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Accurate, high-throughput spatial profiling of whole slide samples with the Paletrra™ multiplexed image analysis pipeline

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Abstract: Multiplexed immunofluorescence (mIF) enables spatially resolved single-cell phenotyping within the tumor microenvironment (TME), which is being increasingly recognized as an important predictor of patient response to immunotherapies. Paletrra™ is a mIF full service provided by NeoGenomics Laboratories, Inc capable of staining and analyzing up to 60 proteins on a single formalin-fixed paraffin-embedded tissue section, with an image-able area of nearly 10 square centimeters. This capability enables profiling of millions of individual cells in a single slide, providing unbiased spatial phenotyping at scale.

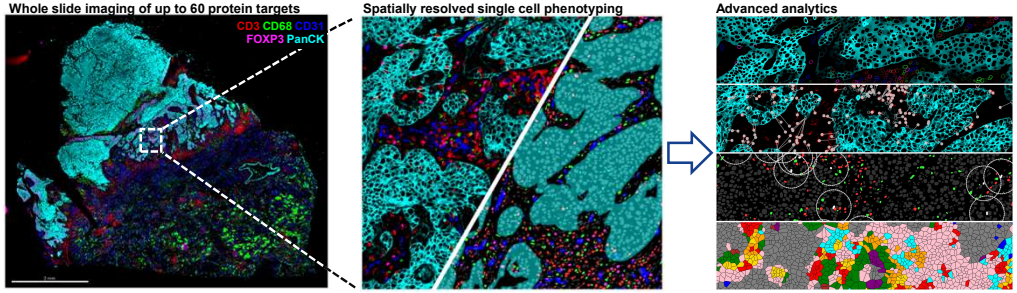
Here we present the Paletrra image analysis pipeline that has been optimized for whole-slide analysis. We demonstrate the pipeline's capabilities using a 16-marker TME panel to spatially characterize a set of 20 non-small cell lung cancer (NSCLC) samples. Accuracy is benchmarked against clinically validated immunohistochemistry (IHC) for a subset of representative immune phenotypes. Cell density and intensity data generated by the image analysis pipeline demonstrated strong concordance with IHC assays for all markers evaluated.

To achieve high throughput whole slide processing while maintaining high accuracy, we use an automated pipelined backed by 100% human quality control (QC). Automation begins with image acquisition, where QC algorithms monitor image collection in real-time to detect imaging failures and trigger corrective action. In post-processing, tissue and stain QC algorithms then identify regions of tissue artifacts such as tissue loss, tissue folding, and necrotic regions to be excluded from downstream analysis.

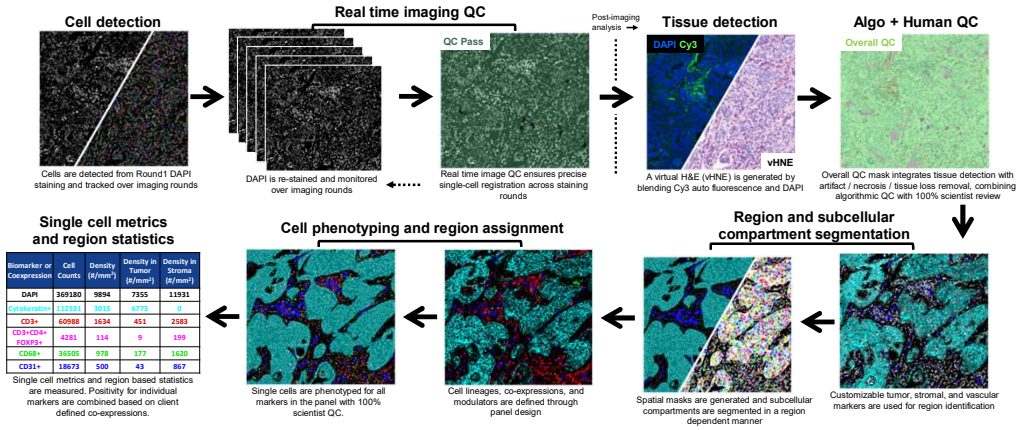
We next transform our mIF images into a single-cell map of the TME by segmenting the tissue into spatial regions and phenotyping on a single cell level. Region segmentation delineates the tissue from surrounding glass, and then further segments the tissue into different regions such as intratumoral, stromal, vasculature, and leading-edge regions. Single cells and subcellular compartments are segmented, which enables accurate intensity quantification over the relevant expression patterns of each marker. Cells are phenotyped using an in-house library of AI algorithms trained on millions of annotated cells – the models integrate morphological and intensity features to accurately distinguish target populations from artifacts and identify phenotypes across diverse tissue types and qualities.

Finally, we demonstrate our comprehensive spatial profiling capabilities on the cohort of 20 NSCLC samples. Cell distributions are quantified through a combination of unsupervised neighborhood clustering, nearest neighbor distances, and tissue region profiling. By mapping critical cell populations—such as tumor-infiltrating lymphocytes, cancer-associated fibroblasts, and other cellular signatures—across distinct tissue regions, we extract salient spatial information that provides biological insight into the TME.

Paletrra transforms tumor samples into single cell maps of the TME



Paletrra AI-enabled image analysis

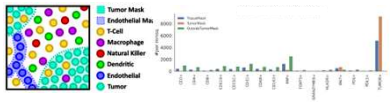


Biomarker or Coexpression	Cell Counts	Density (#/mm²)	Density in Tumor (#/mm²)	Density in Stroma (#/mm²)
DAPI	369180	9894	7355	11931
Cytokeratin	112531	3015	6773	0
CD3+	60988	1634	451	2583
CD3+CD4+ FOXP3+	4281	114	9	199
CD68+	36505	978	177	1620
CD31+	18673	500	43	867

Advanced analytics

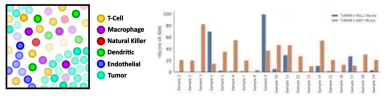
Region Analysis

- Tissue is spatially segmented into biologically functional regions (e.g. tumor, vasculature, etc.)
- The density and count of cell populations is reported within theses spatial regions



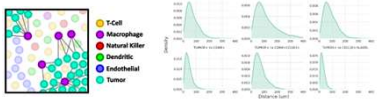
Intensity

- Single cell marker intensities are measured in their relevant subcellular compartment (e.g. nucleus, cytoplasm, whole cell)
- Metrics: single cell intensities, region based mean fluorescent intensity (MFI), and H-scores



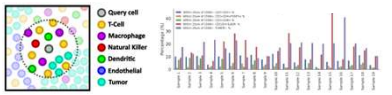
Nearest neighbors

- Distance is calculated from every 'source' cell to nearest 'target cell'.
- Average distances are reported between all 'source' cells to their nearest 'target' cells



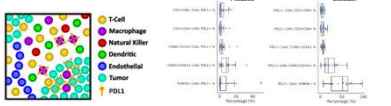
Proximity

- Cells within specified distance of query cell type are identified.
- Positivity of each phenotype-of-interest is quantified, as percent of identified cells



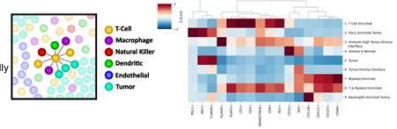
Biomarker profiling

- Prevalence of modulatory biomarker *within* a cell lineage
- Distribution of modulatory biomarker *across* cell lineages



Cell Neighborhood

- Marker expression is tallied across the local ten cell neighborhood
- Neighborhoods are algorithmically clustered and then labelled
- Samples are characterized by neighborhood composition



Tissue scale features

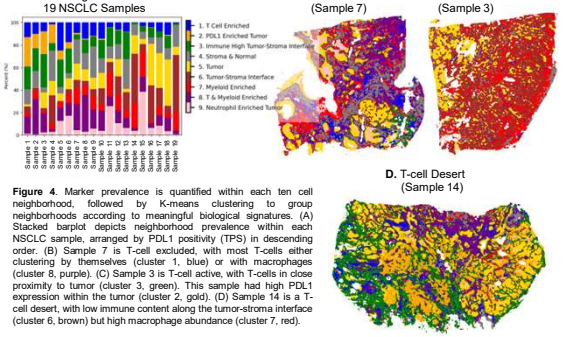
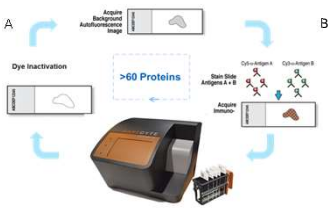


Figure 4. Marker prevalence is quantified within each ten cell neighborhood, followed by K-means clustering to group neighborhoods according to meaningful biological signatures. (A) Stacked barplot depicts neighborhood prevalence within each NSCLC sample, arranged by PDL1 positivity (TPS) in descending order. (B) Sample 7 is T-cell excluded, with most T-cells either clustering by themselves (cluster 1, blue) or with macrophages (cluster 8, purple). (C) Sample 3 is T-cell active, with T-cells in close proximity to tumor (cluster 3, green). This sample had high PDL1 expression within the tumor (cluster 2, gold). (D) Sample 14 is a T-cell desert, with low immune content along the tumor-stroma interface (cluster 6, brown) but high macrophage abundance (cluster 7, red).

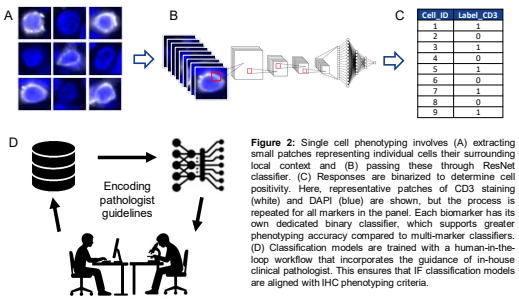
Paletrra staining and imaging workflow



Round	Cy3	Cy5
1	Ki67	CD163
2	CD3	PD-1
3	PanCK	PD-L1
4	HLA-DR	FAP
5	GzB	CD31
6	CD4	CD11b
7	CD11c	FOXP3
8	CD8	CD68

Figure 1: (A) Paletrra Assay Workflow. NSCLC slides were prepared, imaged, and stained using our mIF staining protocol. For each round of staining, conjugated fluorescent antibodies were applied to the slide, followed by imaging acquisition of stained slides. The dye was erased, enabling a second round of staining with another pair of fluorescent antibodies. Samples were imaged using the RareCyte CyteFinder® II HT whole slide imaging platform. (B) A 16plx panel containing immune, stroma, and tumor biomarkers was stained on all NSCLC samples.

Accurate single cell phenotyping



Cell_ID	Label_CD3
1	1
2	0
3	1
4	0
5	1
6	0
7	1
8	0
9	1

Figure 2: Single cell phenotyping involves (A) extracting small patches representing individual cells their surrounding local context and (B) passing these through ResNet classifier. (C) Responses are binarized to determine cell positivity. Here, representative patches of CD3 staining (white) and DAPI (blue) are shown, but the process is repeated for all markers in the panel. Each biomarker has its own dedicated binary classifier, which supports greater phenotyping accuracy compared to multi-marker classifiers. (D) Classification models are trained with a human-in-the-loop workflow that incorporates the guidance of in-house clinical pathologist. This ensures that IF classification models are aligned with IHC phenotyping criteria.

IHC concordant density and intensity outputs

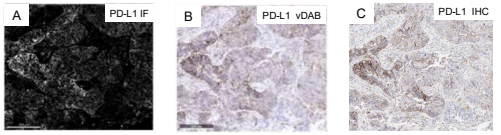
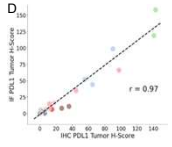


Figure 3. Sequential slides were stained with IF and IHC for a subset of relevant immune markers. Representative IF (A), virtual DAB (vDAB) (B), and IHC (C) images are shown. Stain pattern and appearance appears similar between IHC and IF results. (D-E) Density and percent positivity (all tested markers) and H-score (tumor+ PD-L1 and Ki67) was determined for mIF slides and pathologist approved for all IHC slides. Strong IF/IHC concordance was observed for all metrics.



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	Density (#/mm²)	% Pos	H-score
CD3	0.94	0.94	-
CD8	0.96	0.95	-
CD163	0.87	0.85	-
Ki67	0.91	0.91	0.94
PanCK	0.92	0.93	-
PD-L1	0.94	0.95	0.97

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