Utilizing Image Analysis to Characterize an 8-plex Immunofluorescence Immune Checkpoint Biomarker Panel within the Tumor Tissue Microenvironment

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Background

Multiplex staining and image analysis are being increasingly deployed for immuno-oncology (I/O) evaluations to investigate and understand the complexity of immune cell / tumor cell interactions in the tumor microenvironment (TME) and in discovering novel predictive biomarkers for targeted immunotherapy.

Immune checkpoint inhibitor treatment, guided by immunohistochemically labelled tissue expression of PD-L1 and PD-1, have already been shown to improve outcomes, in the metastatic setting, for non-small cell lung carcinoma (NSCLC) and colorectal cancer (CRC) patients. The purpose of the current study was to phenotype and compare checkpoint and immune cell interactions within NSCLC and CRC tumor types.

Methods

The study included samples from NSCLC, CRC, and normal tonsil tissues. Sections were stained using InSituPlex® multiplex immunofluorescence (mIF), for studying PD-L1/PD-1 checkpoint expression including immune cell markers for CD3, CD4, CD8, CD68, FoxP3, and pan-CK as an epithelial tumor indicator. Slides were scanned at 20X magnification on the ZEISS Axio Scan.Z1 platform, with individual .tiff format files being generated per marker. A stacked OME.tif file was then created from the individual .tiff format files, *per* section. Image analysis was performed using the Visiopharm Oncotopix® platform. Individual marker image layers were aligned, tumor/stroma regions of interest (ROI) classified, and cell formations detected. Markers were quantified across the image layers to evaluate positive cell staining within both cancer indications (Figures 1 and 2).

mIF Marker staining key: CD3 (red), CD4 (yellow), CD8 (green), FoxP3 (pink), CD68 (yellow), CK (cyan), PD-L1 (purple), PD-1 (orange), DAPI (blue)

Cell analysis Phenotype overlay key: CD3 single (red), CD3+ CD8+ PD-1- (green), CD3+ CD8+ PD-1+ (light pink), CD3- CD8+ (purple), CD3+ CD4+ FoxP3+ (lilac), CD68+ PD-L1- (yellow), CD68 PD-1 + (orange), CK+PD-L1+ (dark cyan), CK+PD-L1 (cyan), Negative cells (blue)

Figure 1. Example staining (A and B) and analysis (C and D) for CRC tumor tissue

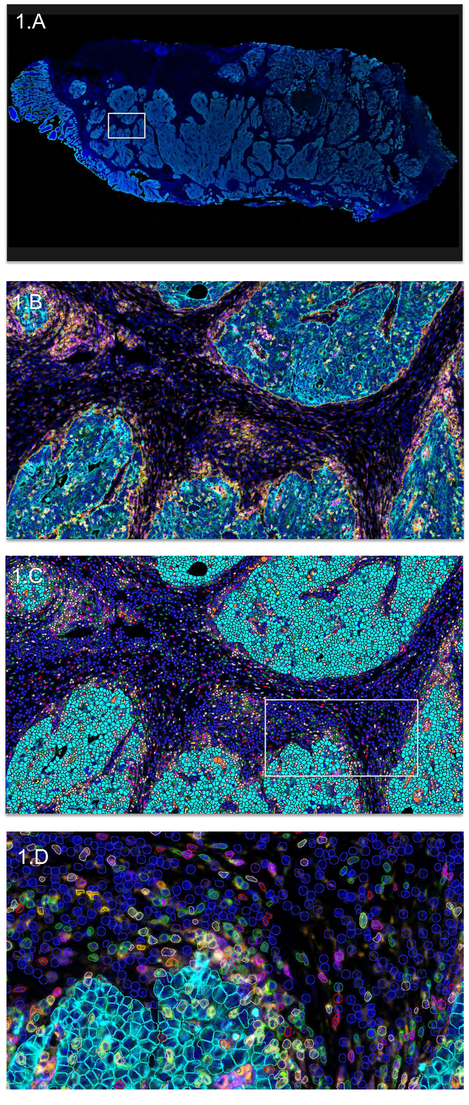
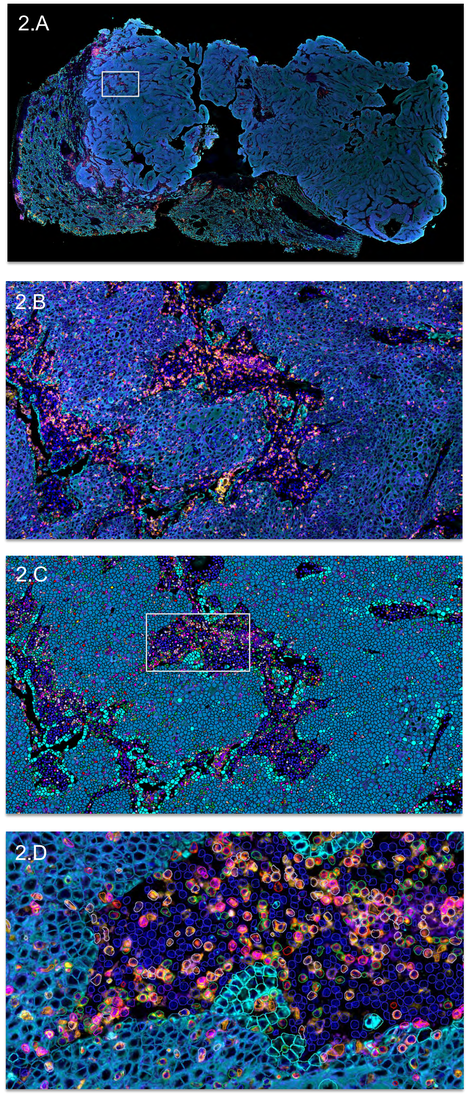


Figure 2. Example staining (A and B) and analysis (C and D) for NSCLC tumor tissue



Results

Quantitative image analysis data generated included total positive and negative cell counts per marker, per mm2 ROI. Co-localization analysis provided relevant immune cell phenotypes (T-cytotoxic, T-helper, T-regs, and macrophages) and PD-1 / PD-L1 cell functional status. Cell spatial analysis relationships were generated, including 'T-cytotoxic PD-1+' cells to 'tumor PD-L1+' and 'Macrophage PD-L1+' cells (Table 1).

Table 1 Example cell analysis data in CRC and NSCLC tumor tissue

Phenotype	CRC		NSCLC	
	Cell per mm² Tumor	Cell per mm² Stroma	Cell per mm² Tumor	Cell per mm² Stroma
Total CD3+	1,397	3,314	795	3,094
CD3+ single	172	932	197	1,050
CD3+ CD8+ PD-1-	280	972	507	1641
CD3+ CD8+ PD-1+	885	994	6	21
CD3+ CD4+ FoxP3+	60	416	85	382
Total CD68+	113	263	168	209
CD68+ PD-L1-	24	46	31	52
CD68+ PD-L1+	89	217	137	157
Total CK +	6,969	N/A	5,579	N/A
CK+PD-L1-	6,796	N/A	796	N/A
CK+ PD-L1+	173	N/A	4,783	N/A

Cell spatial analysis across whole tissue	Avg. Distance (µm) CRC	Avg. Distance (µm) NSCLC
'CD3+ CD8+ PD-1+' to 'CK+ PD-L1+'	57	10
'CD3+ CD8+ PD-1+' to 'CD68+ PD-L1+'	34	31

Conclusions

This study highlighted the benefits of utilizing an 8-plex staining protocol and detailed image analysis approach for the comprehensive assessment of the immune cell types and interactions within the TME. The ability to carry out multiparameter cellular analysis facilitates the delivery of key phenotypic data, thereby leading to greater understanding of tumor biology.

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