Dissecting the myeloid compartment of the tumor microenvironment with SignalStar™ multiplex immunohistochemistry and Phenoplex image analysis

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INTRODUCTION

Myeloid cells, including macrophages, dendritic cells, monocytes, and granulocytes, are integral to the tumor microenvironment (TME). Recent multiomic technologies have identified distinct subpopulations of myeloid cells that regulate tumor progression and metastasis. Spatial biology technologies, such as multiplex immunohistochemistry (mIHC), are essential for investigating myeloid cell phenotype and function within the TME.

METHODS

In this study, we validated and analyzed an 8-plex SignalStar™ mIHC panel from Cell Signaling Technology in squamous cell lung carcinoma FFPE tissue. This panel includes oligo-conjugated antibodies for CD11c, CD68, CD206, CD163, HLA-DRA, PD-L1, SIRPα, and Arginase-1. Fluorescently labeled, complementary oligonucleotides were used to amplify the signal for these eight targets across two rounds of imaging in the same FFPE tissue sample. High-resolution whole-slide images were analyzed using Visiopharm Phenoplex software, which facilitates automated quantification of biomarker co-expression and evaluates the proximity of M1- and M2-polarized myeloid cells in relation to PD-L1 and SIRPα positive and negative cells in the tumor and surrounding TME.

RESULTS

Deep-learning-based software was crucial in several analysis aspects. Image alignment functionality enabled precise overlay of sequential imaging rounds, ensuring consistent localization of target signals. Using both built-in and user-trained algorithms, Phenoplex was trained to recognize distinct cell populations, allowing for accurate identification and quantification of biomarker co-expression. The software also facilitated interactive analysis between tSNE data and image-based data, showcasing cell population distributions. Additionally, the proximity analysis revealed critical spatial relationships between different myeloid subpopulations, including: CD68+CD11c+HLA-DRA+ M1-polarized myloid cells to (SIRPα+ or SIRPα-) cells; and CD68+CD206+Arginase-1+ M2-polarized macrophages to (PD-L1+ or PD-L1-) cells. These insights are essential for understanding how myeloid cells organize and interact to influence the tissue microenvironment, driving disease progression and therapeutic responses.

CONCLUSIONS

The combination of the SignalStar™ mIHC panel and Phenoplex analysis software provides a powerful tool for dissecting the myeloid compartment of the TME. The precise tissue alignment, robust cell population identification, and detailed spatial analysis enabled by this platform offer critical insights into myeloid cell interactions and their role in tumor progression. This integrated approach is pivotal for advancing our understanding of the TME and improving therapeutic strategies targeting myeloid cells.

REFERENCES

1. PMID: 25598292
2. PMID: 25506346
3. PMID: 27869795
4. PMID: 22038213
5. PMID: 24333724
6. PMID: 18978793

Protocol and Panel Design

Staining, imaging, image processing, and analysis

- All stains were performed using the Bond Rx by Leica Biosystems.
- IF whole slide images were collected at 20X magnification using the Akoya PhenoImager HT.
- IF images from consecutive round of imaging were aligned and stacked using TissueAlign™ by Visiopharm.
- Example images were generated and AI-driven image analysis was performed with Phenoplex (Visiopharm).
- Graphs were made using Excel.

Figure 1A

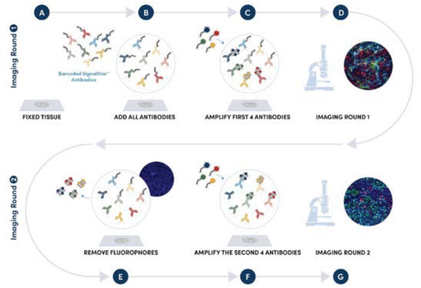


Figure 1B

Primary Antibody Marker (clone)	Purpose	Imaging Round	Fluorophore
CD11c (D3V1E)	M1-like Macropahge[1]	1	488
CD68 (D4B9C)	Pan Macrophage[3]	1	594
CD206/MRC1 (E2L9N)	M2-like Macrophage[2]	1	647
CD163 (D6U1J)	M2-lilke Macrophage[4]	1	750
HLA-DRA (E9R2Q)	M1-like Macropahge[5]	2	488
PD-L1 (E1L3N®)	Inhibitory receptor	2	594
SIRPa/SHPS1 (D6I3M)	Inhibitory receptor	2	647
Arginase-1 (D4E3M)	M2-like Macrophage[6]	2	750

Figure 1. SignalStar protocol and panel design.
A) All 8 uniquely tagged, oligo-conjugated antibodies are added in cocktail in one primary incubation step. Complementary oligos with fluorescent dyes (channels: 488, 594, 647, and 750) amplify the signal of the first 4 antibodies by building oligo-fluorophore constructs attached to the antibody (Imaging Round 1). The first round of oligos and fluorophores are removed, and a second round of amplification is performed to visualize the second 4 antibodies (Imaging Round 2). The 2 images are aligned and fused computationally to generate an image consisting of up to 8 targets.
B) Panel design performed using the SignalStar™ mIHC Panel Builder that can be found at: https://www.cellsignal.com/applications/signalstar-multiplex-ihc-panel-builder

Tissue Alignment

Figure 2A

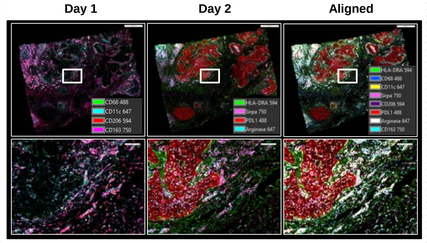


Figure 2B

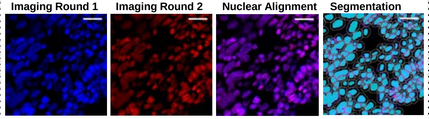


Figure 2C

PD-L1+ tumor: red

Invasive tumor margins
- IM1 0-30μm: cyan
- IM2 30-60μm: yellow

Stroma: transparent, blue outline

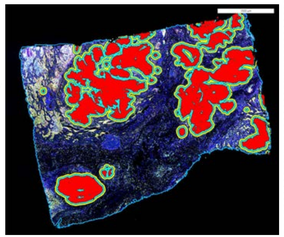


Figure 2. Tissue Alignment, Tissue and Cell segmentation
A) Two SignalStar 5-plex images were cell precise co-registered using the Visiopharm TissueAlign function.
B) The resulting 8-plex biomarker image showed perfect DAPI signal matching of Day 1 and Day 2. Cells were segmented using the pretrained DAPI nucleus detection APP available with Phenoplex.
C) Tissue segmentation algorithm was trained on PD-L1 expressing tumor cells for detection of PD-L1+ SCC tumor cells. Two 30μm invasive margins were grown from the epithelial border into the stromal area to investigate the tumor immune microenvironment in more detail.

Population Analysis

Figure 3A

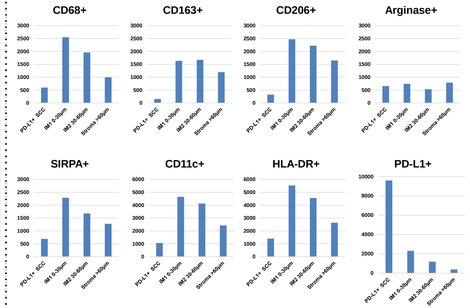


Figure 3B

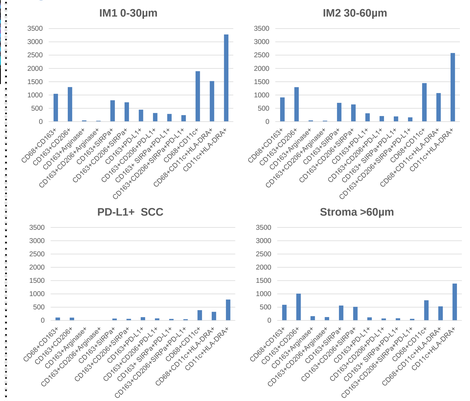


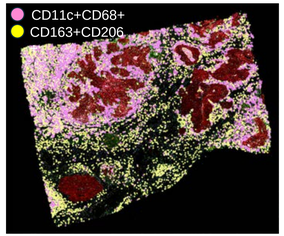
Figure 3. Single biomarker and macrophage population densities within tumor and invasive margins.
A) Individual Biomarker positivity within the cell objects was set using the Phenoplex Guided Workflow. Cell positivity and Phenotypes within the ROIs: PD-L1+ SCC area, Invasive Margin 1 and 2 (IM1 and IM2) and Stroma area >60μm away from the tumor border
B) Aggregated Phenotypes for M1 and M2 macrophage populations are shown within the four ROIs. Data visualizations were compiled using Excel

Spatial Neighborhood analysis

Figure 4A

Neighbor Counts	CD11c+ CD68+	CD11c+ CD68+ HLA-DR+	CD11c+	CD163+ CD206+
CD68+ CD163+	14.59	10.53	32.41	15.38
CD163+ CD206+	11.67	8.35	28.59	14.49
CD68+ CD206+	14.37	10.56	32.27	14.66
CD163+ CD206+ PD-L1+	20.24	16.58	46.36	14.97
CD163+ Arginase+	18.09	13.63	50.75	21.22

Neighbor Counts: CD11c+CD68+ around CD163+CD206+ cells



Neighbor Counts: CD163+CD206+ around Arginase+ CD163+ cells.

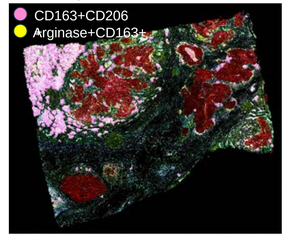
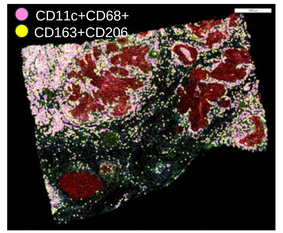


Figure 4B

Nearest Neighbor (distance in μm)	CD11c+ CD68+	CD11c+ CD68+ HLA-DR+	CD11c+	CD163+ CD206+
CD68+ CD163+	1.9	3	1.7	2
CD163+ CD206+	3.3	4.3	2.6	1.9
CD68+ CD206+	2	3.1	1.8	2.3
CD163+ CD206+ PD-L1+	1.3	1.7	0.8	1.8
CD163+ Arginase+	1.6	2.3	0.6	0.9

Nearest Neighbor distance of CD163+CD206+ cells to CD11c+CD68+



Nearest Neighbor distance of Arginase+ CD163+ to CD163+CD206+ cells.

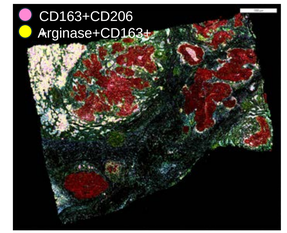


Figure 4: Phenoplex built in spatial neighborhood analysis tools used to quantify neighbors around specified targets within the stromal region. Neighbor counts and nearest neighbor measurements were calculated within a 30μm radius around the target cells (rows).
A) Phenoplex Neighbor Counts showed that CD11c+CD68+ are clustering with CD163+CD206+ M2 macrophages within the invasive margin and the immune cluster (top left corner of the tissue), Arginase+CD163+ are mainly inside the immune cluster surrounded by M2 macrophages.
B) Nearest Neighbor analysis showed that the mean distance of macrophage markers to macrophage markers is very low indicating that these cells highly cluster throughout the tissue.

SUMMARY OF RESULTS

- SignalStar staining is bright and specific.
- The SignalStar assay maintains tissue integrity for accurate image co-registration.
- TissueAlign™ can successfully co-register SignalStar images with optimal nuclear alignment.
- Phenoplex™ analysis software enables quantification and interactive visualization of cellular phenotypes within detailed ROIs, like the tumor margin, to better investigate tumor immune exclusion and biomarker distribution within whole slide images based on user hypothesis

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