# Multiplexed analysis across cancers of diverse origins to unravel spatial interactions within the tumor microenvironment

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## Introduction & Aim

The tumor microenvironment (TME) is heterogeneous and is primarily composed of fibroblasts, extracellular matrix, immune cells, and blood vessels. Importantly, the tumor immune microenvironment (TIME) is a major source of cancer heterogeneity and influences both disease progression and response to therapeutic interventions. Malignant cells typically recruit various cell types such as vascular endothelial cells, cancer-associated fibroblasts (CAFs), and tumor-associated macrophages (TAMs) to promote tumor growth. Therefore, studying such a complex interplay among tumor, stromal cells, and immune cells within the TME necessitates a multiplexed analytical approach to investigate cancerous tissues from diverse origins, to ultimately predict clinical outcomes and design novel therapies. While numerous studies have been focused on investigating the expression patterns of immune-oncology markers within specific tissues, the extent to which such marker expression patterns are shared across tumors originating from various tissues is not adequately understood. In this study, we analyzed a large panel of Cell Signaling Technology (CST®) antibodies that are targeting immune, stromal, epithelial, and vascular markers in cancer tissues derived from various origins including lung, colon, and pancreas. Using multiplexed Cell DIVE imaging, key spatial interactions in the tumor microenvironment that are (1) tissue-specific, and (2) shared across tumors irrespective of origin were determined. Specifically, cluster analysis of a common panel of markers across tissues of different origins uncovered molecular signatures that are common to all cancer tissue types as well as those that are unique to specific tissue types to advance our understanding of the disease, and its progression, shedding light on the intricate interactions within the TME.

## Results

Clustering analysis offers an unbiased approach to identify molecular signature associated with following cancer tissue types- **A. Ovarian Mucinous Cystadenocarcinoma:** Clustering reveals lymphoid cell populations such as Cluster 19 expressing CD56, CD45, along with expression of exhaustion marker, TIM3, and Cluster 4 indicative of NK cells that were observed in the ovarian cancer tissue. Increased expression of CD56 is typically associated with advanced tumor stage in ovarian carcinomas. Furthermore, Cluster 10 indicates a diverse myeloid cell population with potentially immunosuppression and tumor promoting effects. **B. Pancreatic Ductal Adenocarcinoma:** Clusters expressing CD163, CD68, CD11c and TIM3 indicate TAMS and dendritic cell infiltration, associated with immunosuppression and poor prognosis. Cluster 15 representing a combination of cytotoxic T cells and helper T cells, as well as cluster 10 indicative of Tregs also co-express immune checkpoint molecules such as PD1, TIM3, and CTLA4. **C. Large Cell Lung Carcinoma:** Clusters expressing CD45, PD1, CD3, CTLA4 indicate infiltrating T cell expressing immune checkpoint markers, suggestive of immune evasion and poor prognosis. While Clusters showing B cell infiltrating within TME in Cluster 13 are potentially involved in anti-tumor immunity. Clusters expressing CD68, CD163, VIM and TIM3 represent TAMs indicating tumor promoting stroma. **D. Colon Adenocarcinoma:** Co-expression of Foxp3 and CD11b in Cluster 5 suggests potential involvement of regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs) possibly indicating immune suppression within tumor microenvironment. Clusters expressing VIM, CD11c, CD11b, and Foxp3 potentially indicates a subset of TAMs expressing Foxp3 suggesting a regulatory phenotype contributing to tumor growth and immunosuppression. Taken together, the TME for various cancer types reveals differential immune reprogramming revealing unique molecular clusters associated based on tissue type.

Table 1. Study Design: Antibodies and Tissues

| Target | Clone | Conjugate | Concentration | Dilution |
|---|---|---|---|---|
| **SMA** | 1A4 | AF488 | 125 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 |
| **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 |
| **CD11C** | D3V1E | AF750 | 100 µg/mL | 1:100 |

| Slide | Tissue | Catalogue Number |
|---|---|---|
| **1330** | Ovary Mucinous CystAdenocarcinoma | OVA13 |
| **1333** | Pancreatic Ductal Adenocarcinoma | PAC02 |
| **1334** | Lung Large Cell Carcinoma | LUN17 |
| **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 various cancer tissue types. Tissues were 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 1**) were imported, fused, and analyzed using AIVIA 13.1. Using AI-driven analysis, the expression of markers (shown in **Table 1**) was quantified for the segmented phenotypes and used for subsequent clustering analyses (**Figures 2 and 3**).

## Conclusion

Clustering analysis on the CST panel offers insights into the unique cellular composition and immune microenvironment of each cancer type, highlighting potential prognostic implication and therapeutic targets. Cell DIVE multiplexing solution allows further probing to comprehensively characterize the immune, stromal, and epithelial cell types within the tumor microenvironment. Future studies focused on specific sections of the cancerous tissues of various origins delineated by metabolic marker expression can reveal novel spatial characteristics associated with the disease.

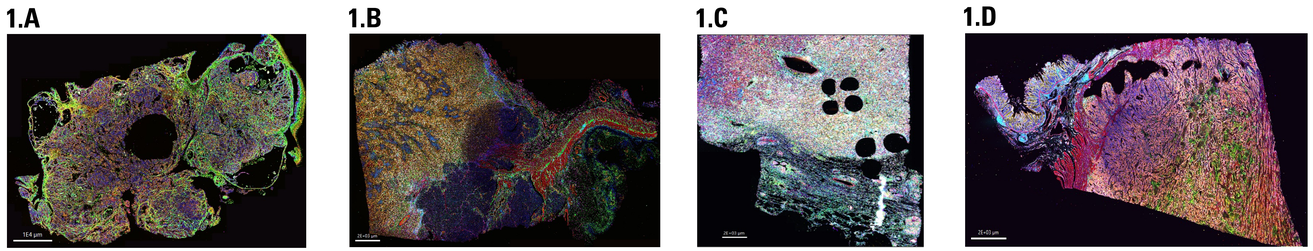


**Figure 1. Multiplexed Cell DIVE imaging in various cancer tissue types. A.** mucinous cystadenocarcinoma of the ovary, **B.** pancreatic ductal adenocarcinoma, **C.** large cell lung carcinoma, and **D.** colon adenocarcinoma

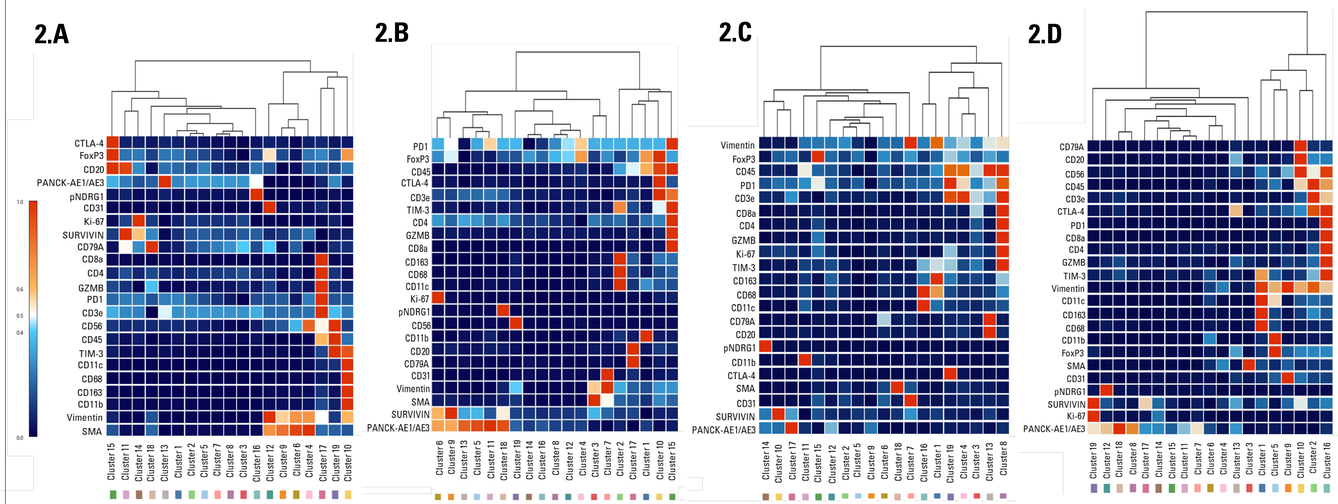


**Figure 2.Identification of clusters containing cells of different immune classes, cell types or subtypes in A.** mucinous cystadenocarcinoma of the ovary, **B.** pancreatic ductal adenocarcinoma, **C.** large cell lung carcinoma, and **D.** colon adenocarcinoma. Based on biomarker expression patterns, clustering of different cell population is determined in each cancer tissue type in a non-biased method to determine interactions that are tissue specific and those that are common to all cancer tissue types within this study.

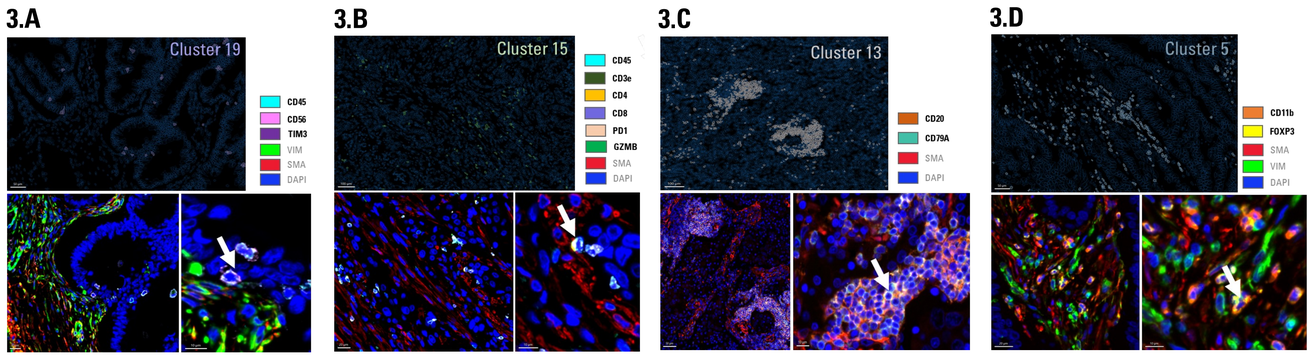


**Figure 3. Multiplexed Cell DIVE imaging** of **selected clusters and unique cell populations identified in A.** mucinous cystadenocarcinoma of the ovary, **B.** pancreatic ductal adenocarcinoma, **C.** large cell lung carcinoma, and **D.** colon adenocarcinoma. Markers are highlighted by color for each cancer type.

**Questions?**
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