

Background

The tumor microenvironment hosts a myriad of cellular interactions that influence tumor biology and patient outcomes. Multiplex immunofluorescence (mIF) provides the ability to investigate a large number of these interactions in a single tissue section, and has been shown to outperform other testing modalities for predicting response to immunotherapies [1].

Multispectral imaging (MSI) improves the capabilities of mIF by providing the ability to spectrally unmix fluorescence signals. This increases the number of markers that can be probed in the same scan and allows for separation of true immunofluorescence signals from tissue autofluorescence background.

Here, we apply MSI to explore spatial interactions observed in lung cancer samples using an end-to-end translational workflow based on the Phenoptics™ platform. The workflow includes:

- A pre-optimized 7-color staining panel kit
- A pre-defined imaging protocol
- A pre-configured analysis algorithm for cell phenotyping

Using tissue microarrays (TMA), we demonstrate the heterogeneity of spatial interactions observed among different lung cancer samples and the improved sensitivity of detection afforded by unmixing multispectral scans.

Methods

Tissue & Staining: Formalin-fixed paraffin-embedded (FFPE) lung cancer TMA contained 120 cores (1.5 mm diameter, US Biomax, Inc., Derwood, MD). The TMA was stained using the MOTiF™ PD1/PD-L1 Panel: Auto LuCa Kit and pre-optimized protocol for the Leica BOND RX™.

Imaging: Whole slide 7-color MOTiF multispectral scan was acquired on Vectra Polaris® using pre-defined parameters. Phenochart™ software was used to identify cores for analysis.

Phenotyping Analysis: Scans were unmixed and analyzed with inForm® software using a pre-configured algorithm tailored to the MOTiF™ PD1/PD-L1 Panel kit. With this algorithm, cells are assigned phenotypes using intensity thresholds for CD8, PD1, FoxP3, CD68, and PanCK signal levels, subject to pre-defined marker priority rules. The rules limit co-positivity to any combination of CD8, FoxP3, and PD1, but no combinations of those markers with CD68 or PanCK, and no combination of CD68 with PanCK. When threshold levels generate excluded combinations, priority is given to calls for CD8/FoxP3/PD1 over CD68, which in turn has priority over PanCK. To explore the dynamic range of PD-L1, it was assessed via expression level (signal intensity), not phenotyping.

Spatial Analysis: Spatial analyses and visualizations were performed in R [2] using the phenoptr and phenoptrReports packages [3], and custom scripts.

Results: 7-color MOTiF PD1/PD-L1 Panel: Auto LuCa Kit Applied to Lung Cancer TMA

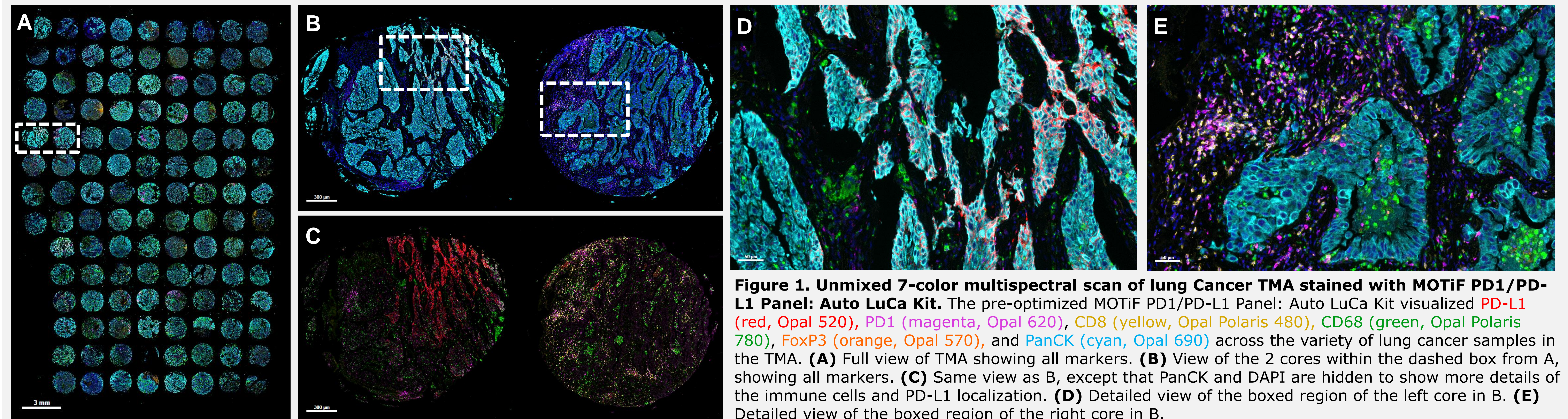


Figure 1. Unmixed 7-color multispectral scan of lung cancer TMA stained with MOTiF PD1/PD-L1 Panel: Auto LuCa Kit. The pre-optimized MOTiF PD1/PD-L1 Panel: Auto LuCa Kit visualized PD-L1 (red, Opal 520), PD1 (magenta, Opal 620), CD8 (yellow, Opal Polaris 480), CD68 (green, Opal Polaris 780), FoxP3 (orange, Opal 570), and PanCK (cyan, Opal 690) across the variety of lung cancer samples in the TMA. **(A)** Full view of TMA showing all markers. **(B)** View of the 2 cores within the dashed box from A, showing all markers. **(C)** Same view as B, except that PanCK and DAPI are hidden to show more details of the immune cells and PD-L1 localization. **(D)** Detailed view of the boxed region of the left core in B. **(E)** Detailed view of the boxed region of the right core in B.

Results: Core-to-Core Heterogeneity in Phenotype Densities and Proximities

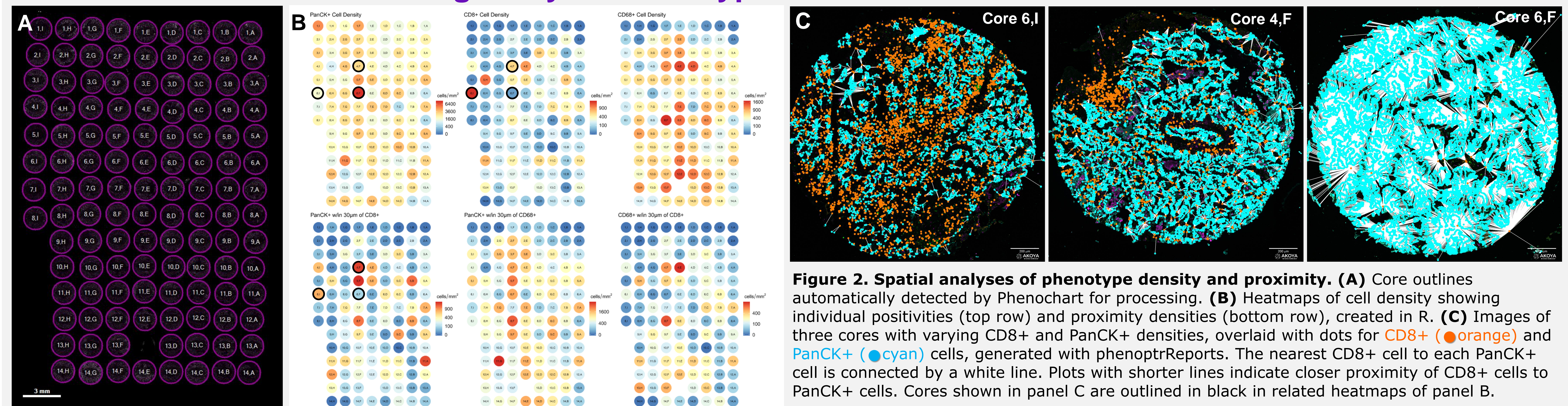


Figure 2. Spatial analyses of phenotype density and proximity. **(A)** Core outlines automatically detected by Phenochart for processing. **(B)** Heatmaps of cell density showing individual positivites (top row) and proximity densities (bottom row), created in R. **(C)** Images of three cores with varying CD8+ and PanCK+ densities, overlaid with dots for CD8+ (orange) and PanCK+ (cyan) cells, generated with phenoptrReports. The nearest CD8+ cell to each PanCK+ cell is connected by a white line. Plots with shorter lines indicate closer proximity of CD8+ cells to PanCK+ cells. Cores shown in panel C are outlined in black in related heatmaps of panel B.

Results: Improved PD-L1 Detection Sensitivity with Unmixing

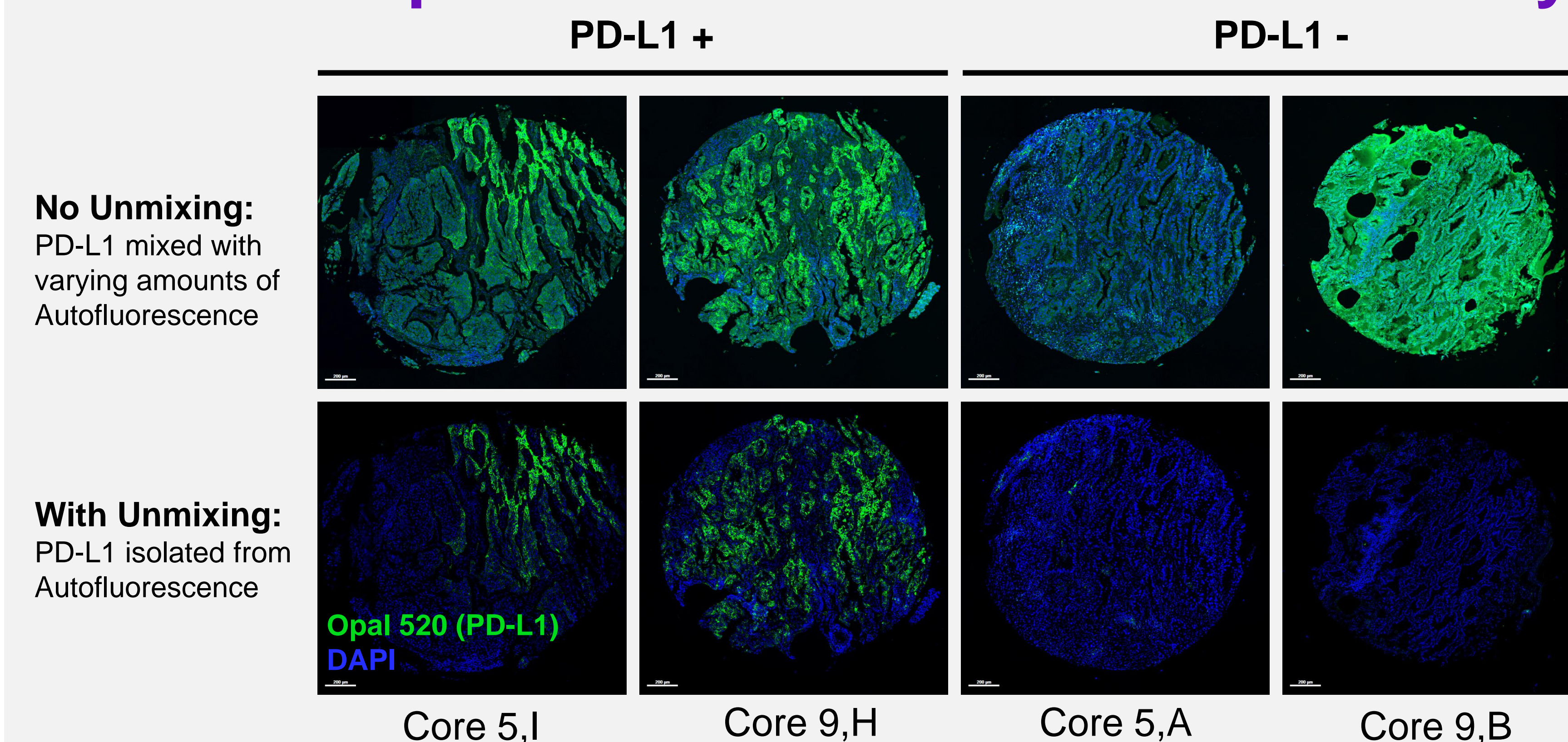


Figure 3. Detecting PD-L1 expression above autofluorescence background. Overlays of PD-L1 (Opal 520, green) and DAPI (blue) signals for PD-L1+ cores (left) and PD-L1- cores (right). Without unmixing (top row), autofluorescence signals can be detected in the Opal 520 channel and PD-L1- cores may be mistakenly categorized as PD-L1+. Spectral unmixing (bottom row) accommodates the wide variety in autofluorescence intensity (as seen by comparing cores 5,A and 9,B) by utilizing the intrinsic spectral signature of autofluorescence to isolate it from true Opal 520/PD-L1 signals.

Conclusions

The end-to-end Phenoptics staining, imaging, unmixing, and spatial analysis workflow described here provides a robust and sensitive platform for exploring the immune landscape within the tumor microenvironment.