

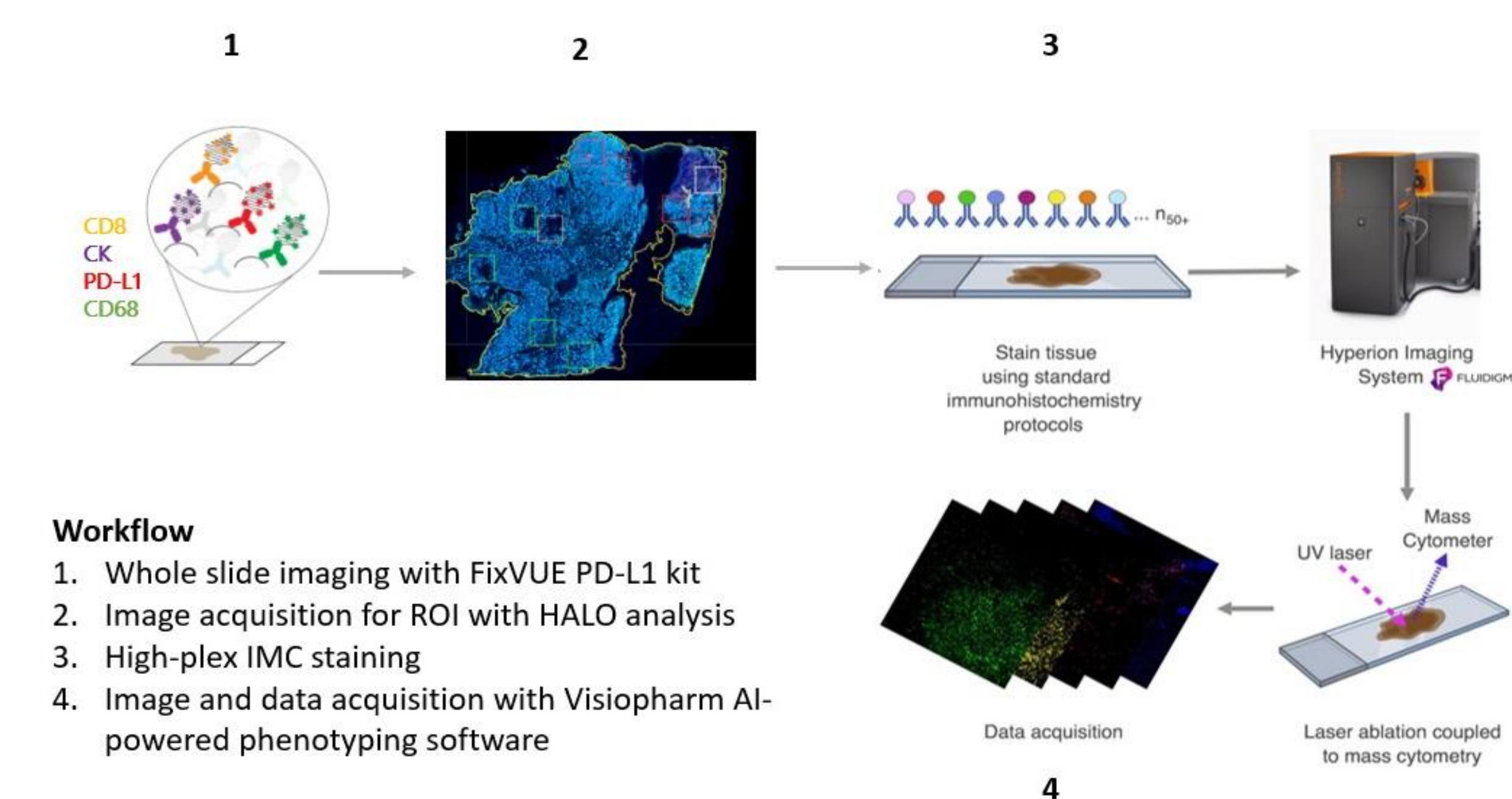
### Introduction

Pancreatic cancer remains a deadly disease due to difficulties hindering its early diagnosis, giving way to metastasis of the tumor and resulting in poor prognosis. While there are many neoplasms of the pancreas, pancreatic invasive ductal adenocarcinoma (PDAC) is the most common, and treatment options are few, with poor overall survival.

The complexities of the tumor microenvironment (TME) have been implicated in the failure of chemotherapy, radiation therapy, and immunotherapy. The tumor microenvironment of PDAC is especially rich with multiple interactions between pancreatic epithelial/cancer cells, stromal cells, immune cells, and the extracellular matrix (ECM). PDACs are characterized by a complex ECM of desmoplastic reaction consisting of an extensive and dense fibrotic stroma that surrounds and infiltrates clusters of malignant epithelial cells, together with the loss of basement membrane integrity and an abnormal vasculature.

In the present study we demonstrate a tissue phenotyping workflow combining three complementary methods that can unravel novel insights in the complex tumor microenvironment. This novel workflow delivers tissue morphology information, spatial phenotyping of immune cell population on whole slides, and high-dimensional imaging in selected regions of interest (ROIs) by combining H&E, multiplex immunofluorescence (mIF), and Imaging Mass Cytometry® (IMC®).

### Workflow



**Figure 1. Three complementary methods to address the complexity of the TME.** Whole slide imaging performed by Ultivue, targeted ROI selection from Standard BioTools and data analysis by Visiopharm.

### Materials and Methods

Serial sections were freshly cut from three FFPE blocks containing pancreatic ductal adenocarcinoma (PDAC) tissue samples obtained commercially from BioIVT. One section from each block was stained with the InSituPlex® (ISP) automated assay protocol by Ultivue using the FixVUE I/O PD-L1 kit. The resulting images were reviewed by a pathologist and multiple 2mm<sup>2</sup> Regions of Interest (ROIs) were selected based on PD-L1 expression and tumor morphology. Based on ISP-guided ROI selection, specific ROIs on two adjacent sections (on each side of the ISP-stained section) were stained using a custom panel from Standard BioTools (SBT) Therapeutic Insights Services Laboratory using the current Imaging Mass Cytometry Staining Protocol for FFPE Sections.

The images obtained using the FixVUE I/O PD-L1 kit were used to select the ROIs for ablation. The annotated images were imported in the instrument software (v.7.1) and aligned with the slide stained for IMC acquisition and the multiplexed data was acquired at 400Hz on the prototype design Hyperion+™ imaging system.

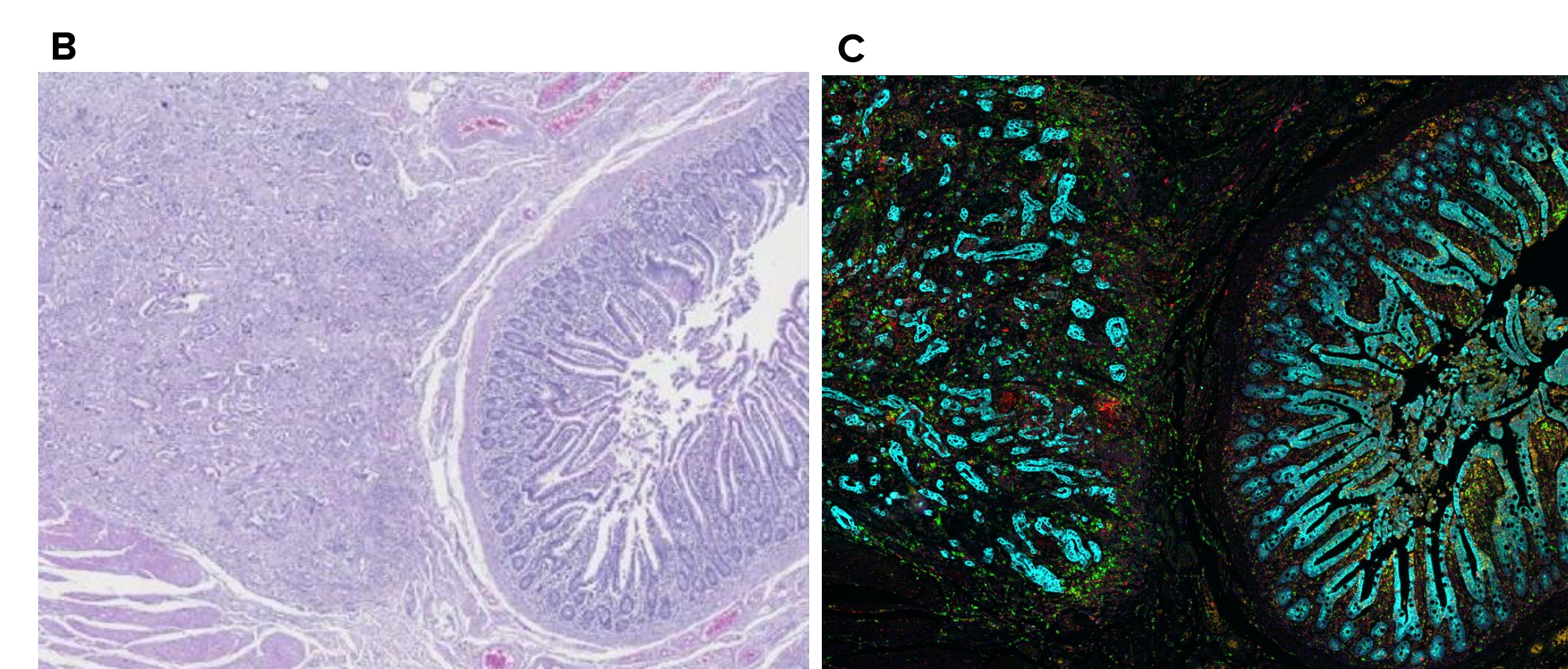
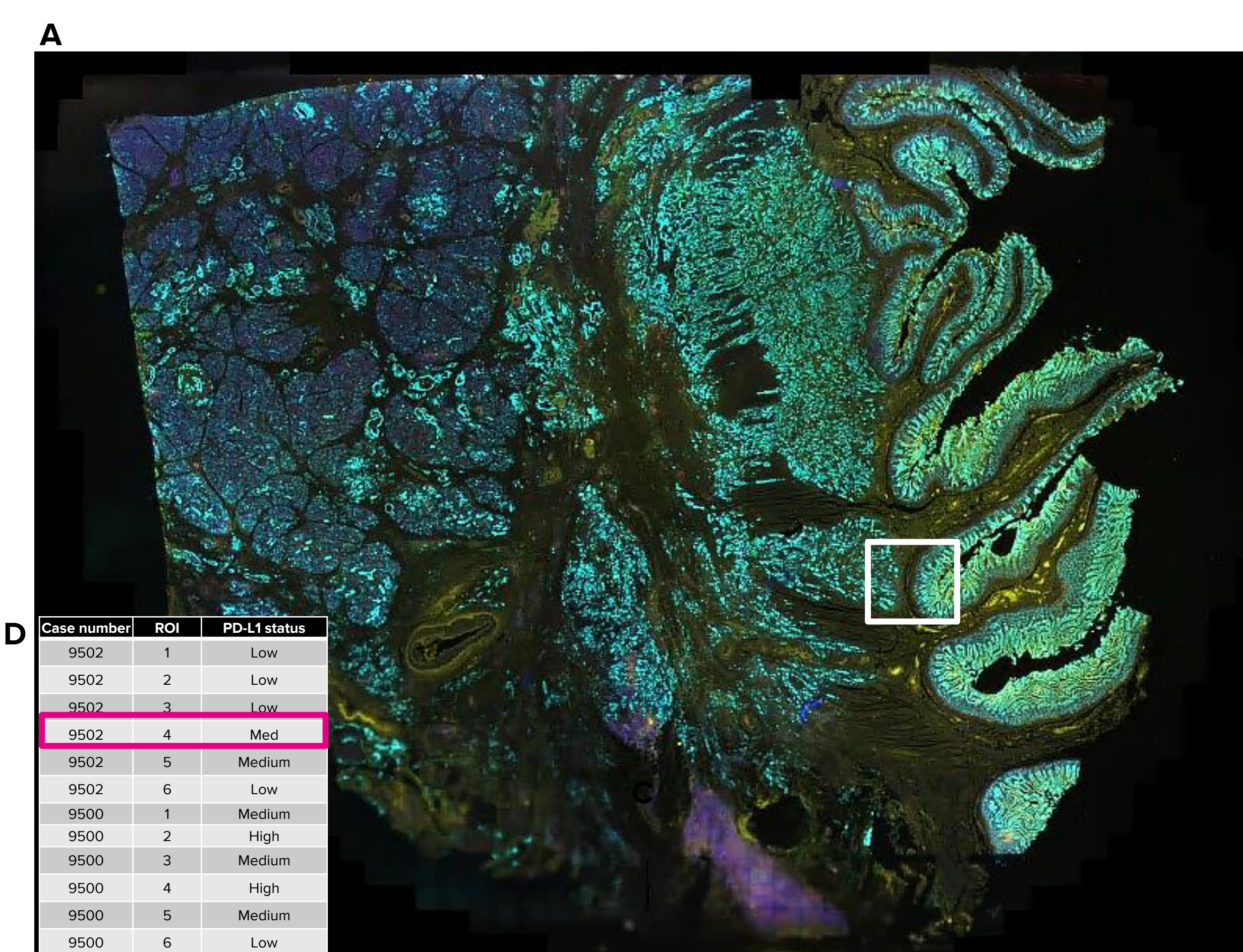
The raw MCD files were imported directly and analyzed in the Visiopharm software platform (Hørsholm, Denmark). Tissue compartments (Tumor, Stroma, and Blood Vessels) were automatically identified using thresholds after combining the Pan-CK + E-Cadherin channels to identify Tumor compartments, and the aSMA + Collagen-1 channels for identifying Stroma compartments and CD31 channel for identifying blood vessels.

For detecting nuclei, a pre-trained Deep Learning algorithm available with the Visiopharm platform was used (U-Net architecture). To account for irregular morphology of heterogeneous cell populations, ICSK channels were included in the classification to improve the delineation of cells boundaries. Visiopharm's Multiplex Phenotyping module was used to automatically identify the range of biomarker combinations in the identified cells. The classification of each biomarker was semi-supervised and gated using two independently controlled parameters: signal intensity and percent coverage.

A second analysis pipeline starts with the raw MCD data was processed, and image analysis stacks were generated using python. Image stack preprocessing was performed in CellProfiler. The preprocessing steps included were hotspot removal and scaling the images by 2x for easier pixel classification.

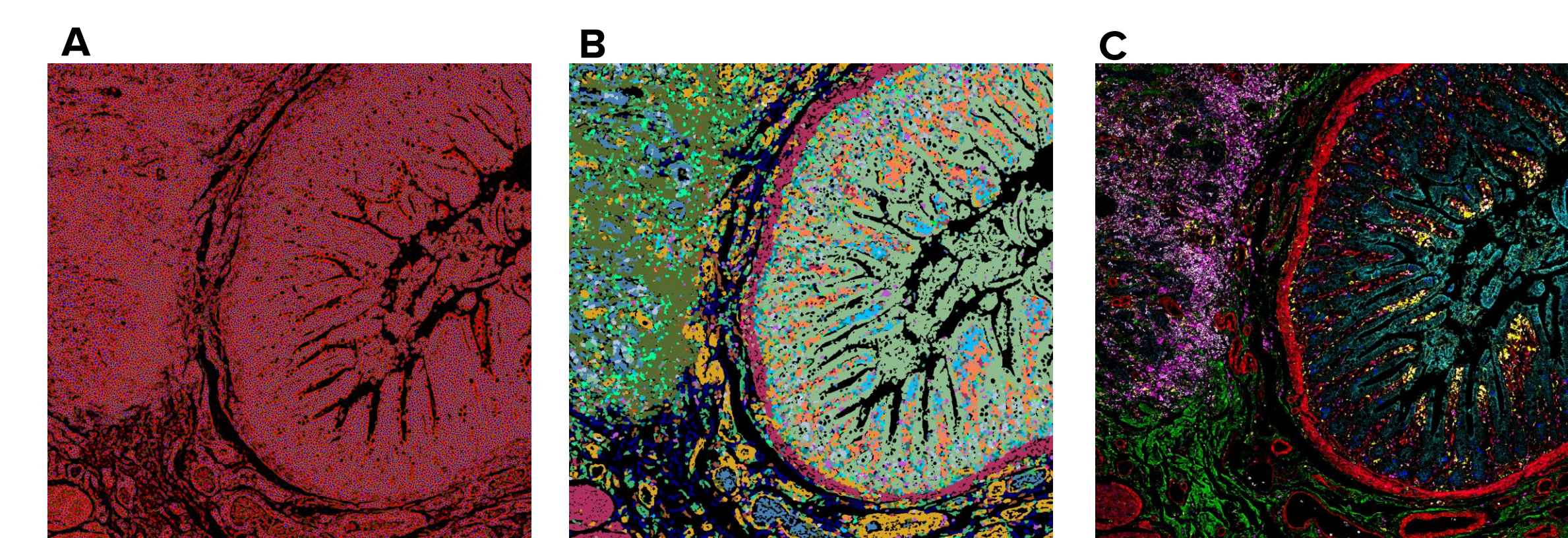
### Results

#### Whole Slide Imaging of PDAC tissue



**Figure 2. Whole slide imaging and ISP-guided ROI selection**  
A. Whole slide image of Case 9502. White box indicating ROI 4. B. H&E image of ROI 4. C. ISP image showing 4 fluorescent markers (PDL-1, PD-1, CD8 and Pan-CK) of ROI 4 and D. Table of Case Number, ROIs selected by Ultivue and their PDL-1 status.

#### Single-Cell Analysis

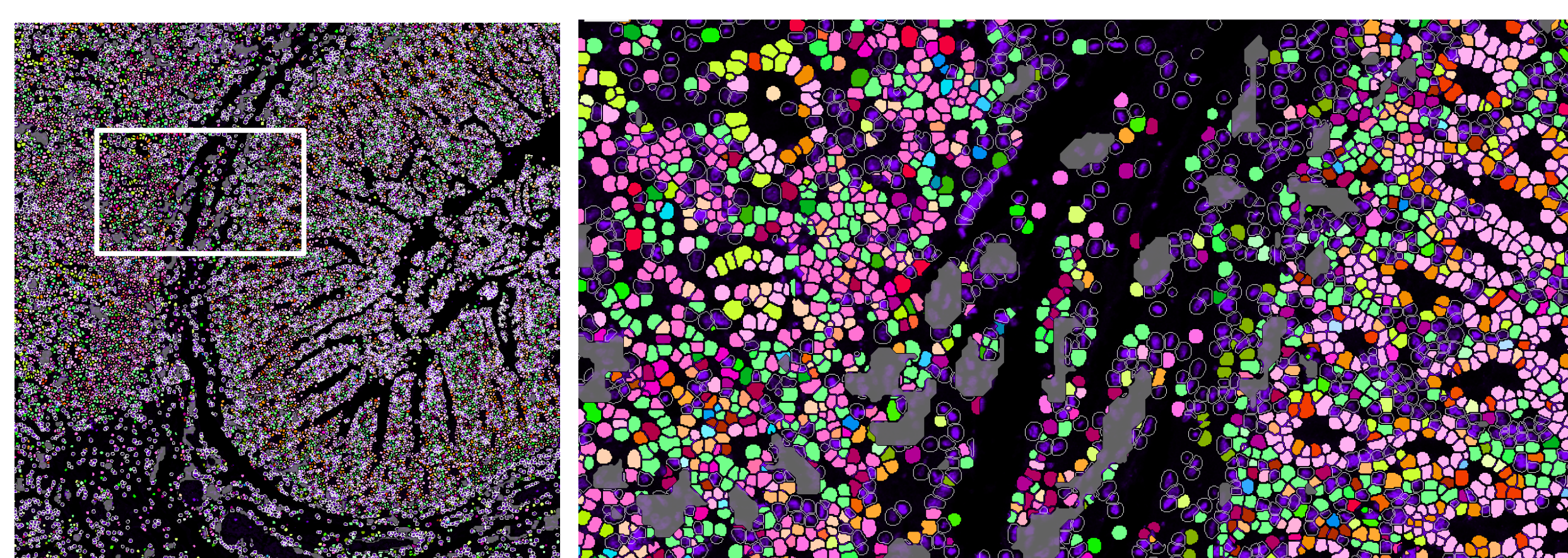


**Figure 3. Cell Segmentation, Clustering Overlay and IMC staining**

A. Pixel-based classification of cell segmentation when using both the Iridium-Blue to identify nuclei and the ICSK markers (Pt194, Pt196, Pt198-combined Red) to demark plasma membrane yields highly reproducible and excellent cellular delineation. B. Clustering overlay of cell phenotypes. C. IMC staining of a 40-marker panel showing 6 markers above, aSMA-Red, HLADR-Yellow, E-Cadherin-Cyan, Collagen 1-Green, CD68-Magenta, iNOS-Blue and Granzyme B-White

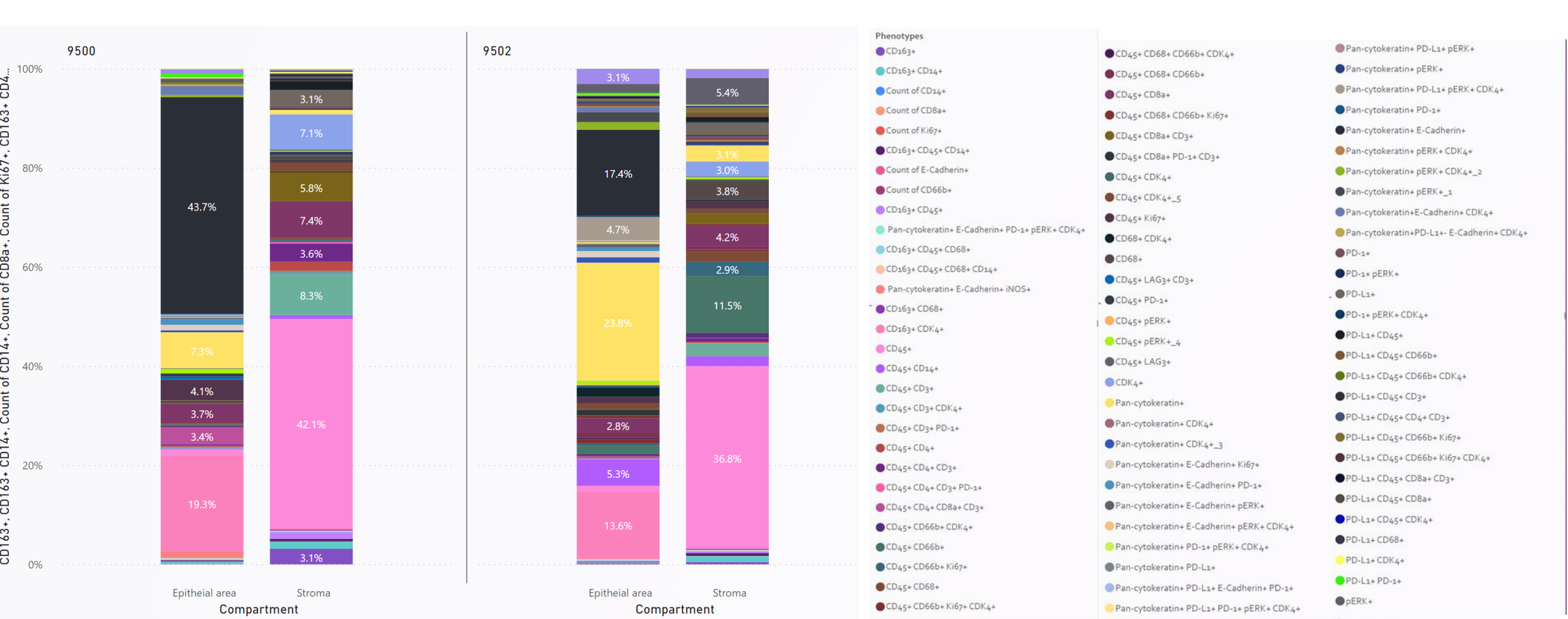
For cell segmentation, an ilastik pixel classifier was trained using the ICSKs and DNA channels. All ROIs were labeled as either DNA, Cytoplasm/Plasma Membrane, or non-cellular. The outputs from this classifier were segmented in CellProfiler. First, the nuclei were segmented, and used as seeds for the cell segmentation. Identified nuclei less than 2.5 microns (5 pixels after 2x scaling) in diameter were discarded. Cell boundaries were determined by propagating outwards from the nuclear seeds along the cytoplasm prediction.

After segmentation, single-cell data was quantified using HistoCAT and clustered using the phenograph algorithm with hyper parameter k = 30.



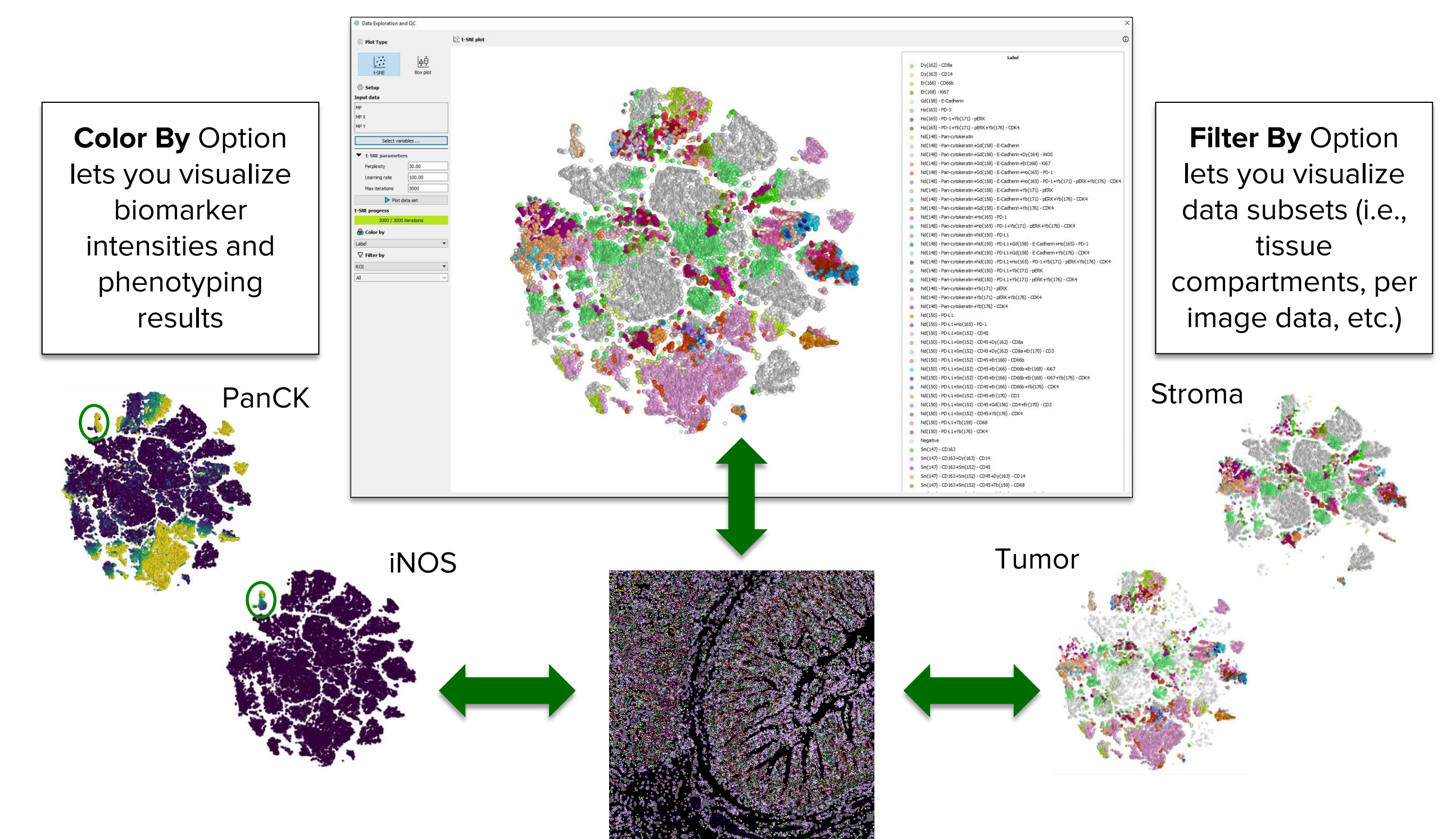
**Figure 4. Visiopharm Cell Detection and classification of PDAC tissue.**

Deep Learning-based nuclear and cell detection using the Ir DNA and ICSK stains to identify nuclei and their boundaries. The outputs from this cell detection algorithm were auto classified using the Visiopharm Phenotyping module. A subset of biomarkers was used for phenotyping the tissue microenvironment: pan-CK, E-Cadherin, Ki67, CD45, CD3, CD4, CD8, CD68, CD163, CD11b, iNOS, pERK, CDK4, PD-1, PD-L1, LAG3, CD14, CD66b



**Figure 5. Cellular phenotypes of PDAC cases 9500 and 9502 in epithelia and stroma tissue compartment**

Analysis of total cellular phenotypes derived from the markers (pan-CK, E-Cadherin, Ki67, CD45, CD3, CD4, CD8, CD68, CD163, CD11b, iNOS, pERK, CDK4, PD-1, PD-L1, LAG3, CD14, CD66b) showed an increase in CD163+ CDK4+ cells in the epithelial area in both cases, 19.3% in 9500 and 13.6% in 9502. The two samples also differ in the presence of CD45+ CD66b+ expressing cells in the stromal area. While 9502 has 11.5% CD45+CD66b+ of all phenotyped stromal cells, 9500 showed rarely this phenotype 0.18%. pERK+ CDK4+ double expressing are more frequent in case 9502 (1.8% in stroma and 3.1% in epithelial area) compared to 9500 (0.2% in stroma and 0.87% in epithelial area)



**Figure 6. Visiopharm Data Exploration and QC Tool.**

An advanced interactive visualization tool is built into the Visiopharm software platform to explore and review your data and results. Visualize biomarker intensities in t-SNE plots to validate co-expression profiles. "Color by" options allow for displaying t-SNE objects according to the auto phenotyping results. "Filter by" options allow for visualization of data subsets (i.e., tissue compartments, per image data, etc.). Bidirectionally interactive plots enable exploration of your data in a GUI interface. For example, clicking on objects in the t-SNE shows the cells in the images; clicking on cells in the image, show the objects in the t-SNE plot.



**Figure 7. Phenotype Matrix in PDAC samples separated by PD-L1 status and tissue compartment**

Plotting of all Phenotype counts in a heatmap matrix separated into PD-L1 ROI status (low, medium, high expression) and tissue compartment epithelial area and stromal area showed a higher count for CD66b expressing cells in 9502 compared to 9500.

### Conclusions

Recent evidence suggests that PDAC progression is affected by the immune-excluded nature of the tumor including a lack of high-quality effector cells (CD4, CD8 T cells, dendritic cells), barriers to effector cell infiltration and a dominance of immune cells such as regulatory T cells, myeloid-derived suppressor cells, and M2 macrophages, resulting in an immunosuppressive tumor microenvironment (TME). Therefore, there is an urgent need to better define the composition of PDAC TME and the key immune players in order to identify new therapeutic targets and strategies. Herein, the use of the InSituPlex® FixVUE I/O PD-L1 kit enabled the streamlined combination and alignment of whole slide H&E and mIF data, leading to the strategic selection of regions of interest (ROI), while IMC technology and Visiopharm analysis enabled downstream imaging of 40 specific protein markers associated with the immune contexture in the selected ROIs to provide a deeper understanding of the tumor microenvironment.

### References

1. Giesen, C., Wang, H.A.O. Schapiro, D., Zivanovic, N., Jacobs, A., Hattendorf, B., Schüffler, P.J., Grolmund, D., Buhmann, J.M., Brandt, S., Varga, Z., Wild, P.J., Günther, D., Bodenmiller, B. "Highly multiplexed imaging of tumor tissues with subcellular resolution by mass cytometry." *Nature Methods* (2014).  
2. Improved understanding of the biology and pathophysiology of the tumor microenvironment in PDAC samples revealed by InSituPlex, Imaging Mass Cytometry, and advanced image processing, SITC Poster, 2021, JTC Abstract Book [https://jitc.bmj.com/content/9/Suppl\\_2/A60](https://jitc.bmj.com/content/9/Suppl_2/A60)