Identification of Distinct Tissue Phenotypes in Lung and Colorectal Cancers by Imaging Mass Cytometry is Consistent Across Serial Sections

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Introduction

Imaging Mass Cytometry™ (IMC)™ is a powerful tool for the study of complex cellular interactions in the tumor microenvironment (TME) and in the discovery of biomarkers that can predict disease outcome or response to therapy. The Hyperion™ Imaging System (Standard BioTools)™ utilizes CyTOF® technology to simultaneously assess 40-plus protein markers at subcellular resolution without spectral overlap or background autofluorescence, thus providing unprecedented insight into the organization and function of the TME. However, despite the advances in staining and imaging methods, developments in analysis software have not kept pace, as we lack a complete, user-defined workflow in a single software package for analysis of high-plex imaging data. Here we present a comprehensive workflow using the Visiopharm® Phenoplex™ platform, designed specifically for high-plex IMC image analysis.

Methods and Materials

This study demonstrates a tissue phenotyping workflow in highly autofluorescent lung and colon cancer tissues using high-plex IMC, which offers the advantage of zero autofluorescence, and hence more reliable results. 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 Kits. The IMC Cell Segmentation Kit (ICSK) was included in the panel for improved nucleus and plasma membrane demarcation. The data analysis pipeline uses Phenoplex software (Visiopharm) for easy, accurate, and quantifiable phenotyping. Analysis consisted of tissue segmentation, nuclear detection using a deep-learning algorithm pre-trained on IMC DNA channels, a threshold-based cellular phenotyping step, and spatial analyses.

Metal	Marker	Clone	Catalog Number
141Pr	αSMA	1A4	3141017D
143Nd	Vimentin	D21H3	3143027D
144Nd	CD14	EPR3653	3144025D
147Sm	MMP-13	OT12D8	Custom conjugation
148Nd	Pan-keratin	C11	3148020D
149Sm	CD1b	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	C8H4B	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
195 Pt	ICSK1		TIS-00001
196 Pt	ICSK2		TIS-00001
198 Pt	ICSK3		TIS-00001

Table 1. Custom Human Immuno-Oncology IMC Panel

Imaging Mass Cytometry workflow

1. **Design** — Design panels using Maxpar antibodies and Maxpar OnDemand™ Antibodies conjugated to metal tags.
2. **Stain** — Stain FFPE tissues using familiar immuno-histochemistry (IHC) protocols.
3. **Image** — Image protein markers at subcellular resolution using the Hyperion Imaging System at 200 Hz with 1 µm pixel size.
4. **Analyze** — Analyze images in minutes using MCDV Viewer and easily export for secondary analysis.

Phenoplex: a new workflow for high-plex image analysis

Visualize — Import images from all major multiplex image formats and set user-defined 7-color channel groups of biomarker combinations.

Classify Tissues — Use Paint-to-Train AI-based tissue segmentation to classify regions in the tissue, subdivide into subregions, and create margins.

Detect Cells — Use our pre-trained deep learning nuclear detection and cell segmentation algorithms and augment with your images if needed.

Phenotype — Choose between an automated unsupervised clustering algorithm and coming early 2023 a user-guided workflow optimized for assays with 8–40 plus markers.

Verify — Multiple data visualization plots allow you to interactively explore your data. Know the cell behind each datapoint to confirm your results.

Explore — Use our comprehensive collection of evaluation plots and calculate distances between phenotypes to check for interaction or count them in specific tissue areas.

Publish — All results, images, and generated outputs can be copy/pasted or exported as standard format raw data files for subsequent 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

Normal colon

Colon mucinous adenocarcinoma

Colon mucinous adenocarcinoma

CD45RO Ki-67 CD45RA EpCAM Collagen 1 αSMA

MMP-13 Pan-CK iNOS Gzm B EpCAM+ E-cadherin

MMP-13 iNOS Pan-CK CD68 EpCAM + E-cadherin

Normal lung

Lung large cell carcinoma

Lung adenocarcinoma

CD45RO αSMA CD68

CD68 Gzm B αSMA CD3 CD20 EpCAM + E-cadherin
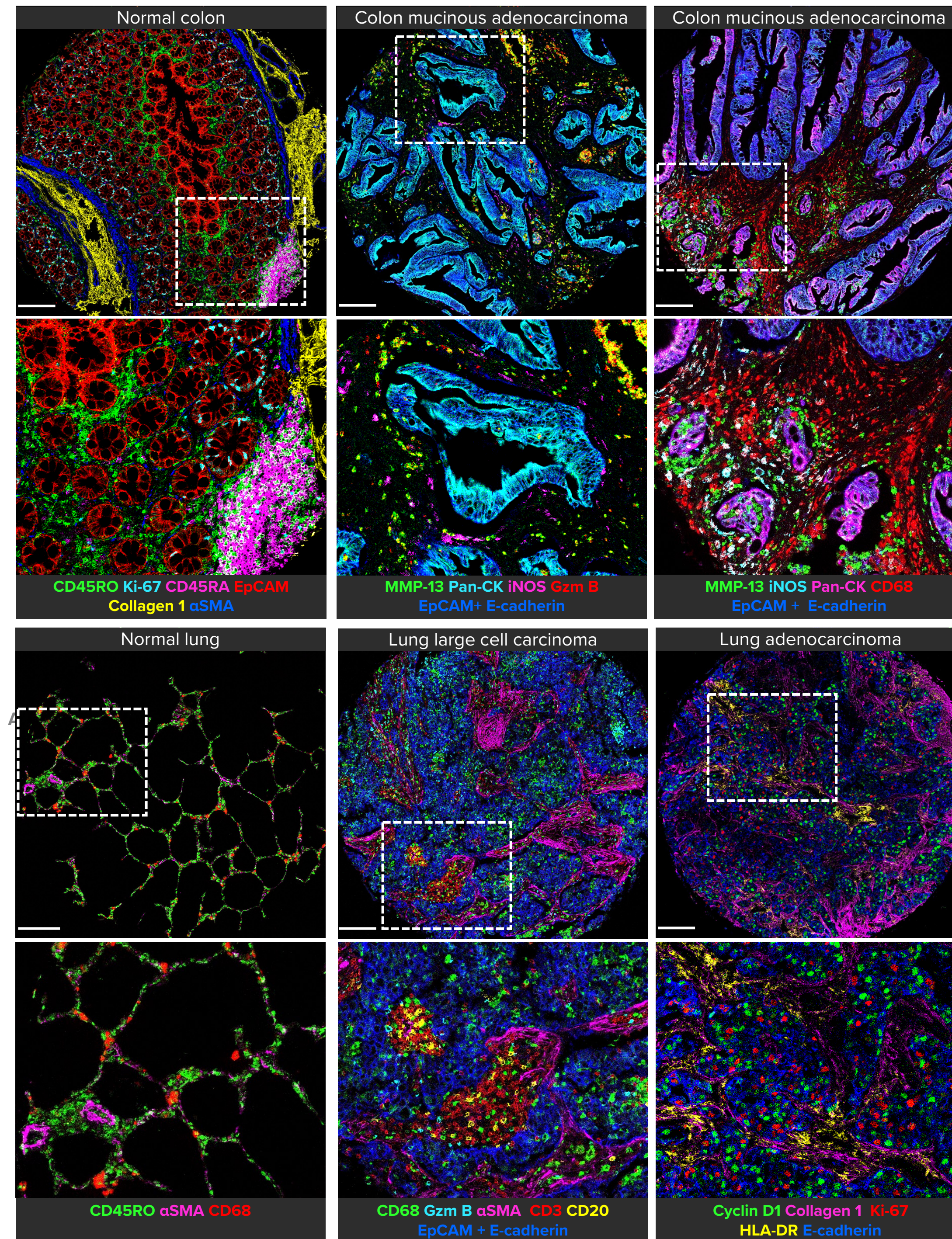
Cyclin D1 Collagen 1 Ki-67 HLA-DR E-cadherin

Figure 1. IMC staining of normal and cancerous colon and lung TMAs. Tumor tissue is identified by EpCAM+E-cadherin and pan-cytokeratin (pan-CK) staining. Mucinous adenocarcinoma of colon shows cells with co-localization of MMP-13 and granzyme B (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.

Cell segmentation using Phenoplex is consistent across serial sections

Lung large cell carcinoma

Tissue segmentation

Cell segmentation

CD68 Gzm B αSMA CD3 CD20 EpCAM + E-cadherin

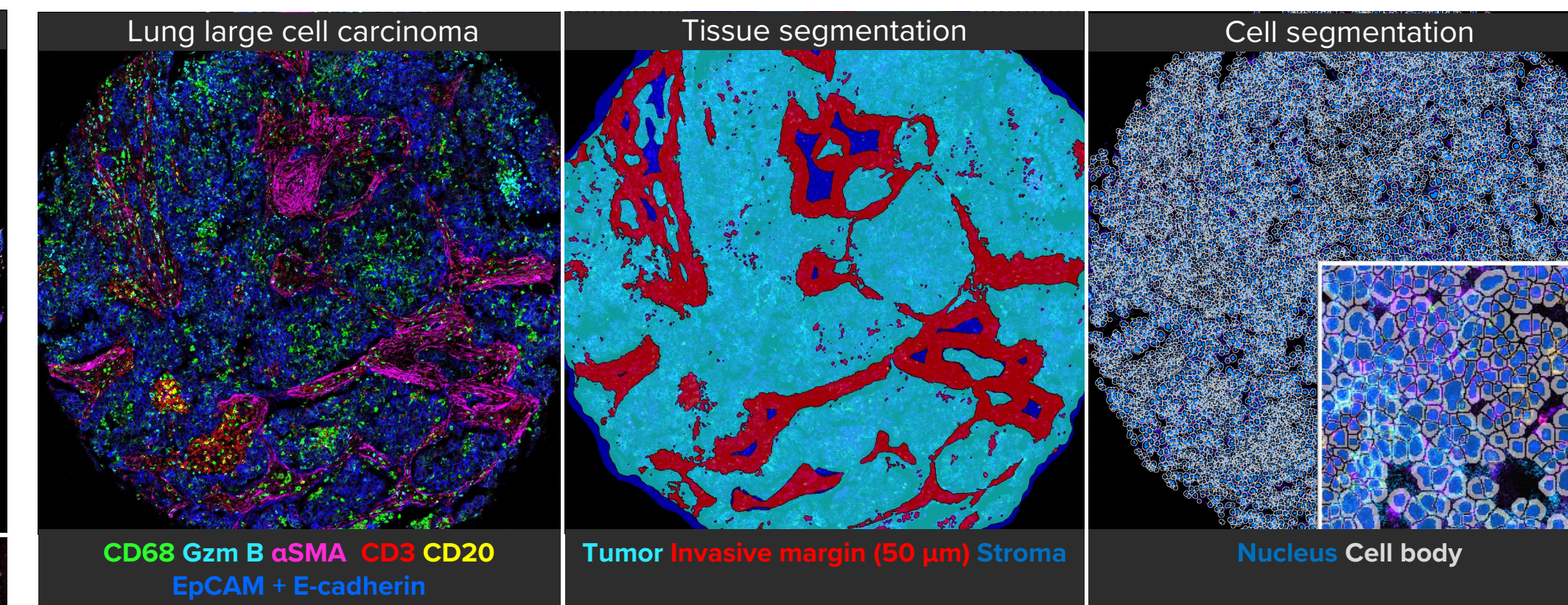Tumor Invasive margin (50 µm) Stroma

Nucleus Cell body

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 depicted around the tumor region. Cell segmentation was based on pre-trained deep learning-based algorithms in the Phenoplex software, augmented with annotations from the IMC Cell Segmentation Kit. The enlarged area of the segmented image clearly shows the nucleus (blue) surrounded by the cellular margins (light gray).
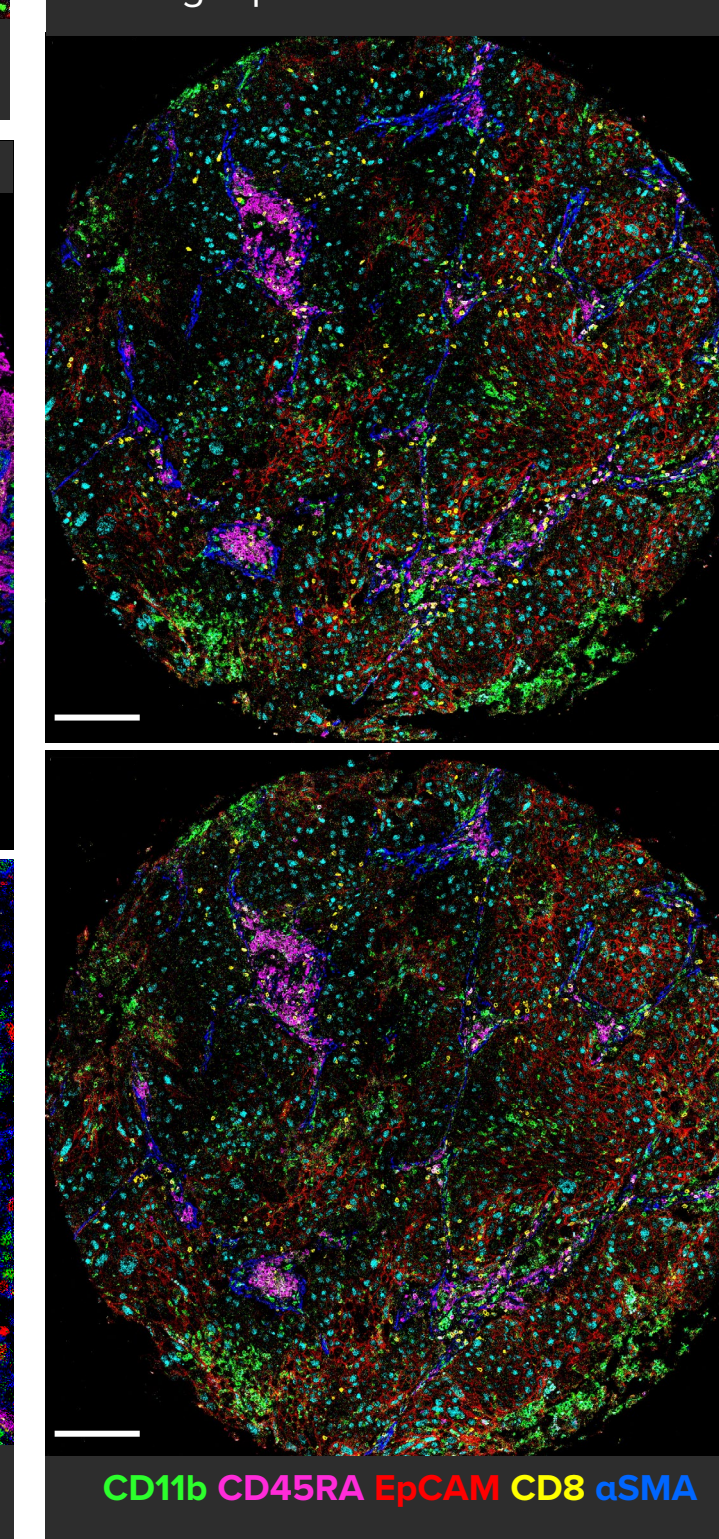

Lung squamous cell carcinoma

Figure 3a. IMC staining of serial sections of lung squamous cell carcinoma. Scale bars are 200 µm.

CD11b CD45RA EpCAM CD8 αSMA

Only 2.7% variability in cell counts across serial sections

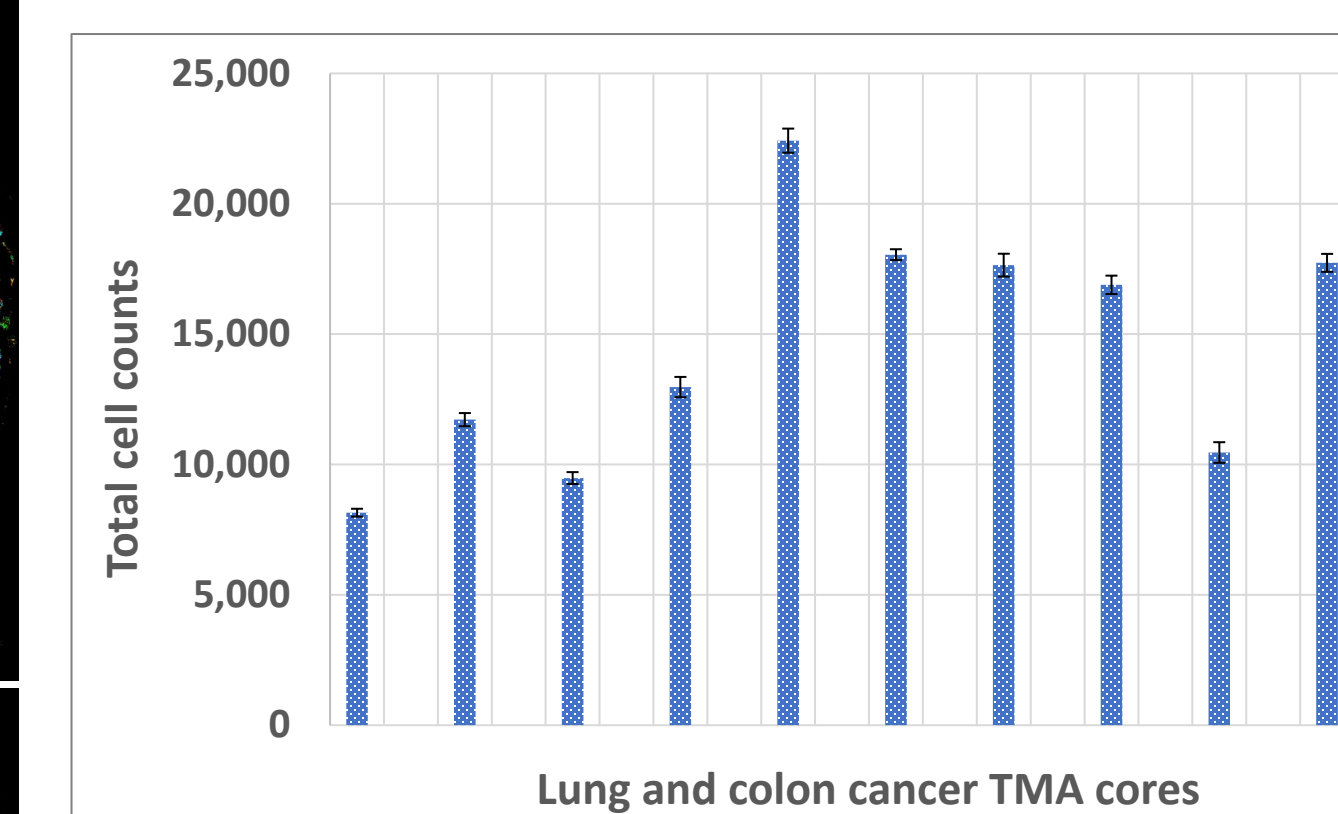Total cell counts — Lung and colon cancer TMA cores

Figure 3b. 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 analysis using Phenoplex software in generating highly consistent segmentation data using serial sections.

Lung large cell carcinoma-cellular clusters split by tissue compartments

Legend

a) All lung cancer cells

b) Negative cells filtered out then split by tissue region

Tumor

Stroma

Margin

Immune cell phenotypes

T cell phenotypes

Ki-67 expressing cells
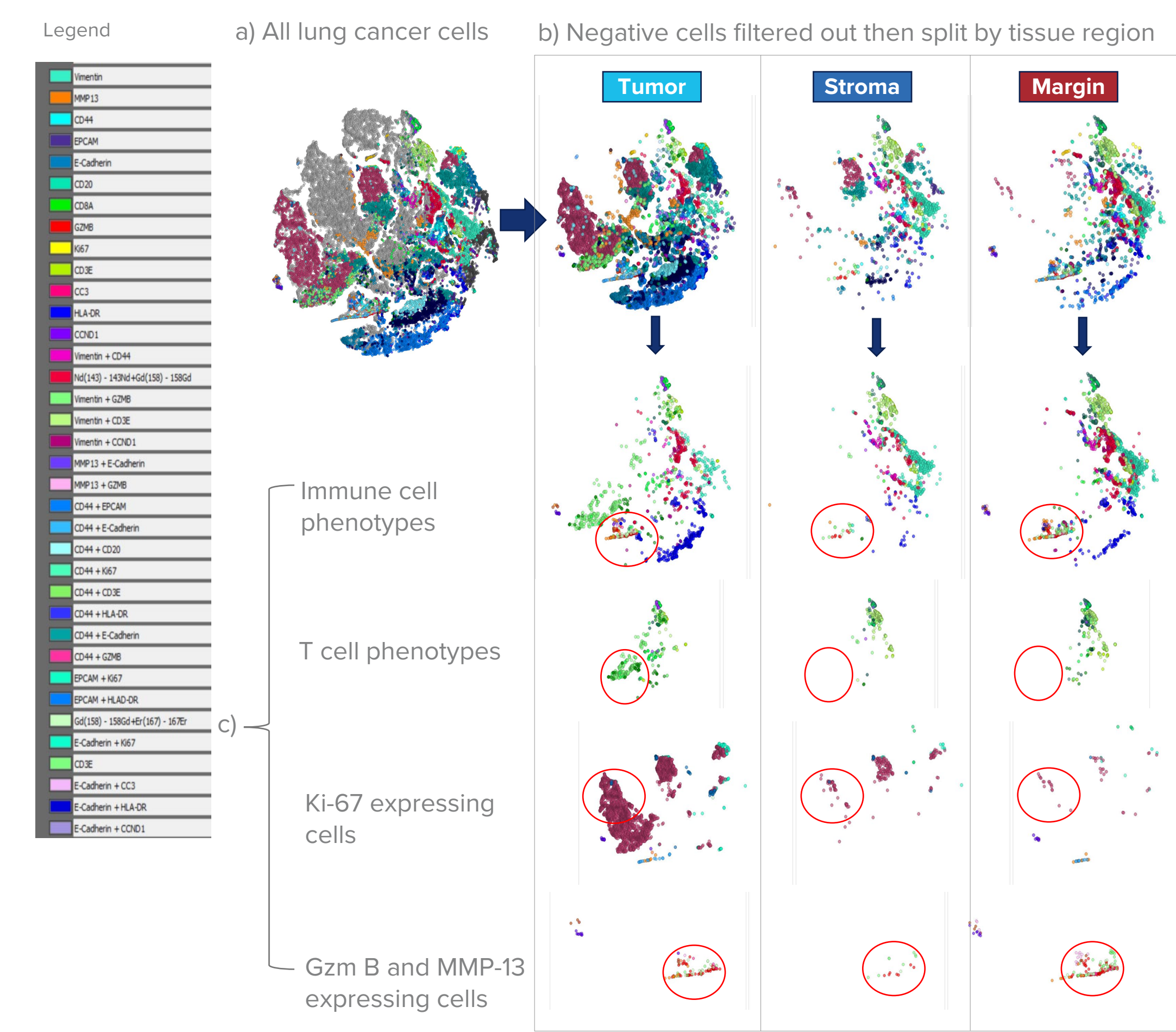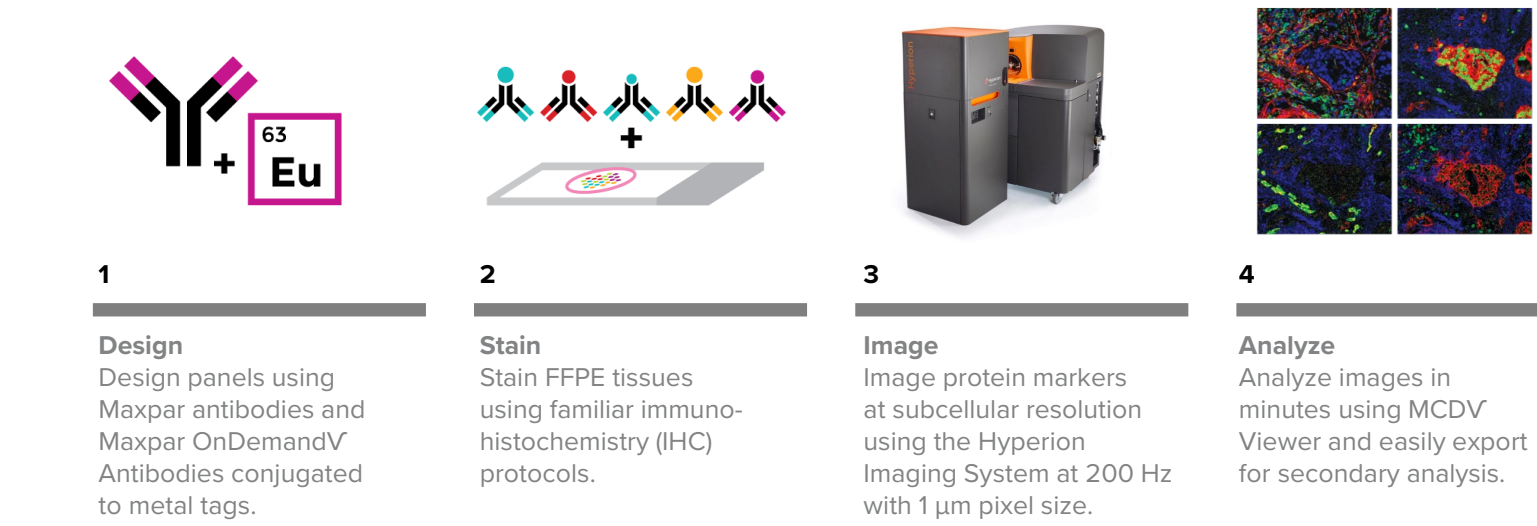
Gzm B and MMP-13 expressing cells

Figure 4. 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 top left t-SNE (a) shows all lung cancer cells, and the top right t-SNE plots (b) show cellular clusters split according to the tissue compartments the cells were in. The bottom 12 t-SNE plots (c) show the cells with differential expression in the tumor, stromal and margin regions. For example, granzyme B and MMP-13 positive cells are in the tumor/margin but not stroma. Ki-67 positive cells are primarily in the tumor. T cell and other immune cell phenotypes vary by tissue region as well.

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, thus empowering IMC users to be confident in biological interpretation of high-dimensional proteomic data.
- This study showcases the capability of IMC technology combined with analysis using Phenoplex software for development of systematic digital profiling of the spatial TME.