# HOST-Factor: A comprehensive platform for single-cell highplex immunofluorescence staining, digital imaging, spatial mapping, and quantitative analysis of the tumor microenvironment.

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## Abstract

Solid tumor complexity extends beyond the genetic and functional landscapes of heterogeneous cancer cells, encompassing the tumor microenvironment (TME). Elucidating the TME's complexity requires a comprehensive assessment of its cellular composition, functional states, and spatial distributions. We developed the Harmonic Output of Stromal Traits (HOST) to identify TME cells and the HOST-Factor to quantify their functional states. The HOST-Factor is a numerical value that reflects the relative contribution of cancer-associated fibroblasts (CAFs) to tumor-suppressive or tumor-promoting functions.

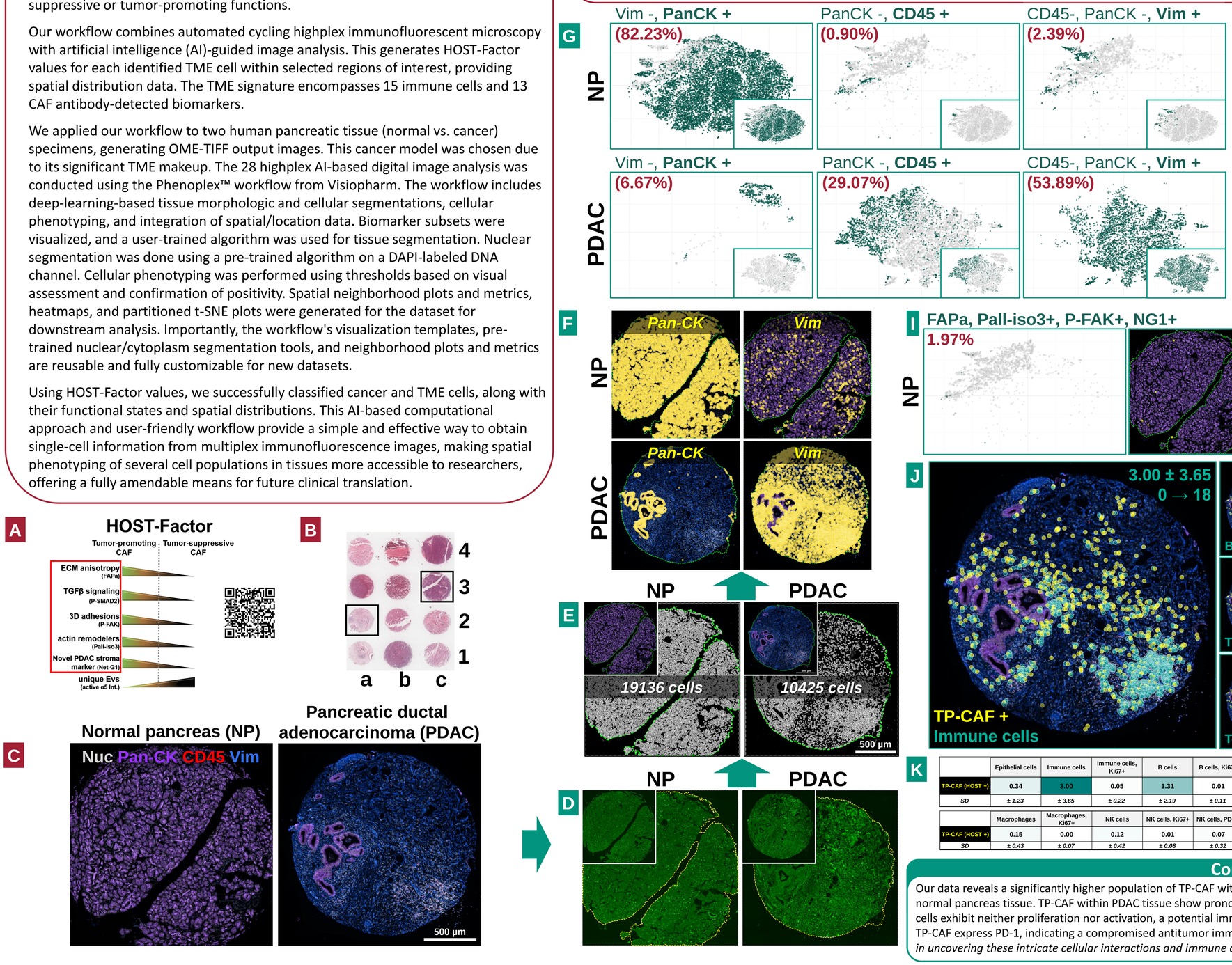
Our workflow combines automated cycling highplex immunofluorescent microscopy with artificial intelligence (AI)-guided image analysis. This generates HOST-Factor values for each identified TME cell within selected regions of interest, providing spatial distribution data. The TME signature encompasses 15 immune cells and 13 CAF antibody-detected biomarkers.

We applied our workflow to two human pancreatic tissue (normal vs. cancer) specimens, generating OME-TIFF output images. This cancer model was chosen due to its significant TME makeup. The 28 highplex AI-based digital image analysis was conducted using the Phenoplex™ workflow from Visiopharm. The workflow includes deep-learning-based tissue morphologic and cellular segmentations, cellular phenotyping, and integration of spatial/location data. Biomarker subsets were visualized, and a user-trained algorithm was used for tissue segmentation. Nuclear segmentation was done using a pre-trained algorithm on a DAPI-labeled DNA channel. Cellular phenotyping was performed using thresholds based on visual assessment and confirmation of positivity. Spatial neighborhood plots and metrics, heatmaps, and partitioned t-SNE plots were generated for the dataset for downstream analysis. Importantly, the workflow's visualization templates, pre-trained nuclear/cytoplasm segmentation tools, and neighborhood plots and metrics are reusable and fully customizable for new datasets.

Using HOST-Factor values, we successfully classified cancer and TME cells, along with their functional states and spatial distributions. This AI-based computational approach and user-friendly workflow provide a simple and effective way to obtain single-cell information from multiplex immunofluorescence images, making spatial phenotyping of several cell populations in tissues more accessible to researchers, offering a fully amendable means for future clinical translation.

## Figure 1

**A.** Harmonic Output of Stromal Traits (HOST) used for classification of carcinoma-associated fibroblasts (CAFs) as tumor-promoting (TP) or tumor-suppressive (TS). **B**. Example of tissue microarray used for assessing the presence of TP-CAF in pancreatic tissue (normal vs. cancer) using HOST-factor **B**. Highplex immunofluorescence-stained tissue images obtained with *Lunaphore technologies' COMET instrument*, illustrating indicated markers **C**. **Visiopharm®**'s AI-guided image analysis employed for mapping images using tissue autofluorescence (**D**) and obtaining single-cell information (**E**) for HOST and immune markers evaluation. Images in (**F**) depict positive cells (yellow) based on pan-cytokeratin (Pan-CK) or vimentin (Vim) marker expression in various tissues. Data obtained using ***Phenoplex™ workflow***: (**G**) Pure epithelial (Pan-CK+) vs. immune (CD45+) vs. fibroblastic (CD45-, Pan-CK-, Vim+) cell populations gated for analysis, shown in t-SNE plots with prevalence percentages in dark red. (**H**) Significant correlative HOST markers co-expression in fibroblastic cell population, with prevalence percentages shown in dark red. (**I**) Identification of TP-CAF (HOST+) population within fibroblastic cells pool, with prevalence percentages provided and corresponding visual localization in tissues (yellow bullets). Using the ***"Neighbor counts" module of Phenoplex™ workflow***: (**J**) Localization of selected immune populations within 10 µm distance from TP-CAF (yellow bullets), with dark green bullets representing neighboring immune populations and other colored bullets indicating specific immune cell phenotypes (e.g., proliferation, PD-1 expression, or activity), with mean ±SD of corresponding cells and minimum to maximum cell counts provided. (**K**) Table showing mean ±SD of selected cells identified in proximity to TP-CAF, with a color scale reflecting low-to-high cell occurrence distribution




**K**

| | Epithelial cells | Immune cells | Immune cells, Ki67+ | B cells | B cells, Ki67+ | Th cells | Th cells, Ki67+ | Th cells, PD-1+ | Tct cells | Tct cells, Ki67+ | Tct cells, PD-1+ | Tct cells, GZMB+ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| TP-CAF (HOST +) | 0.34 | 3.00 | 0.05 | 1.31 | 0.01 | 1.65 | 0.02 | 0.68 | 1.89 | 0.01 | 0.56 | 0.12 |
| *SD* | *± 1.23* | *± 3.65* | *± 0.22* | *± 2.19* | *± 0.11* | *± 2.74* | *± 0.11* | *± 1.43* | *± 3.03* | *± 0.11* | *± 1.29* | *± 0.47* |

| | Macrophages | Macrophages, Ki67+ | NK cells | NK cells, Ki67+ | NK cells, PD-1+ | NK cells, GZMB+ | Dendritic cells | Dendritic cells, Ki67+ |
|---|---|---|---|---|---|---|---|---|
| TP-CAF (HOST +) | 0.15 | 0.00 | 0.12 | 0.01 | 0.07 | 0.05 | 0.58 | 0.01 |
| *SD* | *± 0.43* | *± 0.07* | *± 0.42* | *± 0.08* | *± 0.32* | *± 0.27* | *± 1.49* | *± 0.12* |

Low — High

## Conclusions

Our data reveals a significantly higher population of TP-CAF within the pancreatic ductal adenocarcinoma (PDAC) tissue compared to normal pancreas tissue. TP-CAF within PDAC tissue show pronounced interactions with B cells, T helper cells, and T cytotoxic cells. These cells exhibit neither proliferation nor activation, a potential immune-suppressive microenvironment. Nearly one in two T cells proximal to TP-CAF express PD-1, indicating a compromised antitumor immune response. *Visiopharm's "Neighbor counts" module, was instrumental in uncovering these intricate cellular interactions and immune dynamics.*

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