The importance of spatial analysis and multispectral imaging when evaluating tumor-immune interactions in glioblastoma

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BACKGROUND

Glioblastoma (GBM) is an aggressive and almost universally fatal brain cancer. GBM tumor masses are highly vascularized and infiltrated with cells mediating both innate and adaptive immunity. The presence and organization of immune cells within the tumor microenvironment varies between patients and within individual tumors. Macrophages can be present in different phenotypes including pro-inflammatory (M1-like) and pro-growth (M2-like). The presence and interaction of these different macrophage phenotypes within in the immune landscape influences responses to different treatment modalities, including immunotherapies in other cancer subtypes.

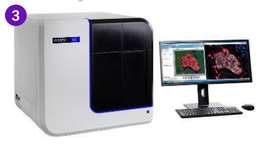
Akoya's MOTiF™ PD-1/PD-L1 Panel is a validated, multiplex immunoassay enabling detection of PD-1, PD-L1, FoxP3, CD8, CD68, and Sox10/S100, and optimized with the PhenoImager HT (previously Vectra Polaris) Automated Quantitative Multispectral Imaging (MSI) system. We have expanded the MOTiF™ PD-1/PD-L1 Panel kit to a 7-plex (8 color) panel in order to further interrogate the innate-immune landscape of GBM, and specifically identify astrocytes with GFAP, and brain-resident microglia and infiltrating macrophages with Iba1, CD163, and CD11c. In this study, we demonstrate the utility of multispectral imaging and spatial analysis of multiple relevant biomarkers in a single tissue sample for characterizing the complex immune landscape of GBM tumors with potential implications for enhancing design and implementation of therapeutics for GBM.

METHODS

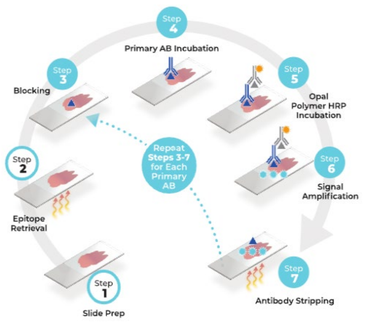
MOTiF PD-1/PD-L1 Automated Panel Kit Workflow

MOTiF Panel Kit + Opal 540

Leica BOND RX



PhenoImager HT System & InForm Software



Iterative Staining Protocol for Opal mIF Assay

Panel 1	Panel 2	
FoxP3	GFAP	CD11c
PD-L1	Iba-1	
Sox10/S100	EGFR	
PD-1	CD163	
CD8	CD8	
CD68	CD68	
DAPI	DAPI	

Previously, human FFPE GBM tissues were stained using the MOTiF™ PD-1/PD-L1 Auto Melanoma kit, designated as Panel 1. Staining was performed on the Leica BOND RX™ automated stainer. For the current study, multiple biomarker swaps and/or additions were made to derive the 8 color (7-plex) panel designed to evaluate the spatial phenotypes within the immune-tumor micro-environment (designated as Panel 2 above).

Whole-slide imaging followed by multispectral field acquisition was performed on the PhenoImager HT (formerly Vectra Polaris®) with optimized acquisition parameters; and scans were unmixed and analyzed with InForm® software v. 2.6. Spatial analyses and visualizations were performed using phenoptr and phenotprReports.

RESULTS: 7-plex Multispectral Imaging of Glioblastoma Tissues

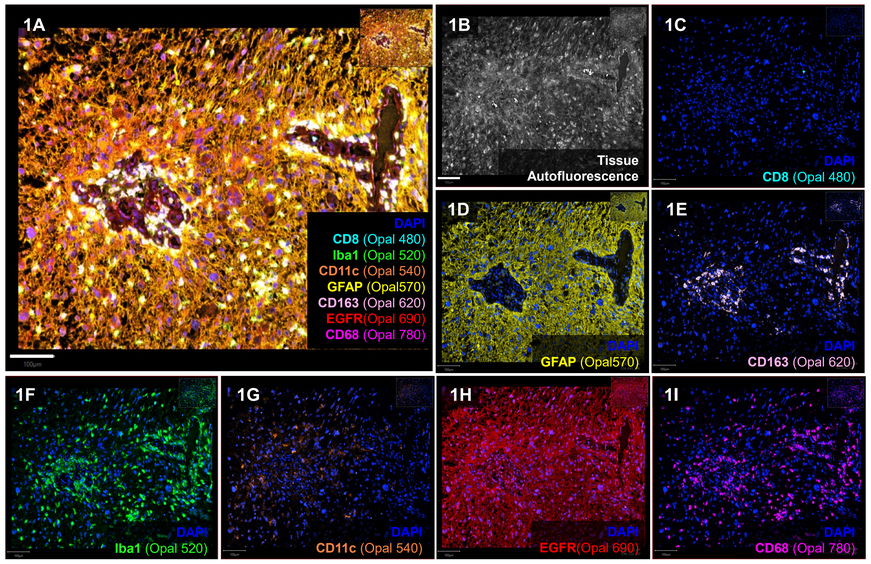
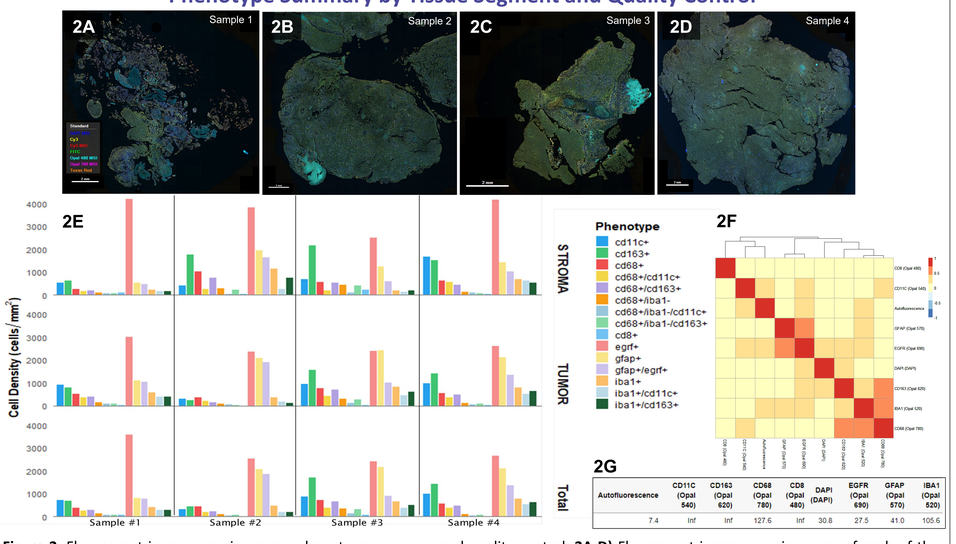


Figure 1. 8 color (7-plex) multispectral image of human, formalin-fixed, paraffin-embedded (FFPE) glioblastoma (GBM, Figure 2B) stained with Opal fluorophores and imaged with PhenoImager HT multispectral fields workflow. The multispectral fields workflow allows for imaging of up to 9 colors and includes a fluorescent overview image (Figure2, A-D) followed by acquisition of multispectral fields by engaging a liquid crystal tunable emission filter. **1A**) Multiplex 8 color image, spectrally unmixed with GBM-specific spectral library. **1B**) Tissue-specific autofluorescence signal unmixed into a separate channel and re-colored white for visualization. **1C-I**) Individually unmixed channels to visualize each marker of interest plus DAPI. Scale bars = 100µm. Scale bar in 1B representative for all single-color images (**B-I**). All images are pseudo-colored and brightness increased for visualization purposes only.

Phenotype Summary by Tissue Segment and Quality Control



2G

Autofluorescence	CD11C (Opal 540)	CD163 (Opal 620)	CD68 (Opal 780)	CD8 (Opal 480)	DAPI (DAPI)	EGFR (Opal 690)	GFAP (Opal 570)	IBA1 (Opal 520)
7.4	Inf	Inf	127.6	Inf	30.8	27.5	41.0	105.6

Figure 2. Fluorescent image overview scan, phenotype summary and quality control. **2A-D)** Fluorescent image overview scan of each of the four GBM tissue samples evaluated. Filters engaged for acquisition are shown in 1A. Scale bars=2mm. **2E)** Cell phenotype density in each tissue segment (tumor vs stroma segmentation algorithm used GFAP+/EGFR+, dapi and autofluorescence signals as inputs) **2F)** Quality Control representative heat map showing clean unmixing of channels with minimal overlap except in channels containing expected co-expressing markers (i.e GFAP (Opal 570) and EGFR (Opal 690)). **2G)** Signal to Noise ratio table for each channel ('inf' indicates background of zero after spectral unmixing).

M1-like to M2-like Macrophages and Spatial Relationships to GFAP+/EGFR+ Tumor Cells

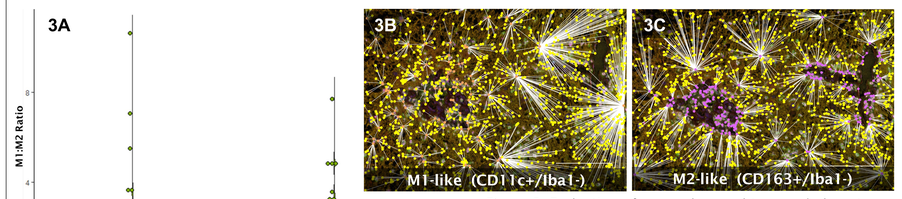


Figure 3. Evaluation of macrophage subtype and phenotypes. **3A**) M1:M2 ratio separated by sample (color) and origin (Iba1+/-). **3B**) Nearest infiltrating M1-like macrophage (CD11c+/Iba1-) to each tumor cell (GFAP+/EGFR+). **3C**) Nearest infiltrating M2-like macrophage (CD163+/Iba1-) to each tumor cell (GFAP+/EGFR+). Note the clustering of M2-like infiltrating macrophages around blood vessels whereas M1-like macrophages show less aggregation.

Nearest Neighbor Analysis

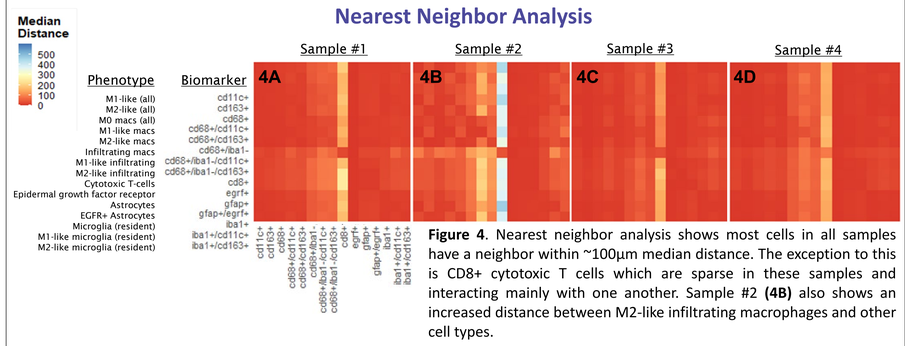


Figure 4. Nearest neighbor analysis shows most cells in all samples have a neighbor within ~100µm median distance. The exception to this is CD8+ cytotoxic T cells which are sparse in these samples and interacting mainly with one another. Sample #2 **(4B)** also shows an increased distance between M2-like infiltrating macrophages and other cell types.

Interacting Complex Phenotypes Can Differentiate Tumor Samples Beyond Nearest Neighbor Analysis

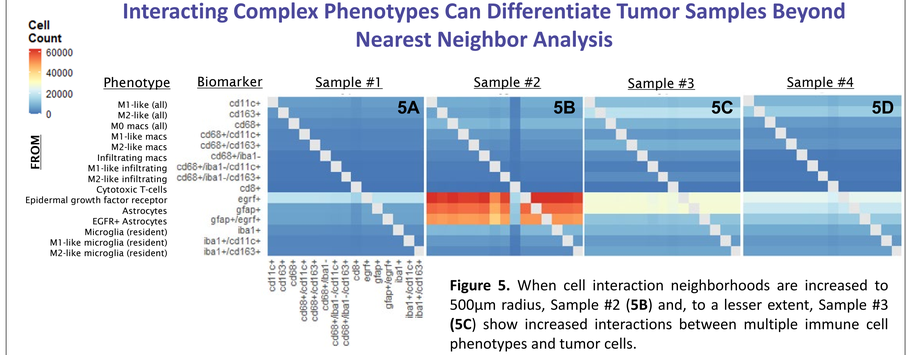


Figure 5. When cell interaction neighborhoods are increased to 500µm radius, Sample #2 (**5B**) and, to a lesser extent, Sample #3 **(5C**) show increased interactions between multiple immune cell phenotypes and tumor cells.

CONCLUSIONS

- Multispectral imaging using Akoya's PhenoImager (previously Phenoptics) technology allows for evaluation of phenotypes by imaging multiple biomarkers on a single tissue sample rather than superposition of serial sections
- Imaging multiple biomarkers on a single tissue sample allows for less ambiguous and more nuanced analyses of complex phenotypes and spatial interactions
- For complex and heterogeneous tumors such as glioblastoma, imaging biomarkers in context allows for analyses beyond immune presence and M1:M2 ratios.
- Insights into cellular relationships and differential landscapes may reveal new spatial signatures to inform patient stratification and alternative treatment options.

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