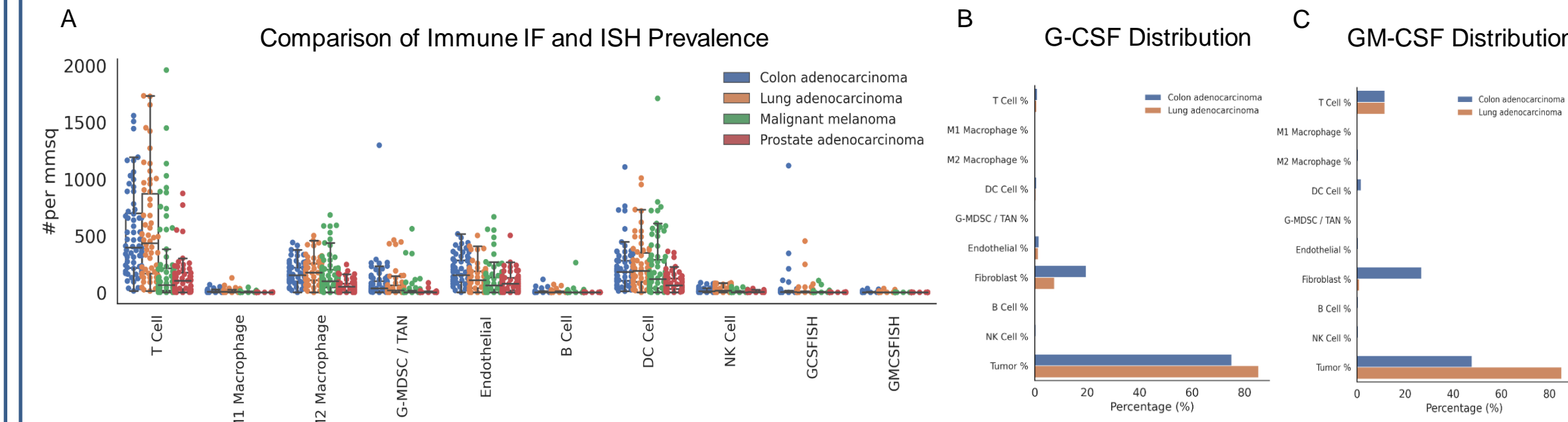
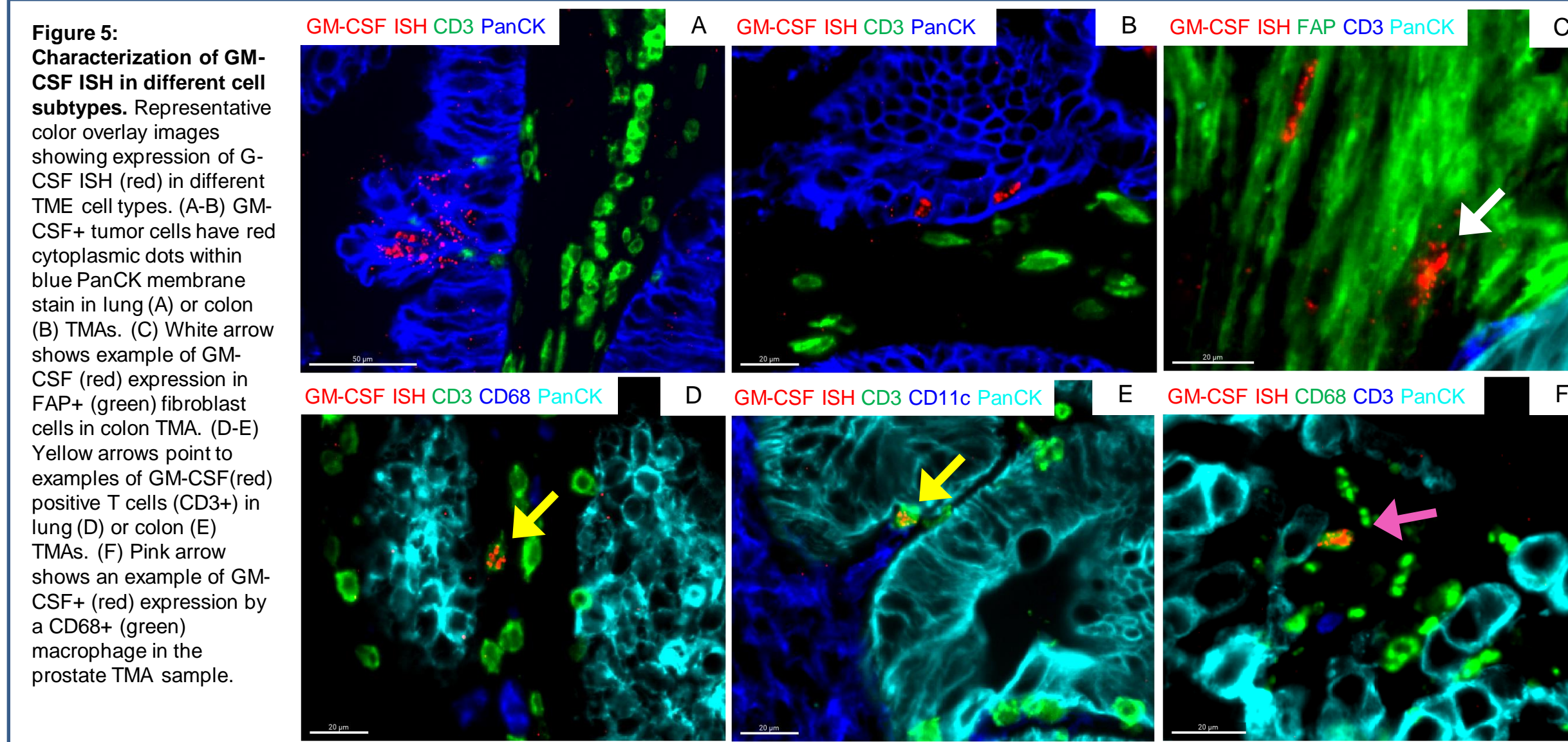
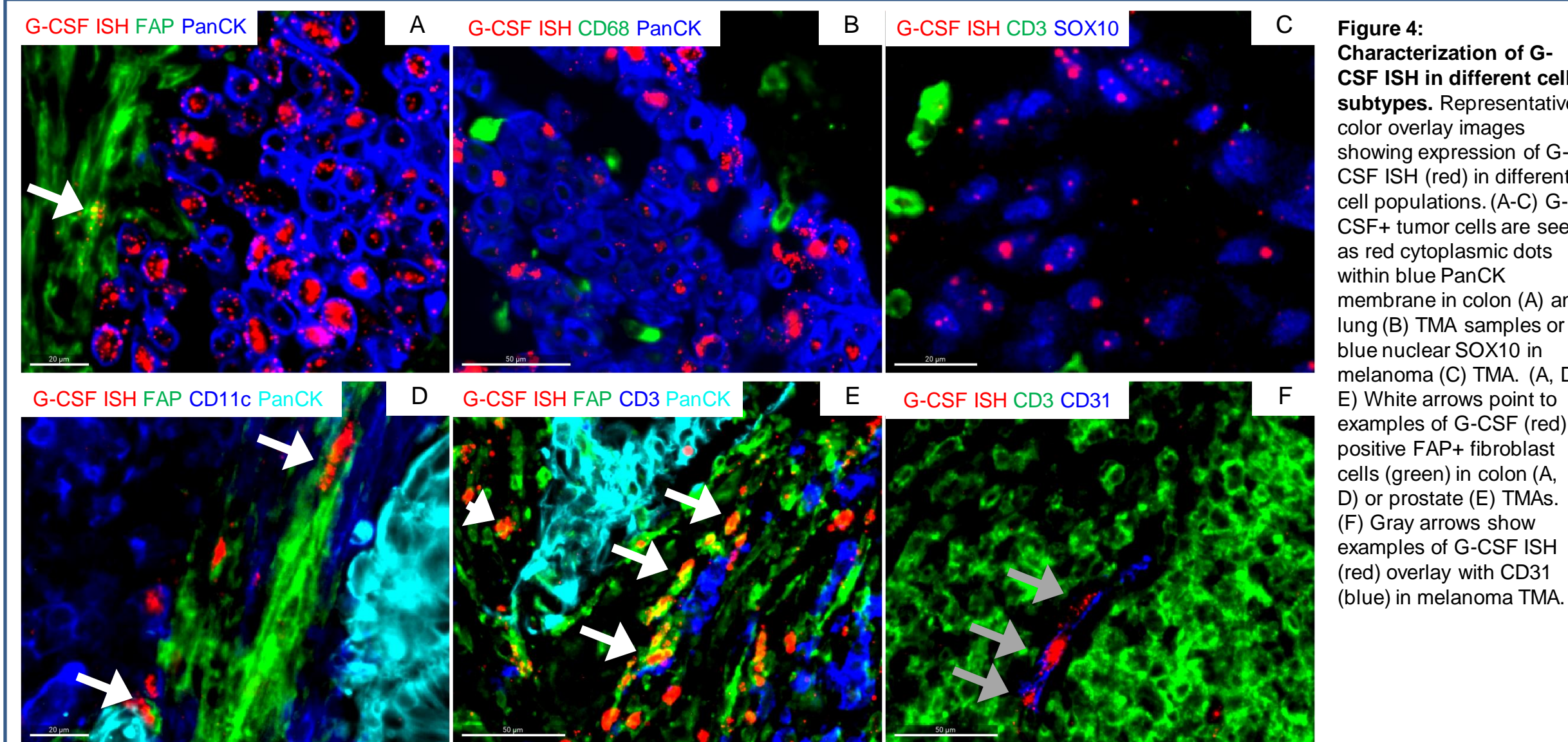
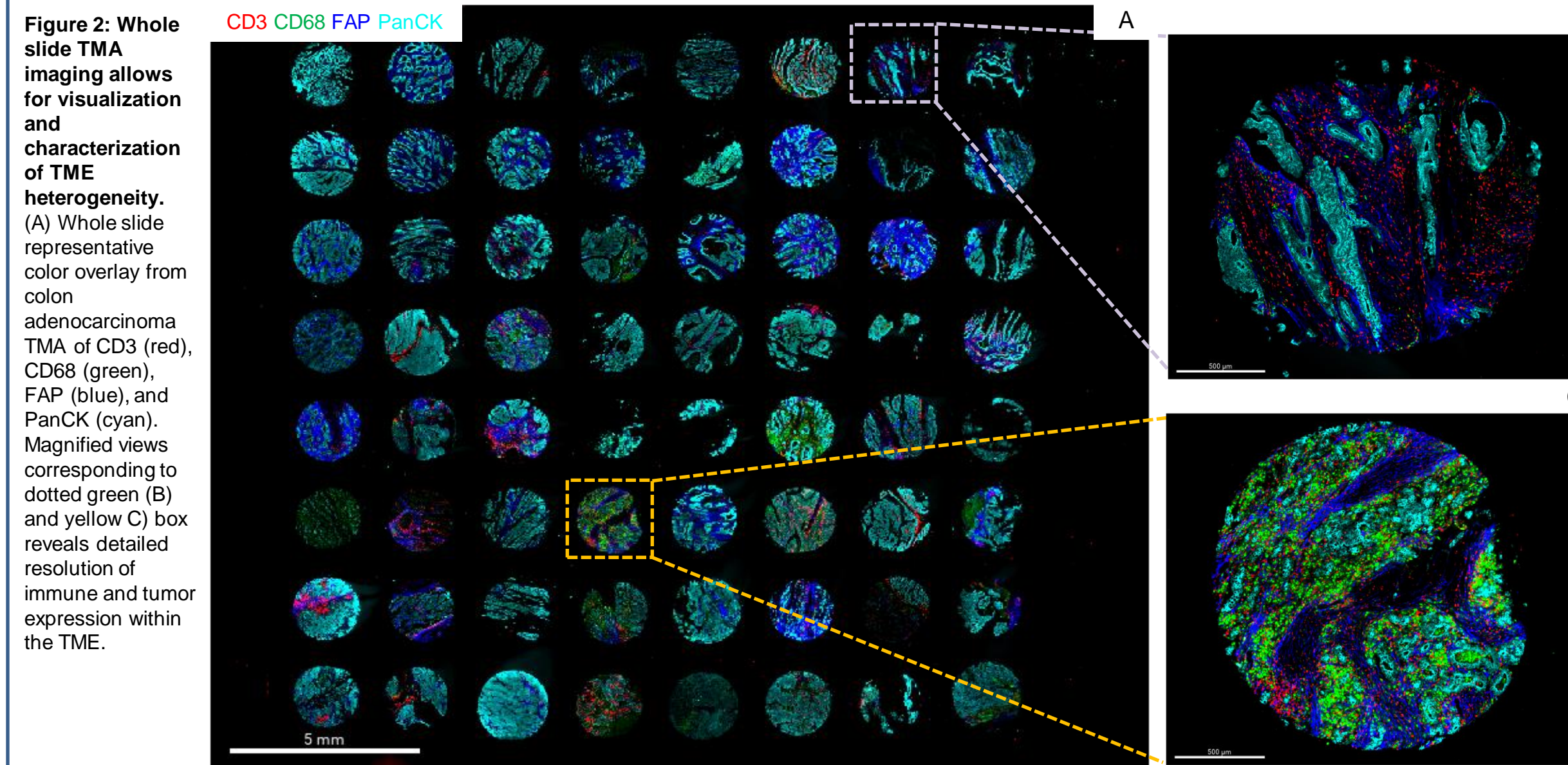
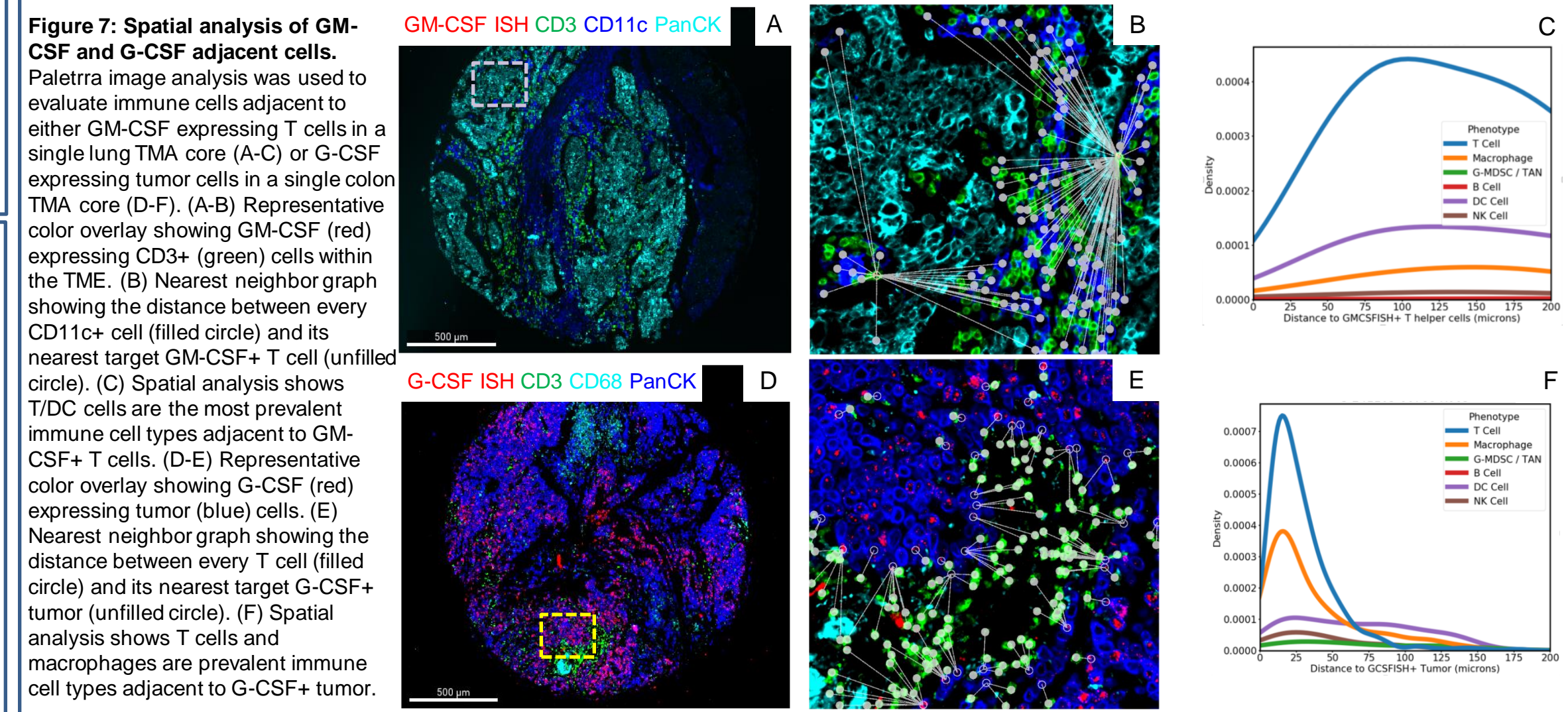
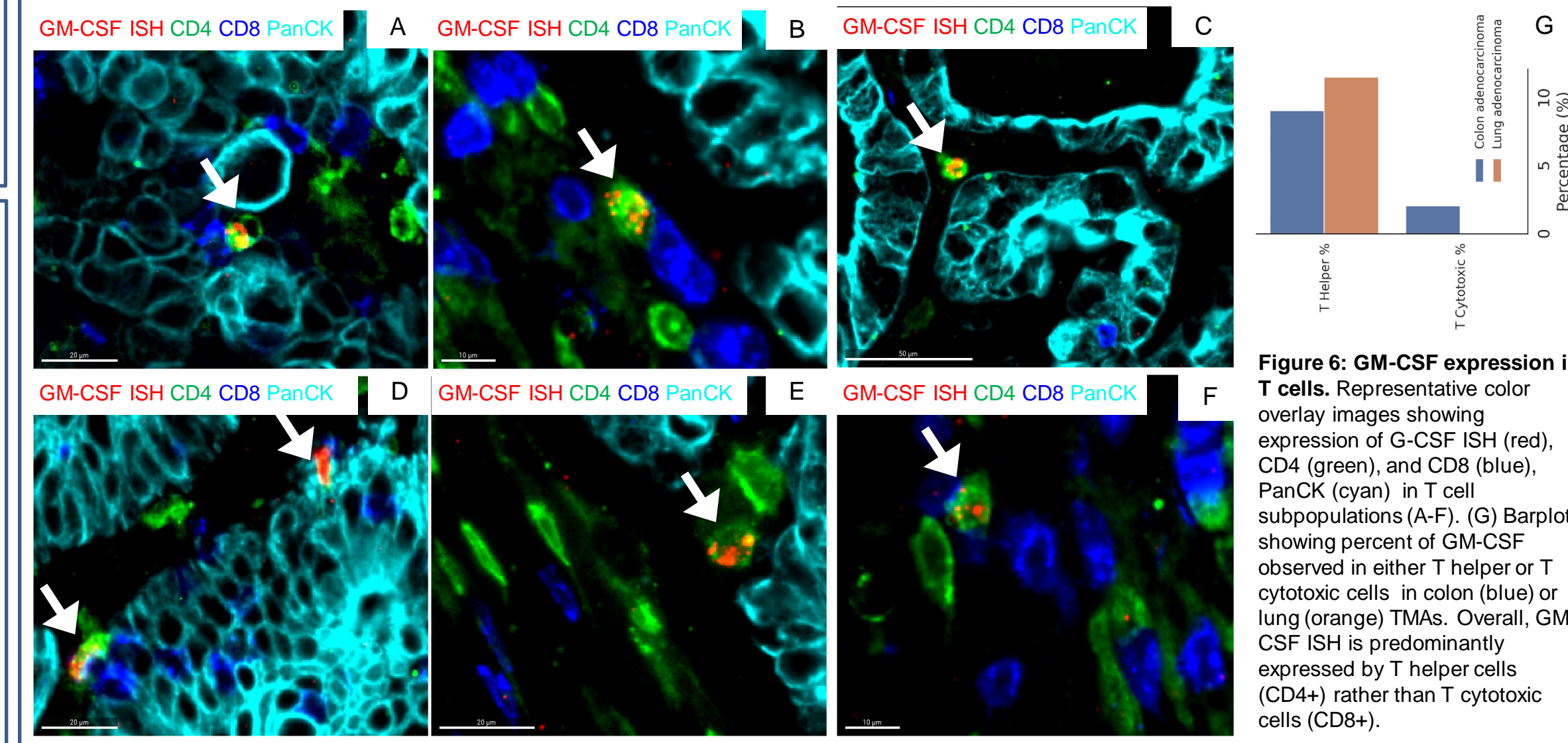


Granulocyte-macrophage colony-stimulating factor (GM-CSF) and granulocyte colony-stimulating factor (G-CSF) are cytokines known to stimulate differentiation and activation of granulocytes, monocytes, macrophages, and dendritic cells (DCs) as well as non-immune cells including endothelial, epithelial and fibroblasts. GM-CSF and G-CSF can be produced by many different cell types, including immune cells, fibroblasts, endothelial cells and have also been shown to be upregulated in multiple human cancers. Studies have further demonstrated the immunomodulatory effects of GM-CSF/G-CSF can promote both anti-tumorigenic or pro-tumorigenic activity through regulation of neutrophils/M1 macrophages/T-effectors or myeloid-derived suppressor cells (MDSCs)/M2 macrophages/Tregs, respectively. Therefore, modulators to promote or reduce the activity of GM-CSF/G-CSF have been explored for tumor therapeutic intervention, often in combination with other therapies such as chemotherapy. However, to further dissect and target anti or pro-tumor GM-CSF/G-CSF modulation requires a better understanding of the distribution and identity of GM-CSF/G-CSF secreting cells within the tumor micro-environment (TME).

To characterize GM-CSF/G-CSF expression in the TME, we use the Integrated Palettra™-RNAscope™ workflow. Palettra (NeoGenomics Laboratories, Inc) is a proprietary multiplex immunofluorescence (mIF) service for the visualization and characterization of up to 60 protein biomarkers in a single formalin-fixed paraffin-embedded (FFPE) section and offers high-resolution spatial and quantitative analysis of protein expression in tissue samples. RNAscope (Bio-Techne) Multiplex is a highly sensitive fluorescent in-situ hybridization (ISH) assay that can detect up to 3 RNA markers in a single FFPE section. The Integrated Palettra-RNAscope assay allows for simultaneous detection of both protein and RNA markers in a single sample. Herein we report the design and use of a novel panel of commercially-available RNAscope ISH probes and IF antibodies broad enough to characterize GM-CSF and G-CSF subpopulations in the TME of a variety of tumor indications including lung, prostate, melanoma, and colon cancer. Using this panel in combination with proprietary deep-learning based image analysis, GM-CSF and G-CSF expressing cells can be characterized into different immune and TME subtypes. Understanding of the variety and phenotype of GM-CSF and G-CSF expressing cells in the TME will help improve the understanding and development of targeted therapies for cancer treatment.

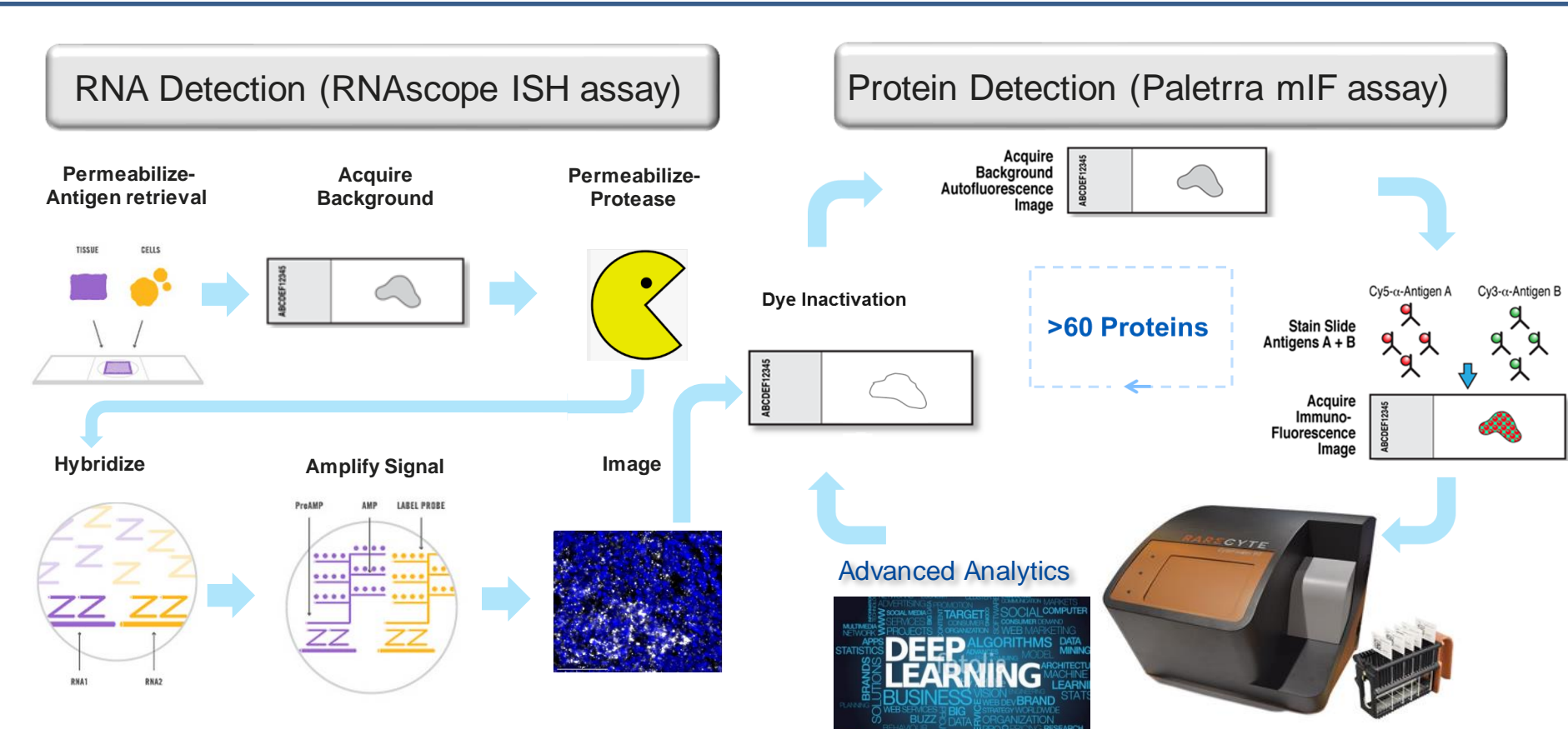


**Figure 3: Immune IF and ISH cell prevalence and distribution between indications.** (A) Scatter plot of density for each immune/ISH population analyzed from all 4 TMA samples. Each dot represents results from a single core from colon (blue), lung (orange), melanoma (green), or prostate (red) TMA. Overall prevalence of GM-CSF ISH and G-CSF ISH was fairly low in all four indications evaluated. In general, G-CSF prevalence was higher than GM-CSF. Colon adenocarcinoma and lung adenocarcinoma TMAs also showed highest prevalence of both G-CSF/GM-CSF. (B-C) Barplots showing overall distribution of immune, stromal, or tumor populations positive for G-CSF (B) or GM-CSF (C) in colon (blue) or lung (orange) adenocarcinoma. G-CSF is mostly expressed by tumor or fibroblasts while GM-CSF is expressed by T cells, fibroblasts, and tumor.



**Summary**

- GM-CSF and G-CSF expressing cells were characterized in the TME of colon, lung, prostate, and melanoma TMAs using the Integrated Palettra-RNAscope assay.
- Both GM-CSF and G-CSF showed overall low prevalence in all TMA indications evaluated, however lung and colon TMAs showed higher prevalence than melanoma and prostate.
- GM-CSF signal was mostly observed in T cells, tumor, and fibroblasts while G-CSF was mostly observed in tumor and fibroblasts.
- The majority of GM-CSF T cell expression was observed specifically in CD4+ T helper cells.
- Palettra spatial analysis quantified proximity of target immune populations to GM-CSF/G-CSF expressing cells.



**Figure 1: Integrated Palettra-RNAscope overview and workflow.** Untreated colon adenocarcinoma (64 cases), lung adenocarcinoma (64 cases), malignant melanoma (73 cases), and prostate adenocarcinoma (52 cases) TMA samples were used in this study. All TMA slides were prepared and stained using the Integrated Palettra-RNAscope workflow, which combines RNAscope ISH and Palettra mIF staining protocols. Slides were first cleared and then undergo pretreatment, which includes both heat activated epitope retrieval and protease steps. Then RNAscope ISH probe hybridization, amplification, and imaging was performed before proceeding to IF multiplexing. For each round of IF staining, conjugated fluorescent antibodies were applied to the slide, followed by imaging acquisition of stained slides. The dye was erased, enabling additional rounds of staining with another pair of fluorescent antibodies. For this study, IF biomarkers were included to characterize multiple immune (CD3, CD68, CD20, Nkp46, CD11c), stromal (CD31, FAP), and tumor (PanCK, SOX10) populations.