Quantifying tumor heterogeneity and mapping complex immune cell interactions with high-throughput, 7-color multispectral slide scans

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1 Background

As the need for multiple biomarker assessment in immune-oncology has become more clear, multiplex fluorescent immunohistochemistry (fIHC) techniques have become integral to immune oncology research.

Multispectral approaches to fIHC improve quantitative performance and signal specificity by ensuring removal of autofluorescence signals and bleed-through between spectrally-adjacent dyes.

We have recently demonstrated a novel high-throughput scanning approach (MOTiF™) that allows acquisition of a 7-color multispectral slide scan in 5–10 minutes (1.5x1.5 cm).

Here, we demonstrate the spatial measurements that are made possible by this high-throughput, translational workflow. These measurements span the scales related to tumor biology from the distance between nearest cell neighbors to the extent of the invasive margin.

2 Methods

Phenoptics™ Workflow

Formalin-fixed paraffin-embedded (FFPE) samples of primary tumors were stained using Opal™ reagents on a Leica BOND RX. Sections from several primary tumor types were stained against 5 key markers in the field of immuno-oncology (CD8, FoxP3, PD-1, PD-L1, CD68) along with a tumor marker (PanCK, PAX8, or Sox10+S100 cocktail) and DAPI counterstain, resulting in 7 colors on each slide.
Conventional and MOTiF™ multispectral scans (0.5 μm/pixel; 2.8 – 7.4 cm²) were acquired on Vectra Polaris® (9 – 26 min scan times). Scans were viewed with a Phenochart™ version modified to divide analysis regions into larger fields to reduce batch analysis times. Cell segmentation and phenotyping were performed in inForm®. Batch results from inForm® were merged and consolidated with the phenoptrReports package[1] in RStudio[2] using R 3.5.2[3]. Tumor margin annotations were drawn and exported with QuPath[4]. Heatmaps, histograms, and density plots were generated from consolidated inForm® results with R scripts under development.

Multiplex Staining

Opal™ reagents allow multiplex fIHC staining of up to 8 antigens with signal amplification and any combination of mouse and/or rabbit primary antibodies.
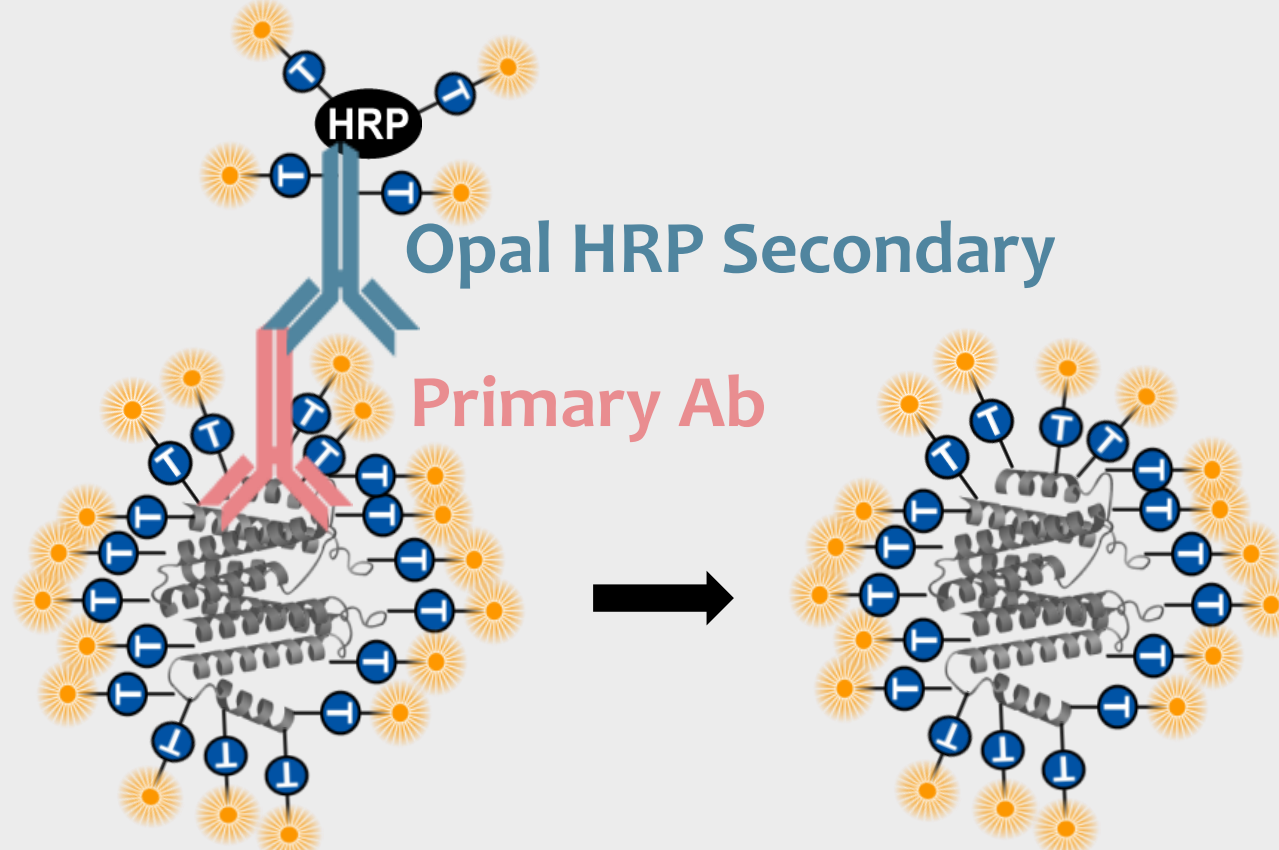
Fig 1. Opal™ Detection. The Opal HRP polymer amplifies IHC detection by covalently depositing multiple Opal fluorophores near the detected antigen. Then, antibodies are stripped to allow for sequential labeling of multiple markers.

Multispectral Imaging and Unmixing

With Vectra Polaris®, different combinations of agile LED bands, bandpass excitation filters, bandpass emission filters, and a liquid crystal tunable filter (LCTF) are used to select narrow spectral bands for imaging. Images from up to 40 narrow spectral bands are layered into a single multispectral image.
Fig 2. Unmixing in inForm® software. (A) A multispectral image cube where each layer is taken at a different spectral range. **(B)** Signals from all spectral bands are linearly unmixed using pure emission spectra for each fluorophore. Intensity values in the resulting unmixed image **(C)** are directly related to the amount of each fluorophore present in each pixel.
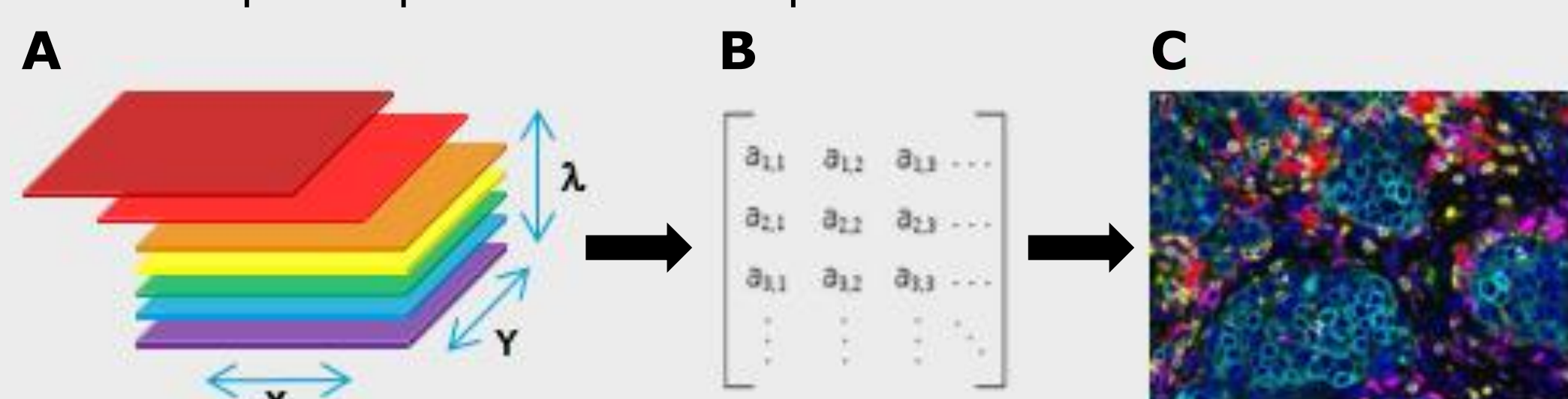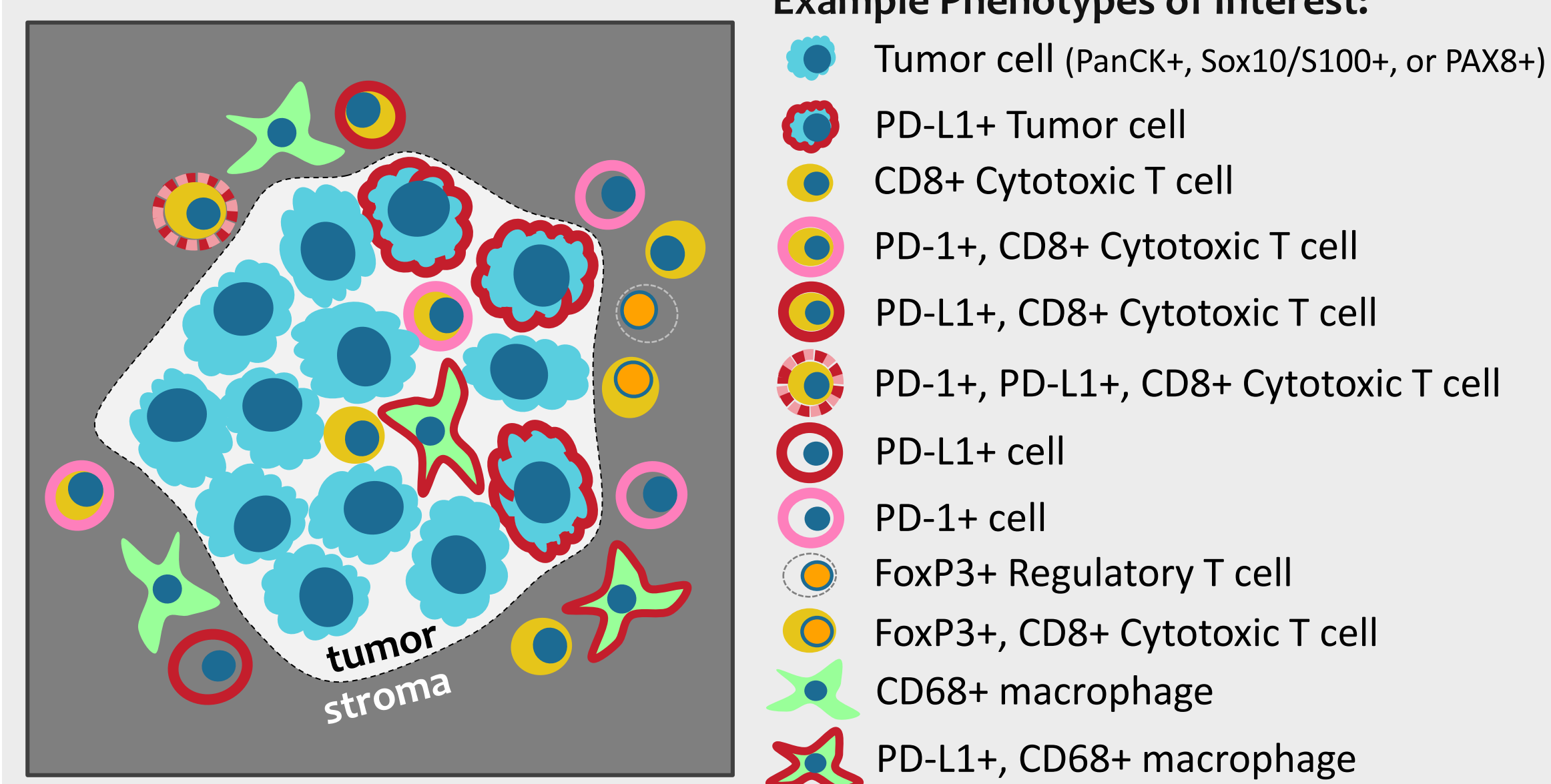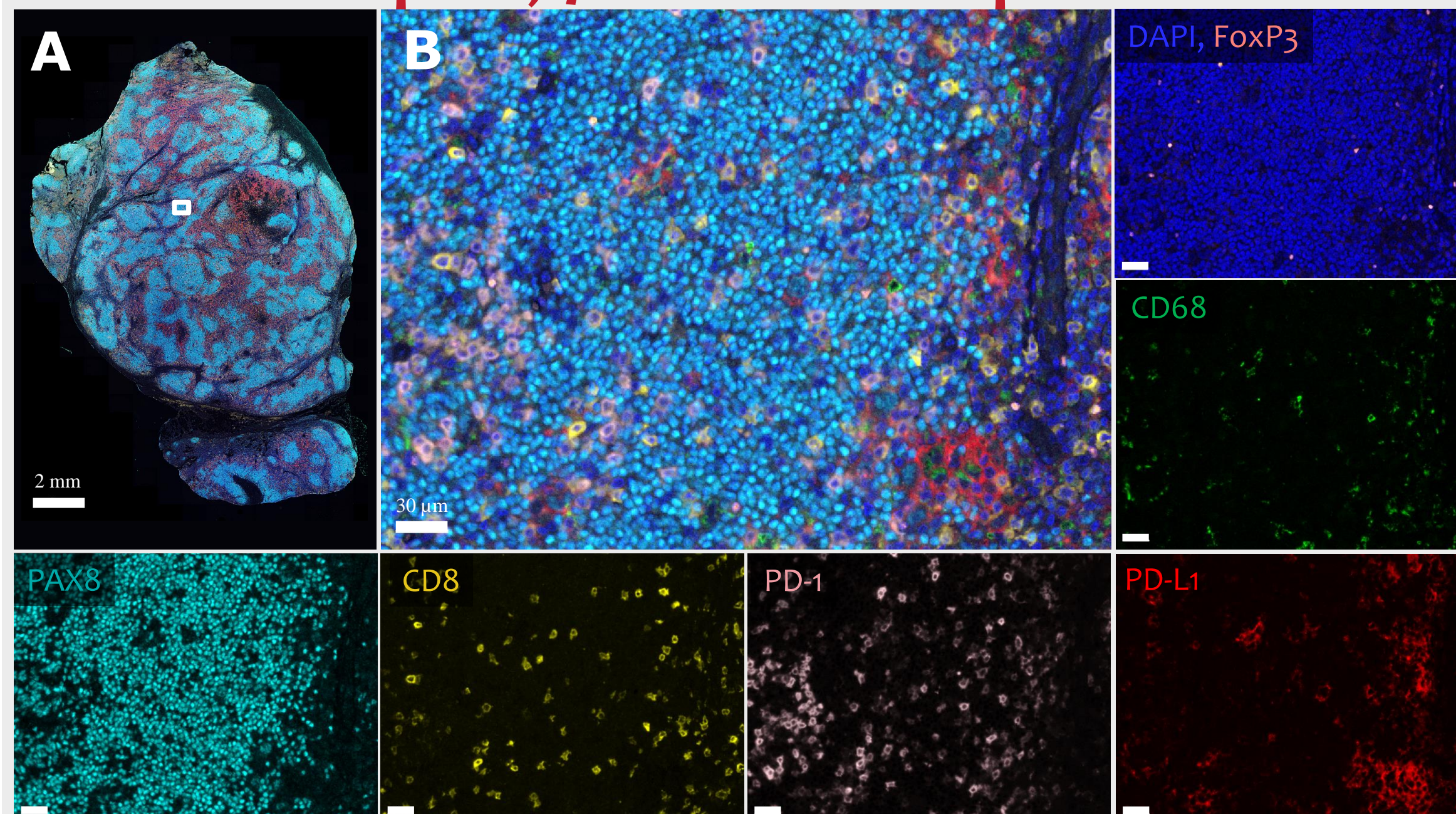
3 7-color Immuno-oncology Panel

FFPE tissue sections were stained with a 6-plex, 7-color Opal™ Polaris panel. The 6 markers in the panel combine to identify at least 12 different phenotypes of interest outlined below.

Phenotype Combinations with 7-color Panel

Panel: PD-L1 PD-1 CD8 Tumor DAPI FoxP3 CD68

Example Phenotypes of Interest:
- Tumor cell (PanCK+, Sox10/S100+, or PAX8+)
- PD-L1+ Tumor cell
- CD8+ Cytotoxic T cell
- PD-1+, CD8+ Cytotoxic T cell
- PD-L1+, CD8+ Cytotoxic T cell
- PD-1+, PD-L1+, CD8+ Cytotoxic T cell
- PD-L1+ cell
- PD-1+ cell
- FoxP3+ Regulatory T cell
- FoxP3+, CD8+ Cytotoxic T cell
- CD68+ macrophage
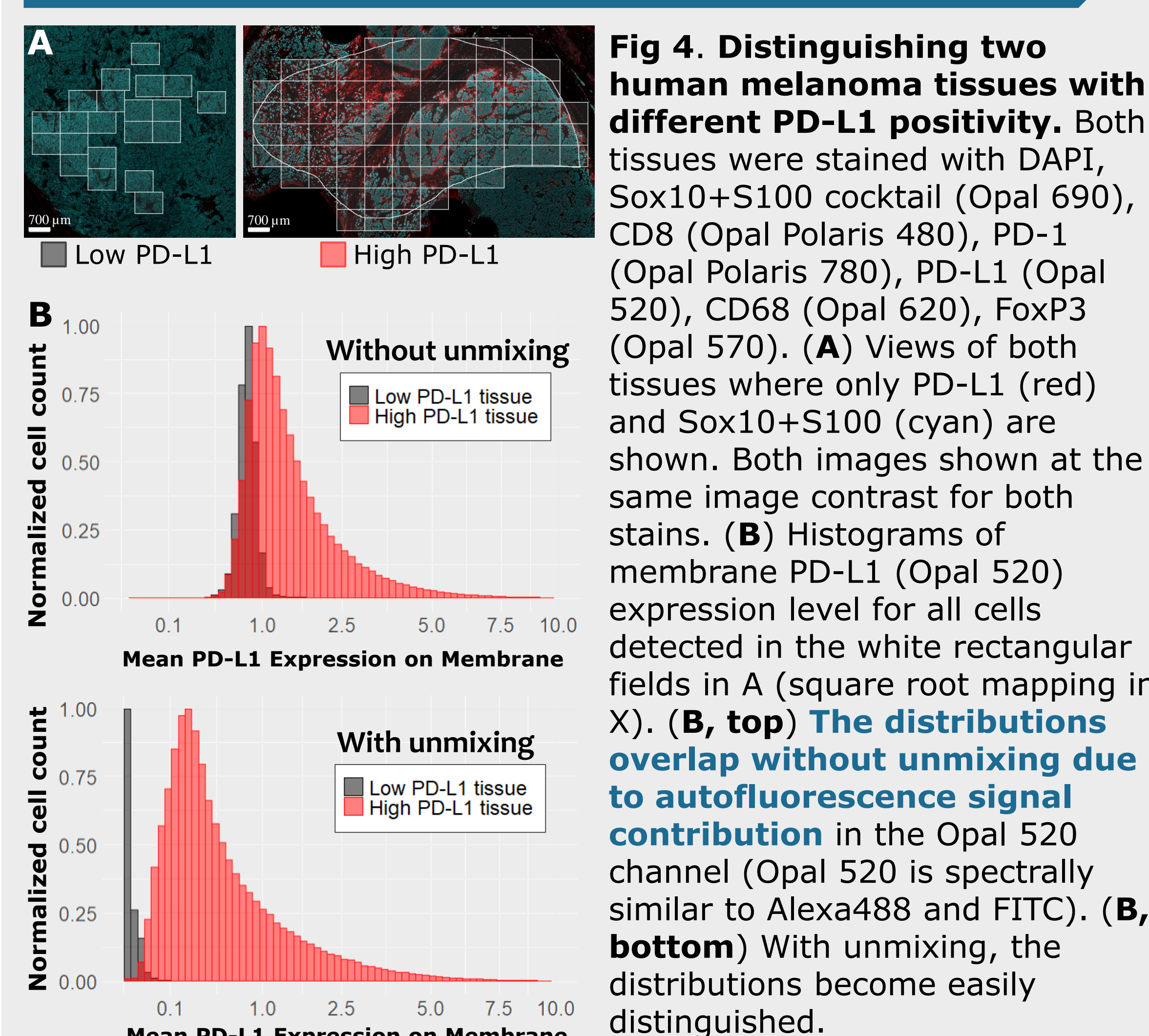- PD-L1+, CD68+ macrophage

tumor
stroma

Unmixed 6-plex, 7-color Multispectral Scan

Fig 3. Multispectral scan of human lymphoma tissue stained with 7-color Opal™ panel: DAPI, PAX8 (Opal 690), CD8 (Opal Polaris 480), PD-1 (Opal Polaris 780), PD-L1 (Opal 520), CD68 (Opal 620), FoxP3 (Opal 570). (A) Whole slide unmixed view from Phenochart™. (B) View of region outlined by white rectangle in A. Remaining panels show separate planes that comprise B (scale bar, 30 μm).
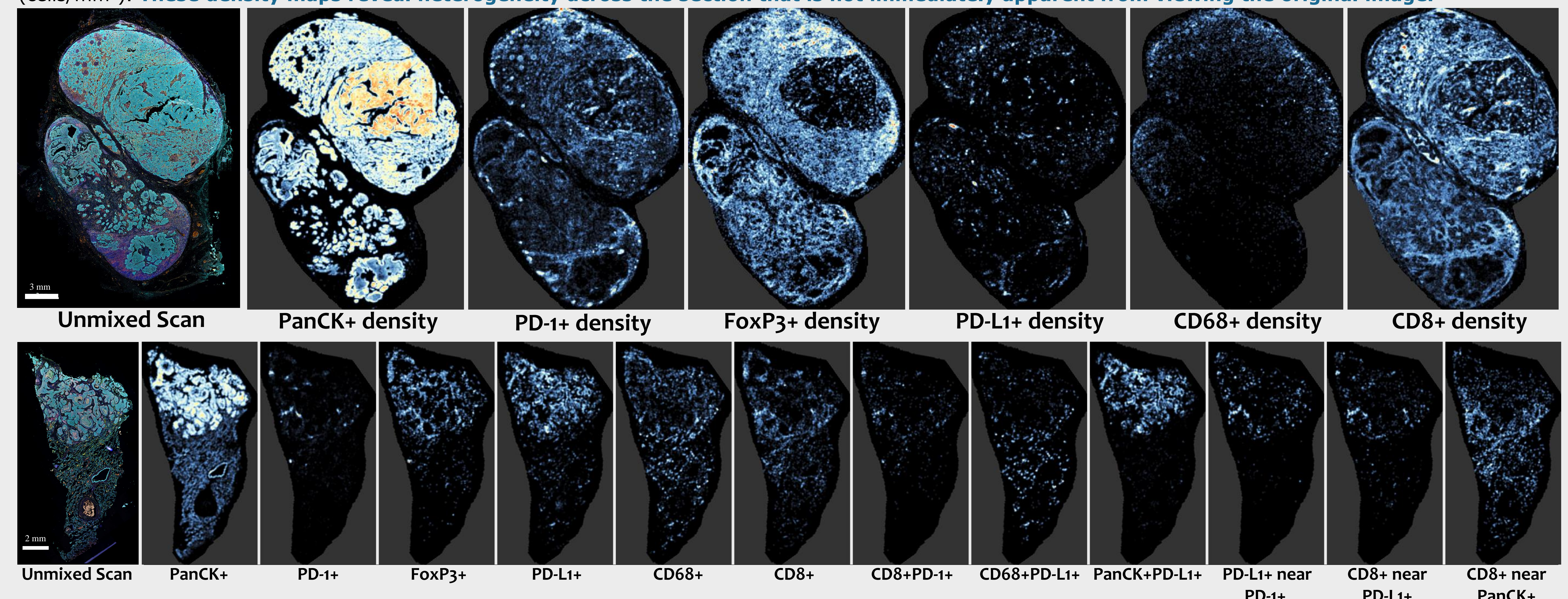
4 Unmixing Improves Sensitivity

Fig 4. Distinguishing two human melanoma tissues with different PD-L1 positivity. Both tissues were stained with DAPI, Sox10+S100 cocktail (Opal 690), CD8 (Opal Polaris 480), PD-1 (Opal Polaris 780), PD-L1 (Opal 520), CD68 (Opal 620), FoxP3 (Opal 570). **(A)** Views of both tissues where only PD-L1 (red) and Sox10+S100 (cyan) are shown. Both images shown at the same image contrast for both stains. **(B)** Histograms of membrane PD-L1 (Opal 520) expression level for all cells detected in the white rectangular fields in A (square root mapping in X). **(B, top)** The distributions overlap without unmixing due to autofluorescence signal contribution in the Opal 520 channel (Opal 520 is spectrally similar to Alexa488 and FITC). **(B, bottom)** With unmixing, the distributions become easily distinguished.

Low PD-L1   High PD-L1

Without unmixing
Low PD-L1 tissue
High PD-L1 tissue

With unmixing
Low PD-L1 tissue
High PD-L1 tissue

Normalized cell count
Mean PD-L1 Expression on Membrane

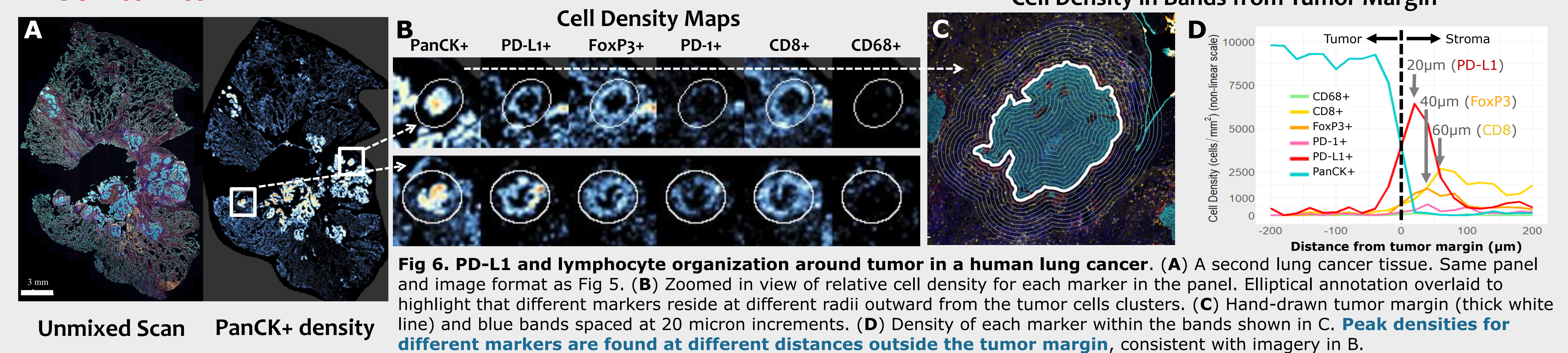## 5 Characterizing the Tumor and Tumor Microenvironment

Visualizing Heterogeneity within a Tissue Section

Unmixed images were processed with inForm® software to phenotype each marker in the panel. Resulting phenotype data can be plotted as heatmaps of cell density (cells/mm²). **These density maps reveal heterogeneity across the section that is not immediately apparent from viewing the original image.**
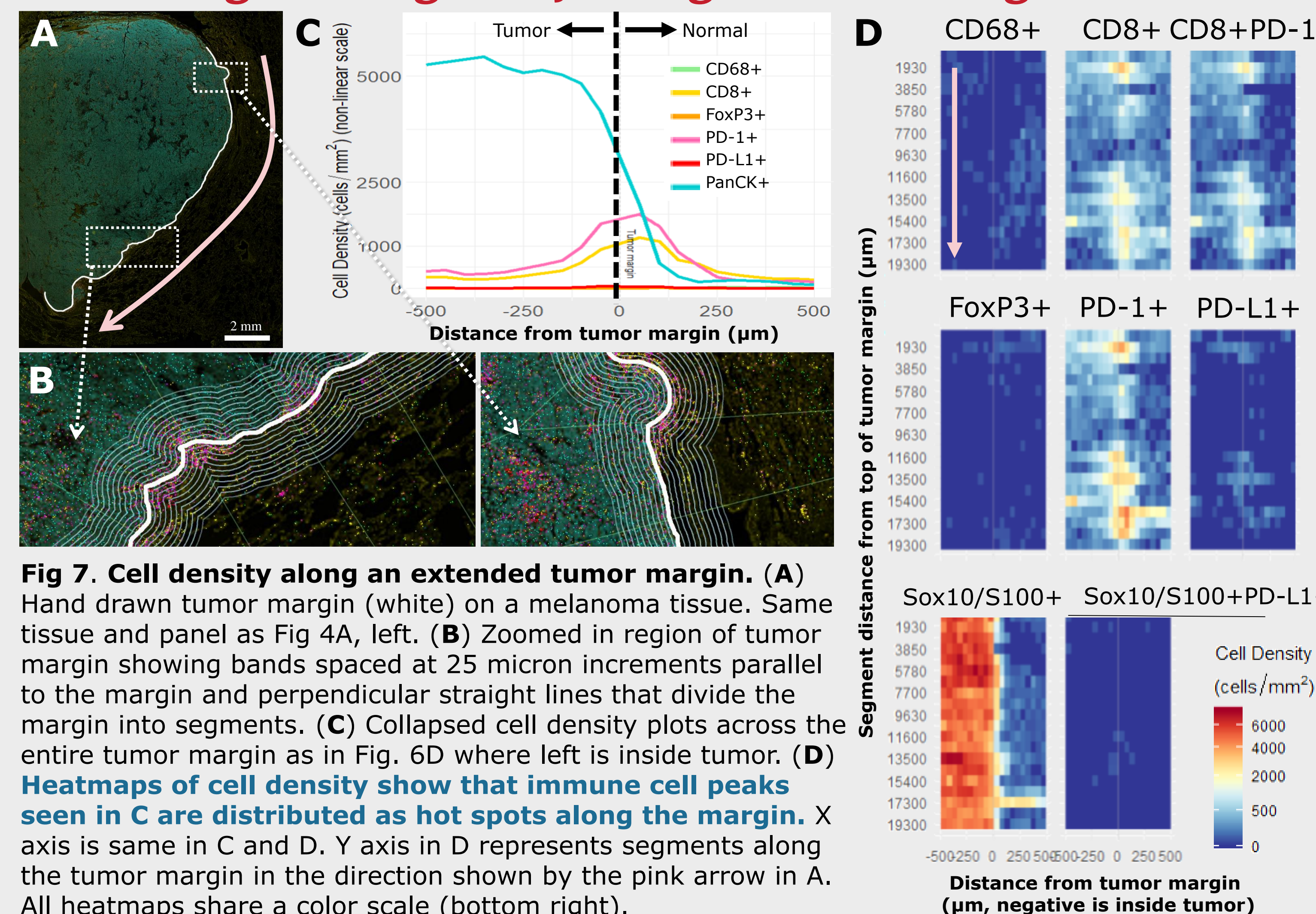
Unmixed Scan | PanCK+ density | PD-1+ density | FoxP3+ density | PD-L1+ density | CD68+ density | CD8+ density

Unmixed Scan | PanCK+ | PD-1+ | FoxP3+ | PD-L1+ | CD68+ | CD8+ | CD8+PD-1+ | CD68+PD-L1+ | PanCK+PD-L1+ | PD-L1+ near PD-1+ | CD8+ near PD-1+ | CD8+ near PanCK+

Fig 5. Cell density maps of a head and neck cancer tissue (top) and lung cancer tissue (bottom). 7-color Opal™ panel: DAPI, PanCK (Opal 690), CD8 (Opal Polaris 480), PD-1 (Opal Polaris 780), PD-L1 (Opal 520), CD68 (Opal 620), FoxP3 (Opal 570). Left-most panel is the unmixed multispectral scan. Remaining panels show heatmaps of cell density for different phenotypes (listed below each panel). Each panel has a different internal color scale. 'Near' indicates within 30 microns.

Mapping Organization within the Tumor Microenvironment

Unmixed Scan | PanCK+ density

Cell Density Maps
PanCK+ | PD-L1+ | FoxP3+ | PD-1+ | CD8+ | CD68+

Cell Density in Bands from Tumor Margin
Tumor ← → Stroma
20μm (PD-L1)
40μm (FoxP3)
60μm (CD8)
Cell Density (cells/mm²) (non-linear scale)
Distance from tumor margin (μm)

Fig 6. PD-L1 and lymphocyte organization around tumor in a human lung cancer. (A) A second lung cancer tissue. Same panel and image format as Fig 5. **(B)** Zoomed in view of relative cell density for each marker in the panel. Elliptical annotation overlaid in A highlight that different markers reside at different radii outward from the tumor cells clusters. **(C)** Hand-drawn tumor margin (thick white line) and blue bands spaced at 20 micron increments. **(D)** Density of each marker within the bands shown in C. **Peak densities for different markers are found at different distances outside the tumor margin**, consistent with imagery in B.

Visualizing Heterogeneity Along a Tumor Margin

Tumor → Normal
CD68+
CD8+
FoxP3+
PD-1+
PD-L1+
PanCK+
Cell Density (cells/mm²) (non-linear scale)
Distance from tumor margin (μm)

CD68+ | CD8+ | CD8+PD-1+
FoxP3+ | PD-1+ | PD-L1+
Sox10/S100+ | Sox10/S100+PD-L1+
Cell Density (cells/mm²)
Distance from tumor margin (μm, negative is inside tumor)
Segment distance from top of tumor margin (μm)

Fig 7. Cell density along an extended tumor margin. (A) Hand drawn tumor margin (white) on a melanoma tissue. Same tissue and panel as Fig 4A, left. **(B)** Zoomed in region of tumor margin showing bands spaced at 25 micron increments parallel to the margin and perpendicular straight lines that divide the margin into segments. **(C)** Collapsed cell density plots across the entire tumor margin as in Fig 6D where left is inside tumor. **(D) Heatmaps of cell density show that immune cell peaks seen in C are distributed as hot spots along the margin.** X axis is same in C and D. Y axis in D represents segments along the tumor margin in the direction shown by the pink arrow in A. All heatmaps share a color scale (bottom right).

6 Conclusions

Spatial analyses of MOTiF™ multispectral scans showed wide variety among the tumor sections and within individual tumor sections. We observed differences in the overall density of individual markers, and further distinctions when looking at the local proximities of marker combinations that could not be observed with a conventional 3- or 4-color slide scan.

Furthermore, when compared with results from analogous scans analyzed without spectral unmixing, the MOTiF™ imagery showed a marked improvement in the dynamic range of detected positive cells.

These tools for spatial analyses of MOTiF™ high-throughput 7-color multispectral slide scans provide translational researchers the ability to probe a whole slide section with the necessary breadth and depth to describe the complex heterogeneity of the tumor microenvironment.

1. https://akoyabio.github.io/phenoptrReports/
2. RStudio Team (2015). RStudio: Integrated Development for R. RStudio, Inc., Boston, MA URL http://www.rstudio.com/.
3. R Core Team (2019). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/.
4. Bankhead, P. et al. QuPath: Open source software for digital pathology image analysis. Sci. Rep. 7, 16878 (2017) https://www.nature.com/articles/s41598-017-17204-5