concentration	Dilution
SMA	1A4	AF488	125 µg/mL	1:100
SOX9	D8G8H	AF488	100 µg/mL	1:100
GAPDH	14c10	AF488	100 µg/mL	1:100
NaK	D4Y7E	AF488	100 µg/mL	1:100
PANCK-AE1/AE3	53-9003-82	AF488	100 µg/mL	1:100
CD68	D4B9C	AF555	4.5 µg/mL	1:100
CD79A	D1X5C	AF555	100 µg/mL	1:100
CD3E	D7A6E™	AF555	100 µg/mL	1:100
CD4	D7D2Z	AF555	200 µg/mL	1:100
GZMB	D6E9W	AF555	200 µg/mL	1:100
CD31	89C2	AF555	47 µg/mL	1:100
CD4	D7D2Z	AF555	200 µg/mL	1:100
CD20	E7B7T	AF647	100 µg/mL	1:100
CD163	D6U1J	AF647	100 µg/mL	1:100
Ki-67	D2H10	AF647	100 µg/mL	1:100
FOXP3	D2W8E™	AF647	500 µg/mL	1:100
TIM-3	D5D5R	AF647	100 µg/mL	1:100
CD56	E7X9M	AF647	200 µg/mL	1:100
PDL-1	E1L3N	AF647	200 µg/mL	1:100
CTLA-4	E2V1Z	AF647	200 µg/mL	1:100
pNDRG1	D98G11	AF647	25 µg/mL	1:100
SURVIVIN	71G4B7	AF647	50 µg/mL	1:100
Vimentin	D21H3	AF750	100 µg/mL	1:100
CD8α	D8A8Y	AF750	100 µg/mL	1:100
CD45	D9M8I	AF750	100 µg/mL	1:100
PD-1	D4W2J	AF750	100 µg/mL	1:100
CD11B	D6X1N	AF750	200 µg/mL	1:100
LAG3	D2G4O	AF750	200 µg/mL	1:100
CD11C	D3V1E	AF750	100 µg/mL	1:100

Slide	Tissue	Catalogue Number
1339	Adenocarcinoma, Colon	COL04

Methods & Materials

CST antibodies undergo a vigorous validation process to ensure antibody performance on FFPE tissue. All antibodies in this study were direct conjugates (**Table 1**). Following preliminary validation, conjugated antibody solutions with the optimum degree of labeling and concentration were randomly assigned to a round, without optimization and used for subsequent staining of CAC tissue. Tissue was obtained from a commercial source (Pantomics; **Table 1**). Slides were imaged on the Cell DIVE imager using four channels plus DAPI, with automatic AF removal, corrections, and stitching. Imaging rounds were conducted over a 2-week period. At round 10 slides were stored for long term at 4°C for future experiments. Fully stitched images (**Figure 1A**) were imported, fused, segmented, and analyzed using AIVIA 13.1 (**Figure 1B**). Using AI-driven analysis, the expression of markers (shown in **Table 1**) was characterized through clustering and dimensionality reduction (UMAP) methods (**Figure 2**).

Conclusion

Iterative staining and imaging of CAC tissue with CST antibodies and the Cell DIVE multiplexed imaging solution enabled spatial characterization of a highly heterogeneous tumor environment. This study revealed that tumor cells in the CAC sample deregulate cellular metabolism, heighten proliferative signaling, drive angiogenesis, and activate tumor-promoting inflammation, all while evading destruction by the immune system. Notably, our analysis revealed crucial insights about the immune landscape within the CAC tissue, for example, TIM3 expression in tumor-associated macrophages which may serve a promising therapeutic target.

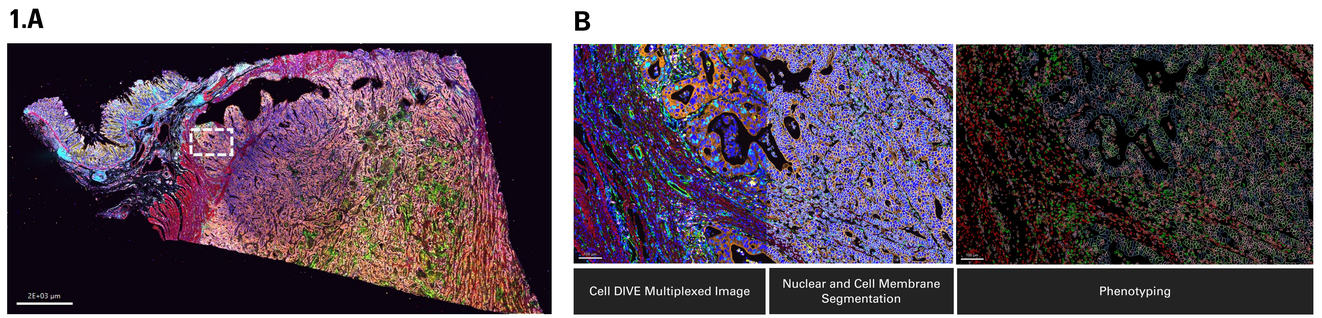

Figure 1. Cell DIVE + AIVIA multiplexing and analysis workflow to understand the heterogeneous tumor microenvironment. Multiplexed Cell DIVE imaging of **A.** Colon Adenocarcinoma (CAC) tissue. A panel of approximately 30 biomarkers targeted towards various leukocyte lineages, epithelial, stromal, and endothelial cell types was utilized to characterize the tumor immune microenvironment in human colon adenocarcinoma (CAC) tissue. **B.** The final image was utilized to perform cell membrane and nuclear membrane segmentation to identify individual cells using an AI-powered cell detection recipe, followed by phenotyping to assign phenotypic markers to individual cells.

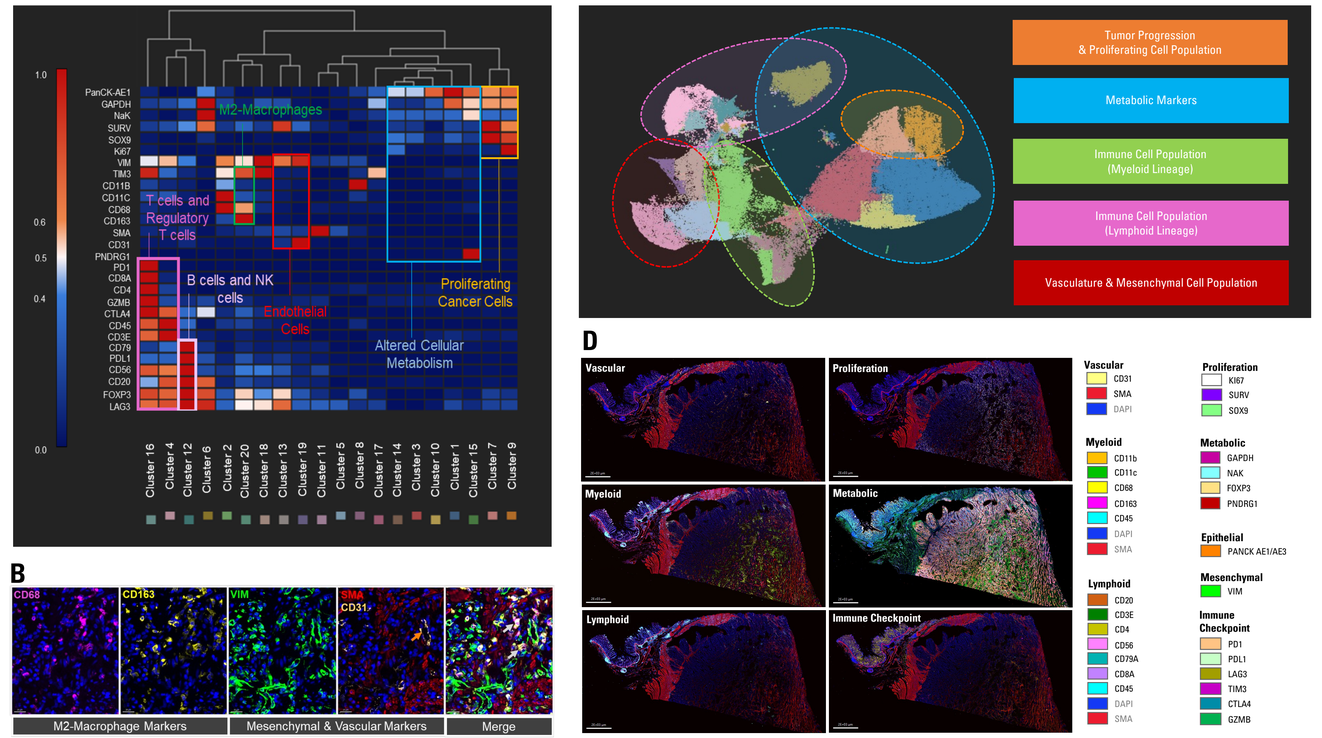

Figure 2. Understanding the heterogeneous spatial landscape in CAC. A. Clustering analysis within the CAC tumor reveals hierarchical relationships between markers. Heatmap indicates a measure of marker strength within a given cluster. 20 clusters were identified using PhenoGraph-Leiden algorithm on AIVIA to identify complex and non-linear relationships between markers in CAC tissue. **B.** Presence of Tumor-associated macrophages (TAMs shown using white arrows) co-expressing CD68 and CD163 in close proximity to mesenchymal and vascular makers including VIM, SMA, and CD31 (CD31 shown using orange arrow). **C.** Dimensionality reduction (UMAP) indicates all the identified phenotypic clusters and groups these clusters while preserving the local and global relationships within the multiplexed Cell DIVE CAC tissue. Various markers and groups of markers within the CAC tissue clustered broadly in the form of tumor progression markers (orange), myeloid markers (green), vascular markers (red), lymphoid markers (pink), and cancer metabolic markers (blue). **D.** Spatial organization of markers associated with these categories reveal complex relationships within the tumor immune microenvironment.

Questions?
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