Development of multiplex immunofluorescence workflows for characterizing tumor-immune and stromal compartments for pharmacodynamic assessments of solid tumors

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Abstract: A117

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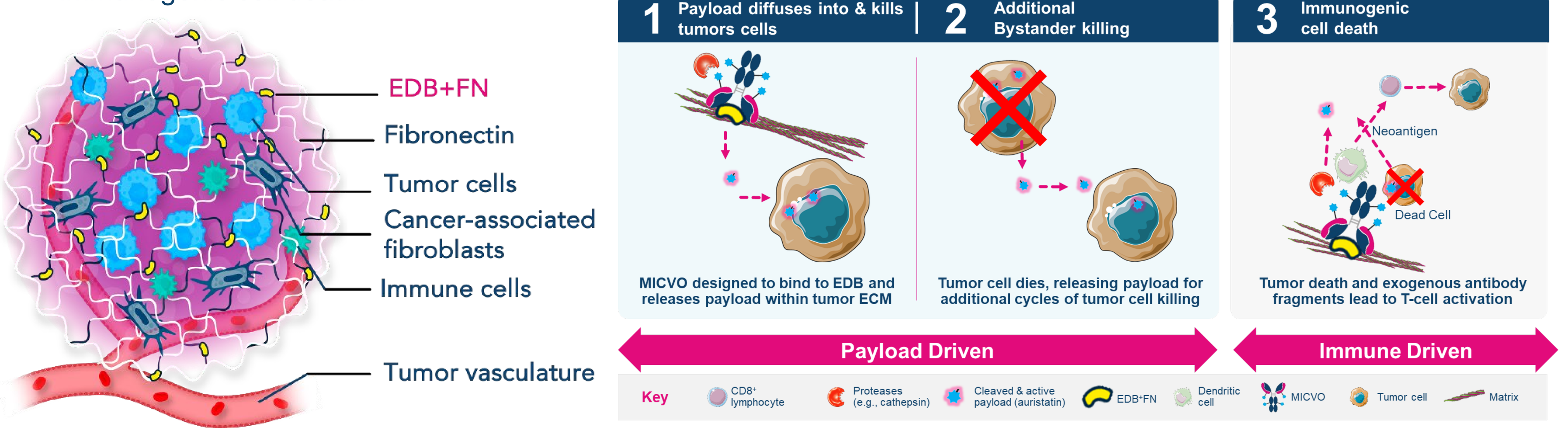
Background

- Micvotabart pelidotin (MICVO, aka PYX-201) is a first-in-concept antibody-drug conjugate (ADC) targeting extradomain-B of fibronectin (EDB+FN), a non-cellular structural component within the tumor extracellular matrix (ECM) that is highly expressed in tumors compared to normal adult tissues. (1, 2) EDB+FN, a splice variant of fibronectin, is known to be involved in tumor angiogenesis, proliferation, and metastasis.
- MICVO is designed to achieve anti-tumor activity via three mechanisms of action: 1) the cytotoxic, cell-permeable Auristatin-0101 payload directly kills tumor cells through disruption of microtubule formation, 2) the payload promotes additional tumor cell killing via the bystander effect, and 3) release of neoantigens from dying tumor cells induces immunogenic cell death.

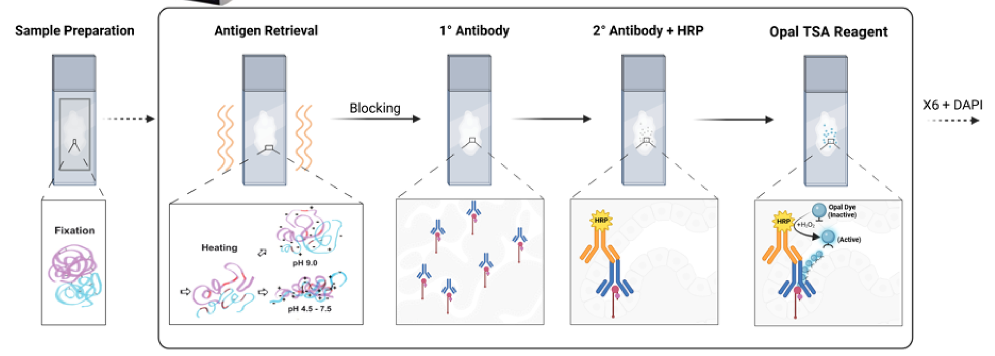


- MICVO is currently being evaluated in a Phase 1 monotherapy trial (**NCT05720117**) and a Phase 1/2 combination trial with pembrolizumab (**NCT06795412**) for advanced solid tumors.
- Histological methods to characterize trial participants' tumors before and after treatment with MICVO are needed; multiplex immunofluorescence (mIF) enables high-resolution phenotyping of tissue architecture and cellular interactions.
- **The objective of this poster is to establish mIF methodologies for integrated spatial assessment of tumor–immune interactions and stromal architecture, enabling characterization of microenvironmental variation across tumor types for biomarker discovery and evaluation of pharmacodynamic responses to MICVO.**

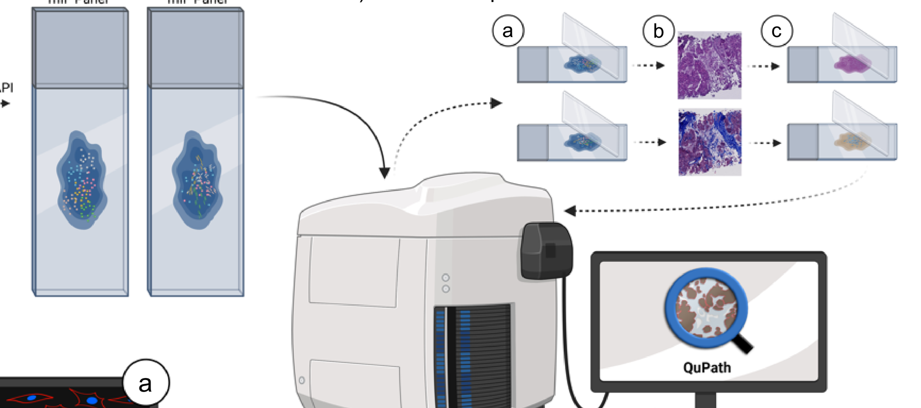
Methods

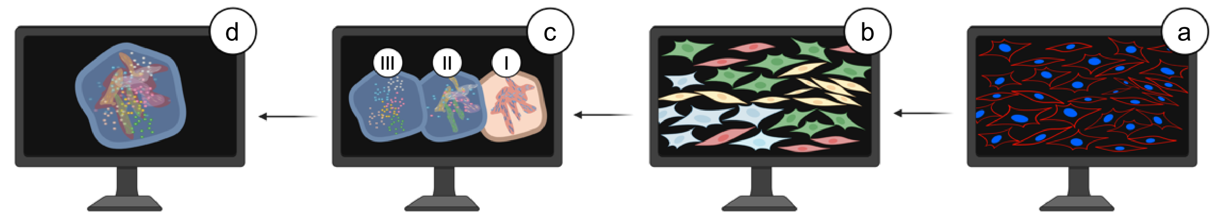
Whole-tissue slides of head and neck squamous cell carcinoma (HNSCC) and pancreatic ductal adenocarcinoma (PDAC) were commercially sourced to validate the workflow.

1. Sequential antigen retrieval and mIF staining:
- Optimized two custom 6-marker panels + DAPI (Opal 6-Plex, Akoya)
  - Panels: 1) tumor-immune and 2) stromal
- Applied to serial sections of nonclinical HNSCC and PDAC samples

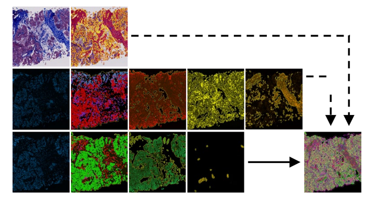


2. Same-slide colorimetric staining:
a) Remove coverslips
b) Perform colorimetric staining
   - H&E on tumor-immune panel slide
   - Masson's trichrome on stromal panel slide
c) Re-coverslip and scan





3. Digital pathology:
QC (annotation of tissue bounds and artifacts)
a) Cell segmentation
b) Object/pixel classification
c) Alignment of scans and annotation transfer
   I. Masson's (Collagen)
   II. Stromal mIF
   III. Tumor-immune mIF
d) Export data for cleaning and processing



4. Data cleaning and feature calculation:
- Filter artifact data and samples where no alignment was possible
- Calculate features, test statistical significance, and generate plots

Stain	PDAC	HNSCC	Total
Stromal mIF and Masson's	19	15	34
Tumor-immune mIF and H&E	19	14	33
Aligned	17	12	30

mIF panel target selection and sample overview

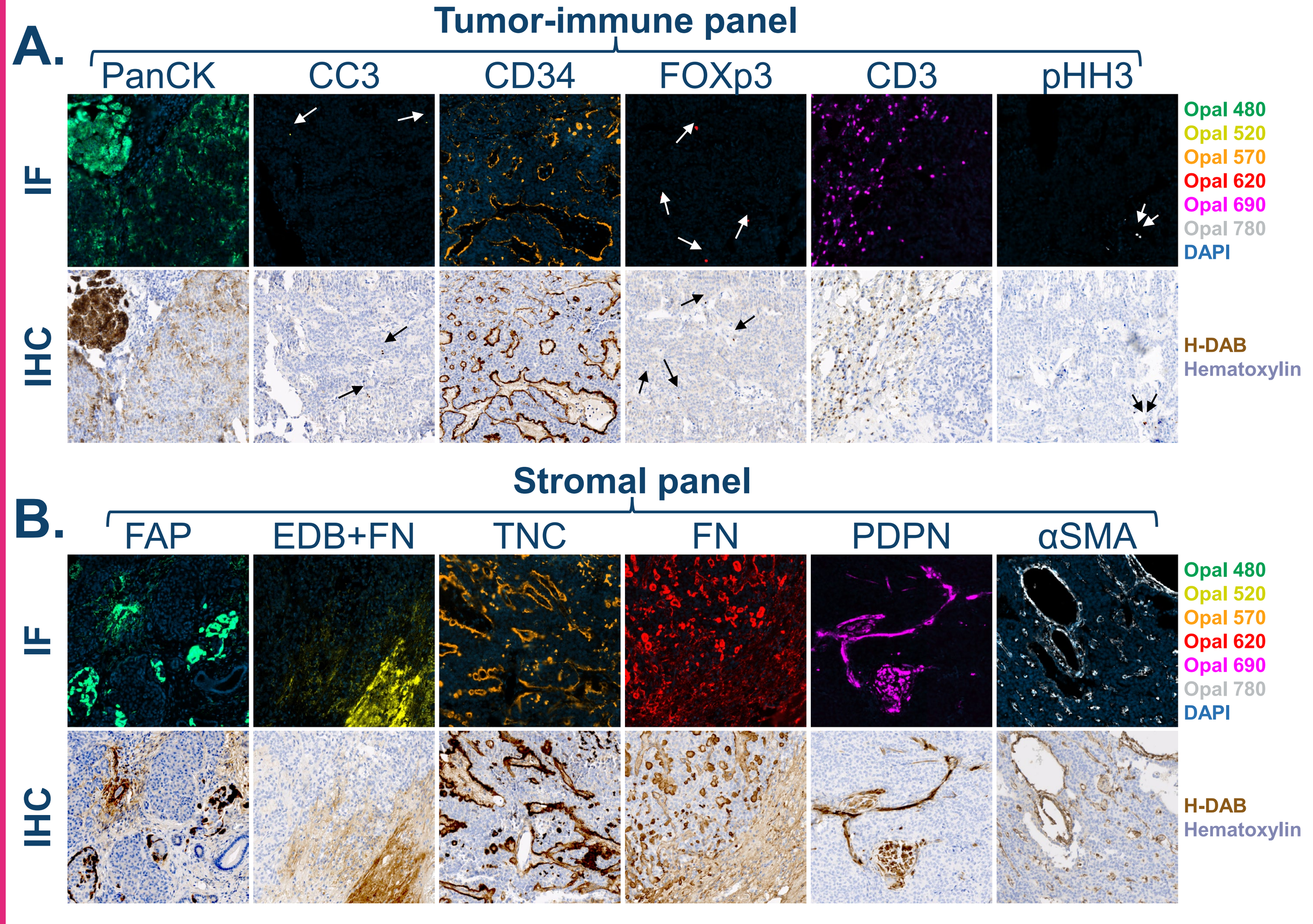


Figure 1: Tumor–Immune & Stromal Markers in HNSCC vs PDAC. Serial sections of a representative sample were stained by either IHC or IF for each marker to confirm consistency of staining patterns. The tumor-immune mIF panel **(A)** contains antibodies for pan-cytokeratin (PanCK; epithelial), cleaved-caspase 3 (CC3; apoptotic), CD34 (endothelial progenitor), FOXp3 (Treg), CD3 (Pan-T cell) & phospho-histone H3 (pHH3; mitotic). The stromal mIF panel **(B)** contains antibodies for fibroblast activation protein (FAP; active fibroblasts), EDB+FN (MICVO target), tenascin C (TNC; ECM glycoprotein), fibronectin (FN; ECM component), podoplanin (PDPN; lymphatic endothelial) & alpha-smooth muscle actin (αSMA; myofibroblast).

Higher Collagen/Stroma in PDAC vs HNSCC

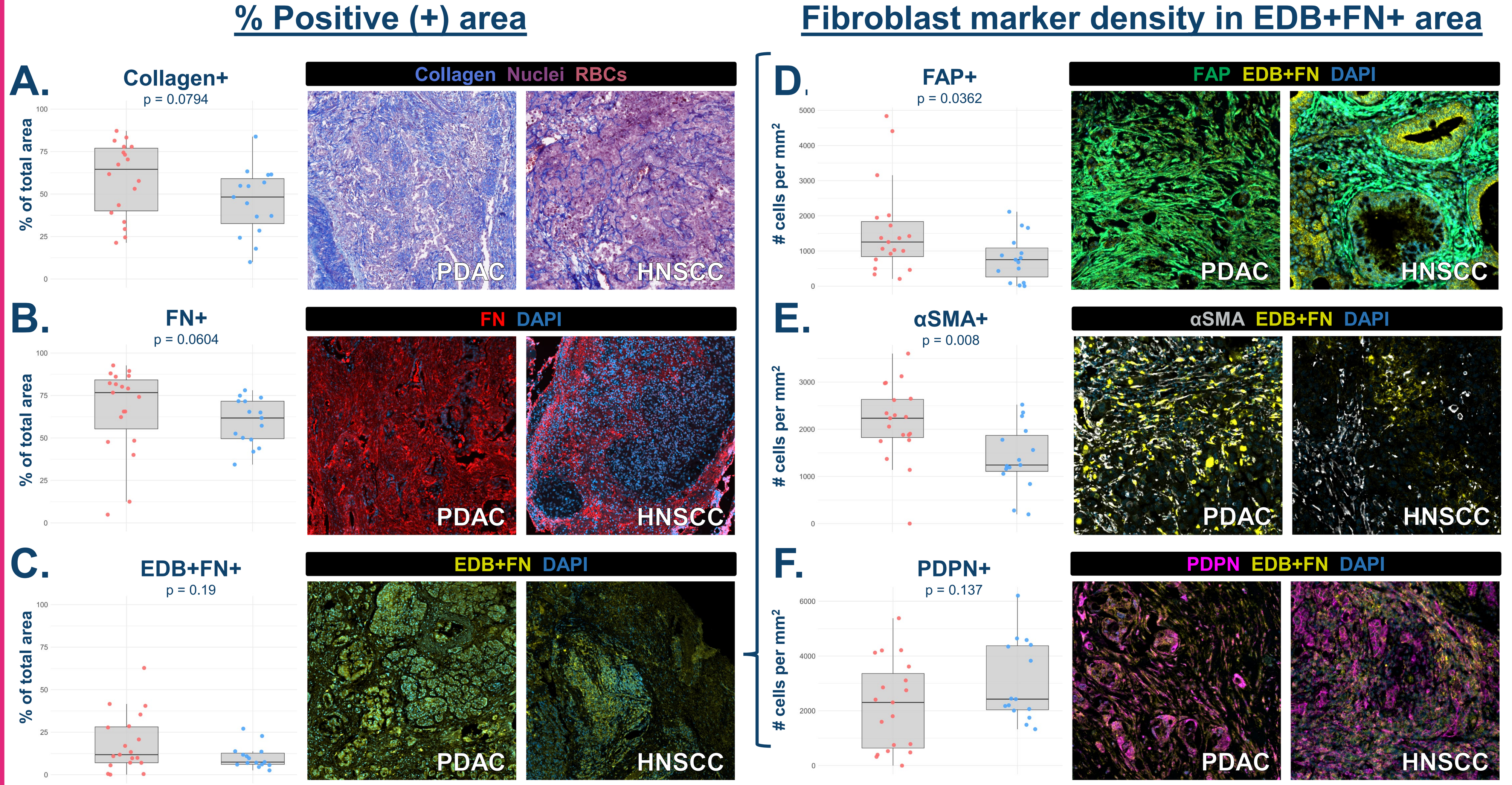


Figure 2. Chromogenic and mIF staining reveal differences in ECM-component abundance and densities of fibroblast markers in EDB+FN+ regions. Analysis of Masson's trichrome staining **(A)** showed a trend toward higher percentage of collagen+ area across whole tissue in PDAC. The percentages of FN+ **(B)** and EDB+FN+ **(C)** area also trended toward higher levels in PDAC than HNSCC. Densities of FAP+ **(D)**, αSMA+ **(E)**, and PDPN+ **(F)** cells in EDB+FN+ regions showed more FAP+ and αSMA+ cells in PDAC, but higher PDPN+ density in HNSCC. Representative images are shown.

HNSCC tumors show greater CD3+ infiltration than PDAC tumors

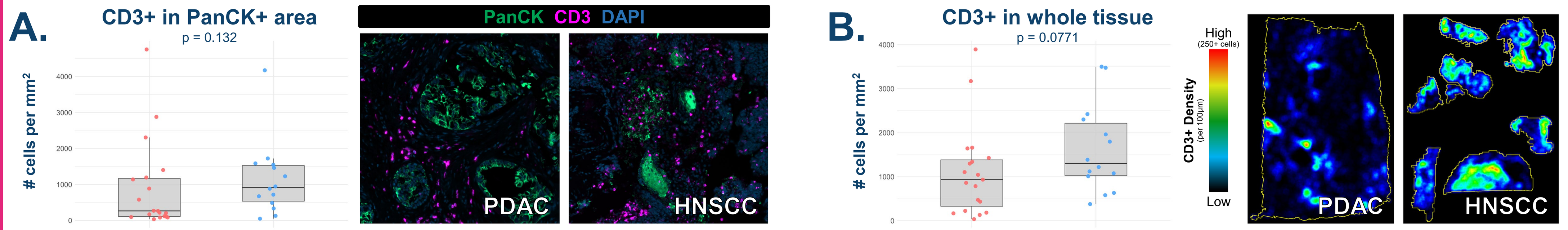


Figure 3: HNSCC tumors show higher CD3+ density in PanCK+ regions and whole tissue. mIF with the tumor-immune panel revealed higher CD3+ density in PanCK+ regions **(A)** of HNSCC vs. PDAC. CD3+ density was also higher across whole tissue **(B)** of HNSCC compared to PDAC. Representative images and density map with color scales reflecting cell abundance per 100µm radius are shown. Yellow lines denote tissue boundaries.

HNSCC tumor stroma is more favorable for immune access than PDAC

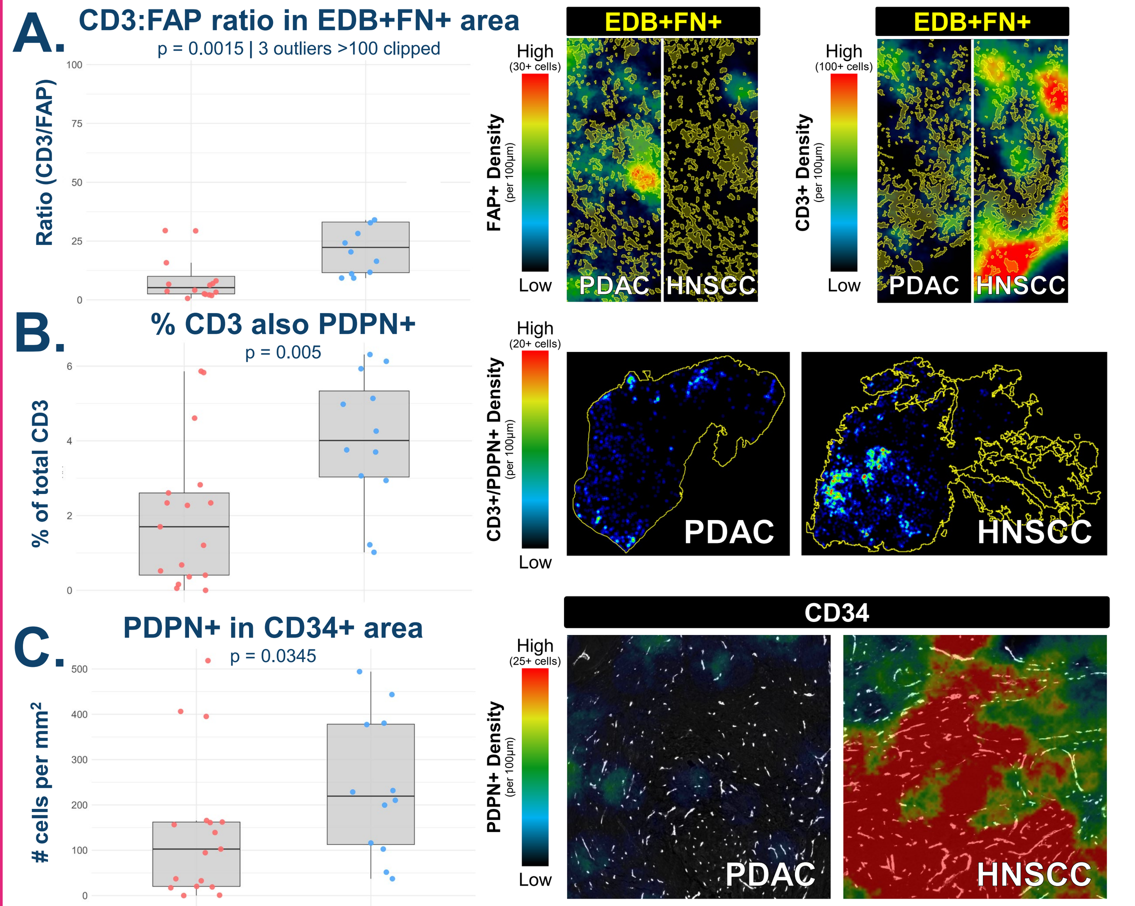


Figure 4: HNSCC tumors show higher CD3:FAP ratios and immune–stromal associations than PDAC. Ratio of CD3+ to FAP+ cell abundance in EDB+FN+ regions **(A)**, percentage of CD3 overlapping PDPN (yellow lines denote tissue boundaries) **(B)**, and PDPN density within CD34+ areas **(C)** were all higher in HNSCC, indicating more immune-accessible and vascularized stroma. Representative images and density maps are shown.

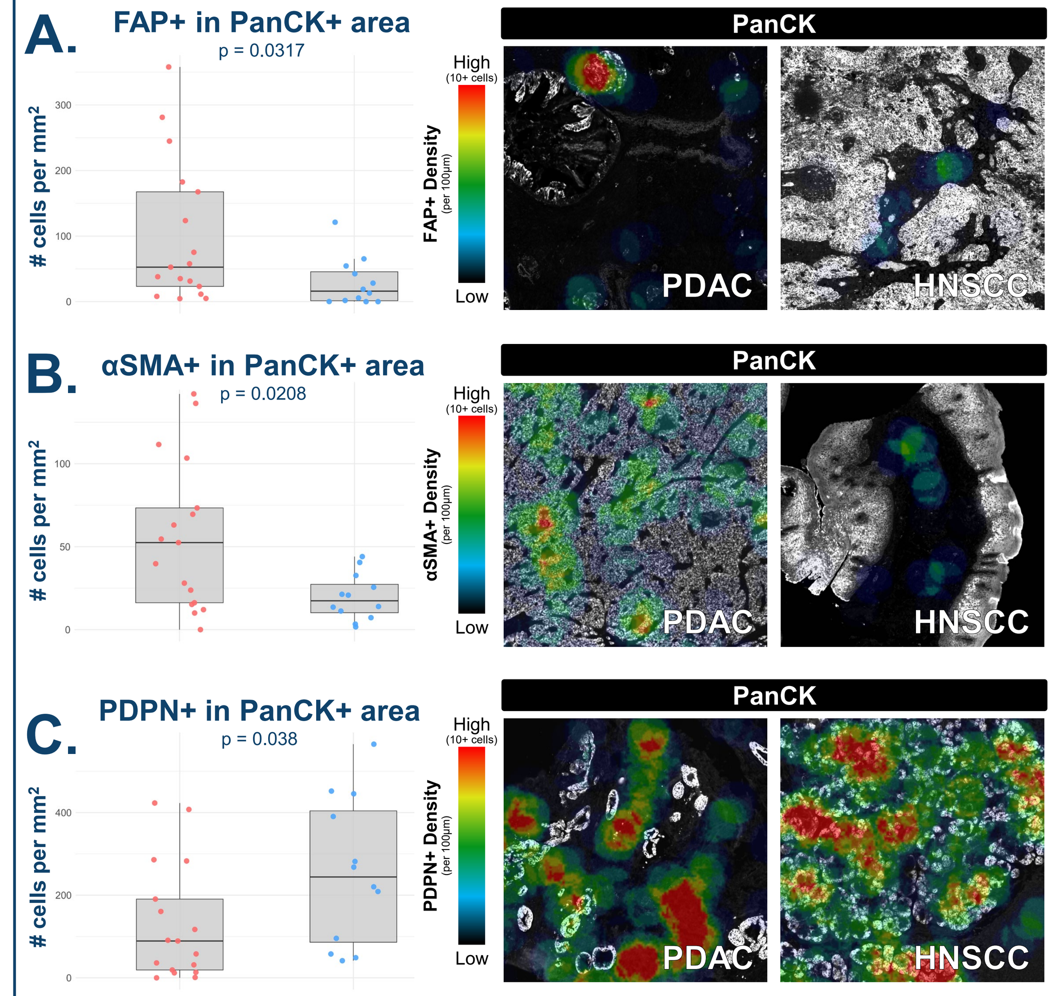


Figure 5: Fibroblast populations show differential enrichment at tumor-stroma interface. αSMA+ cell density **(A)** and FAP+ cell density **(B)** were higher within PanCK+ areas of PDAC compared to HNSCC, whereas PDPN+ cell density **(C)** was higher within PanCK+ areas HNSCC. Representative images and density maps are shown.

Conclusions

- Two complimentary mIF panels were developed and integrated with digital spatial analysis to allow for characterization of tumor, stroma, and immune characteristics across serial sections and sequential same-slide staining of solid tumors.
- Together, these data reveal distinct tumor-immune features – such as higher CD3+ and PDPN+ cell density in the EDB+FN+ and PanCK+ areas of HNSCC tumors – as well as differences in stromal architecture and composition – such as higher proportion of FN+ and collagen+ areas and higher FAP+ and αSMA+ cell density in EDB+FN and PanCK+ areas of PDAC tumors – which can be further evaluated for correlation to MICVO response in preclinical and clinical samples.
- The mIF capabilities developed for this study have been deployed on clinical tumor biopsy samples obtained from participants treated with MICVO to characterize pharmacodynamic changes in the tumor-immune and stromal compartments (Abstracts: A113 and A114).

References: [1] Lewandowski S, et al. Cancer Res. 2024 Mar;84(6):2908. [2] Hooper AT, et al. Mol Cancer Ther. 2022 Sep;21(9). Servier Medical Art and BioRender for figure design.

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