Imaging Mass Cytometry Enables Identification of Distinct Tissue Phenotypes in Highly Autofluorescent Lung and Colon Cancer Tissues, Producing Consistent Data Across Serial Sections

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Introduction

Successful implementation of immunotherapy requires a deep understanding of the spatial interactions between various cell types in the tumor microenvironment (TME). Most fixed tissues are autofluorescent and staining with cyclic immunofluorescence methods often produces data that are difficult to analyze due to the challenges of background subtraction. Imaging Mass Cytometry™ (IMC™) is a powerful tool for high-plex imaging, utilizing CyTOF® technology to simultaneously assess 40-plus protein markers at subcellular resolution without spectral overlap or background autofluorescence. This study demonstrates a tissue phenotyping workflow in highly autofluorescent lung and colon cancer tissues using high-plex IMC, which produces reliable data that can be easily analyzed. A comprehensive workflow using Visiopharm's Phenoplex™ platform, designed specifically for high-plex IMC image analysis, is presented here.

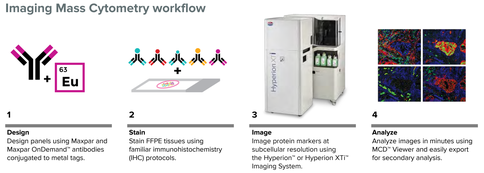
Methods and Materials

Serial sections of lung and colon cancer tissue microarrays (TMAs) were stained with a 26-marker panel comprised of structural, tumor, stroma, and immune cell markers (Table 1). The panel is an easily customized version of the Maxpar® Human Immuno-Oncology IMC Panel Kit. The Maxpar IMC Cell Segmentation Kit (ICSK) was included in the panel for improved nucleus and plasma membrane demarcation. The data analysis pipeline used Phenoplex software (Visiopharm®) for easy, accurate, and quantifiable phenotyping. The analysis workflow consisted of tissue segmentation, nuclear detection using a deep-learning algorithm pre-trained on IMC DNA channels, cell segmentation, a threshold-based cellular phenotyping step, and spatial analyses. Statistical analyses of the reproducibility between serial sections were performed using a paired t-test.

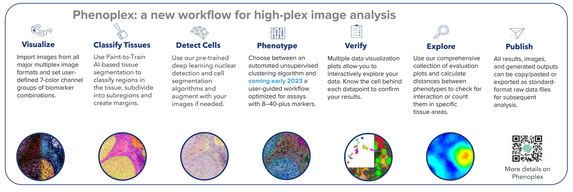
Metal	Marker	Clone	Catalog No.
141Pr	αSMA	1A4	3141017D
143Nd	Vimentin	D21H3	3143027D
144Nd	CD14	EPR3653	3144025D
147Sm	MMP-13	OTI2D8	Custom conjugation
148Nd	Pan-keratin	C11	3148020D
149Sm	CD11b	EPR1344	3149028D
151Eu	CD31	EPR3094	3151025D
152Sm	CD66b	BLR111H	91H033152
153Eu	CD44	IM7	3153029D
154Sm	EpCAM	EPR20532-222	91H024154
155Gd	FoxP3	PCH101	3155018D
156Gd	CD4	EPR6855	3156033D
158Gd	E-cadherin	24-E-10	3158029D
159Tb	CD68	KP1	3159035D
161Dy	CD20	H1	3161029D
162Dy	CD8a	C8/144B	3162035D
164Dy	iNOS	SP126	91H025164
166Er	CD45RA	HI100	3166031D
167Er	Granzyme B	EPR20129-217	3167021D
168Er	Ki-67	B56	3168022D
169Tm	Collagen 1	Polyclonal	3169023D
170Er	CD3	Polyclonal	3170019D
172Yb	Cleaved caspase-3	5A1E	3172027D
173Yb	CD45RO	UCHL1	3173016D
174Yb	HLA-DR	LN3	3174025D
175Lu	Cyclin D1	SP4	Custom conjugation
195Pt	ICSK1		201500
196Pt	ICSK2		201500
198Pt	ICSK3		201500

Table 1. Custom Human Immuno-Oncology IMC Panel

Imaging Mass Cytometry workflow



Phenoplex: a new workflow for high-plex image analysis



Results

In this work, we have shown that analysis of IMC images from lung and colorectal cancer tissues can uncover tissue phenotypic signatures of the TME through the determination of immune cell types found in the vicinity of cancerous cells. Moreover, cell counts and tissue phenotypes were highly consistent across the serial sections, demonstrating the power of IMC in generating robust data.

Profiling the tumor microenvironment of colon and lung cancers

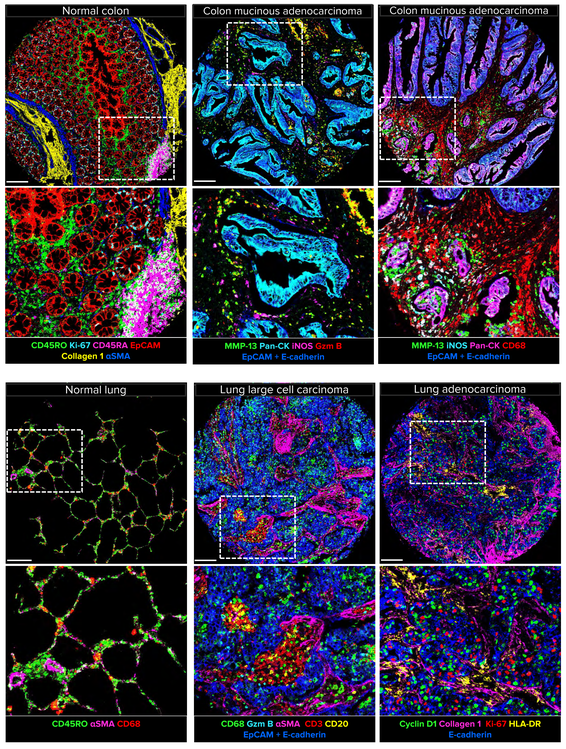


Figure 1. IMC staining of normal and cancerous colon and lung TMAs. Tumor tissue is identified by EpCAM + E-cadherin and pan-cytokeratin staining. Mucinous adenocarcinoma of colon shows cells with co-localization of MMP-13 and granzyme B (appearing as yellow) surrounding the tumor cells, as do cells producing iNOS. Lung adenocarcinoma shows the presence of cyclin D1 and Ki-67 positive cells within the tumor. Scale bars are 200 µm. Enlarged versions of the selected areas are shown in the lower panels.

Easy cell and tissue segmentation and cell phenotyping using Phenoplex

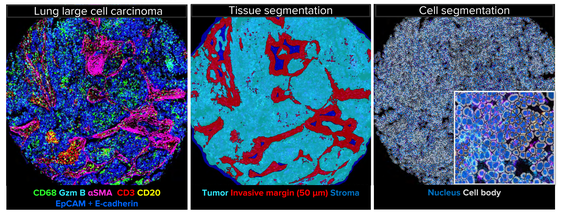


Figure 2. Tissue segmentation and cell segmentation using Phenoplex. To count cells in different tissue compartments, the tissue was differentiated into tumor (cyan) and stroma (blue). A 50 µm invasive margin (red) was calculated around the tumor region. Cell segmentation was based on an IMC specific pre-trained deep learning-based algorithm in the Phenoplex software, augmented with annotations from the Maxpar IMC Cell Segmentation Kit. The enlarged area of the segmented image clearly shows the nucleus (blue) surrounded by the cellular margins (light gray).

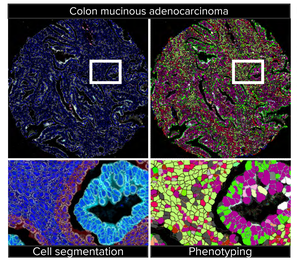


Figure 3. Phenotype cells using Phenoplex. Once cell boundaries are established, biomarker intensities are used to phenotype cells. Phenoplex uses a guided workflow to set thresholds for biomarker positivity using a visual assessment of which cells are positive and which cells are negative. This is done for each biomarker, after which each cell has a phenotype based on these positivities. There are several interactive software tools to help verify that positivity thresholds are optimal. Once a phenotype is established for a cell, it can be plotted in a variety of ways.

Cell counts using Phenoplex are consistent across serial sections

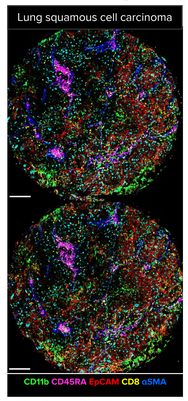


Figure 4a. IMC staining of serial sections of lung squamous cell carcinoma. Scale bars are 200 µm.

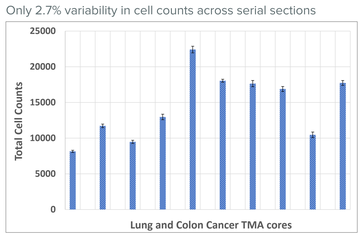


Figure 4b. Serial sections of 10 different lung and colon cancer TMA cores were analyzed by the Phenoplex software. Cell segmentation showed equivalent number of cells between serial sections, with only 2.7% variability. This variability is not much greater than the 2% biological variability observed across serial sections in general. This result emphasizes the power of IMC in combination with Phenoplex analysis in generating highly consistent segmentation data using serial sections.

Verification of cellular phenotyping using the co-occurrence matrix

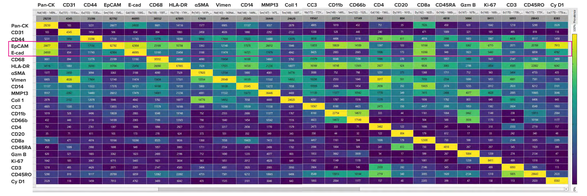


Figure 5. Verify that the phenotyping results match the actual cellular expression patterns. Among other interactive tools, a biomarker co-expression table is a good way to investigate co-expressing cells. This matrix indicates the presence of myeloid, and cytotoxic T cells in the EpCAM+ E-cadherin+ pan-cytokeratin positive tumor compartment. The tumor cells also show granulocyte activation and high levels of proliferation due to upregulation of cyclin D1 and Ki-67.

Lung large cell carcinoma cellular clusters split by tissue compartments

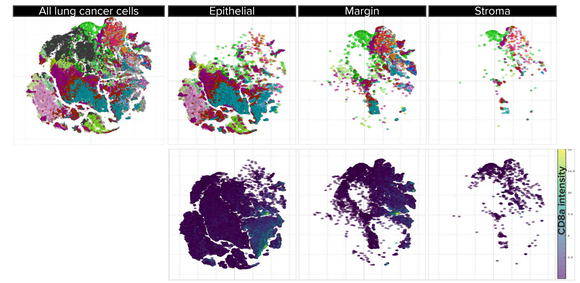


Figure 6. Single-cell analysis of lung large cell carcinoma using Phenoplex can create t-SNE (t-distributed stochastic neighbor embedding) plots split by tissue compartments. The all lung cancer cells t-SNE on the left is split into the cells from different tissue compartments (epithelial, margin, stroma). The sub-t-SNE plots are colored in one of two ways: The upper row shows the cells colored by their computed phenotype, and the bottom row shows the cells colored by the intensity of CD8a in each cell. This can be used to explore differences in biology between tissue compartments. In this case, it highlights that CD8+ cells are found primarily in the epithelial region, with only a few in the tumor margin.

Conclusions

- This work demonstrates that even for highly autofluorescent tissues, IMC can generate high-quality data, consistent across serial sections.
- IMC data can be easily and accurately analyzed using Visiopharm's single software package (Phenoplex), empowering IMC users to be confident in biological interpretation of high-dimensional proteomic data.
- This study showcases the capability of IMC technology combined with Phenoplex analysis for development of systematic digital profiling of the spatial TME.

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