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Deep phenotyping and spatial interrogation of the NSCLC tumor microenvironment using the fully validated Next Generation MultiOmyx™ Platform

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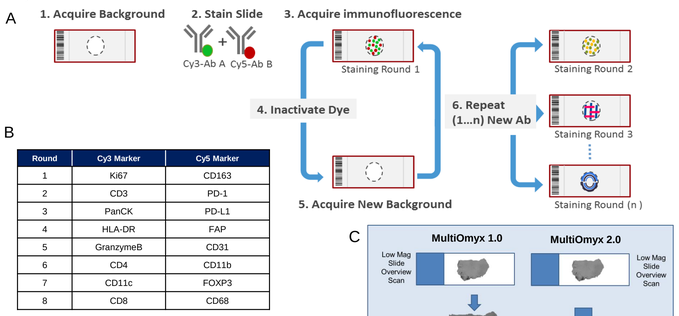
Background: Multiplexed Immunofluorescence (mIF) is a powerful tool for spatially characterizing and phenotyping cells within the tumor microenvironment (TME). MultiOmyx™ (MO) (NeoGenomics Laboratories, Inc) has been one of the leading platforms for generating multiplexed immunofluorescence data to support translational and clinical research for more than a decade. However, MO and other similar platforms are often hampered by a limited imaging area due to either restricted staining areas or being cost prohibitive due to excessive imaging times. Here, we demonstrate the capabilities of an improved MO platform which can be used to generate whole-tissue data from the iterative MO multiplexing process using the CyteFinder® II microscope and a customized software package co-developed by RareCyte, Inc. and NeoGenomics Laboratories, Inc.

Methods: A 16-marker TME panel was used to fully characterize the spatial context in a set of 20 non-Small cell lung cancer (NSCLC) samples. The expression and spatial distribution of each marker in TME panel were analyzed with the proprietary deep learning based NeoLYTX™ image analysis pipeline. Serial sections of each sample were stained via clinically validated immunohistochemistry (IHC) for a subset of biomarkers of the panel for accuracy assessment.

Results: The TME panel successfully identified key tumor infiltrating lymphocytes, cancer associated fibroblasts, and other cellular denizens within the TME as well as their spatial relationship to tumor cells. Detailed pathologist annotations, unsupervised neighborhood analysis and nearest neighbor distances were used to quantify the distribution of cells within the TME. To assess staining accuracy, correlation coefficients were calculated using cell density data generated by mIF benchmarked to gold standard IHC assays; direct correlation was observed for the markers evaluated. To demonstrate the repeatability and reproducibility of the platform, data was generated from three NSCLC samples ran in triplicate in three separate batches and inter-run and intra-run coefficients of variability were calculated for cell density and intensity.

Conclusions: The new NeoLYTX image analysis pipeline in conjunction with the whole-tissue image output of the next generation MO platform allows for improved interaction with pathologists, better histological context, and unbiased spatial analysis.



B

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

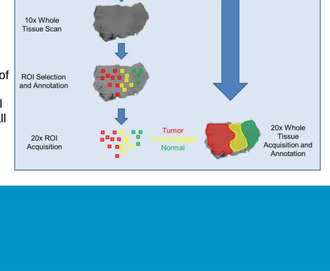


Figure 1: MultiOmyx overview and workflow. (A) MO Assay Workflow. All NSCLC samples were analyzed by MO mIF assay. NSCLC slides were prepared and stained using MO mIF staining protocol. For each round of staining, conjugated fluorescent antibodies were applied to the slide, followed by imaging acquisition of stained slides. The dye was erased, enabling a second round of staining with another pair of fluorescent antibodies. (B) A 16plx panel containing immune, stroma, and tumor biomarkers was stained on all NSCLC samples. (C) Comparison of MO 1.0 and MO 2.0 imaging workflows for MO mIF. The MO 2.0 workflow allows for whole tissue acquisition and greater precision for annotations.

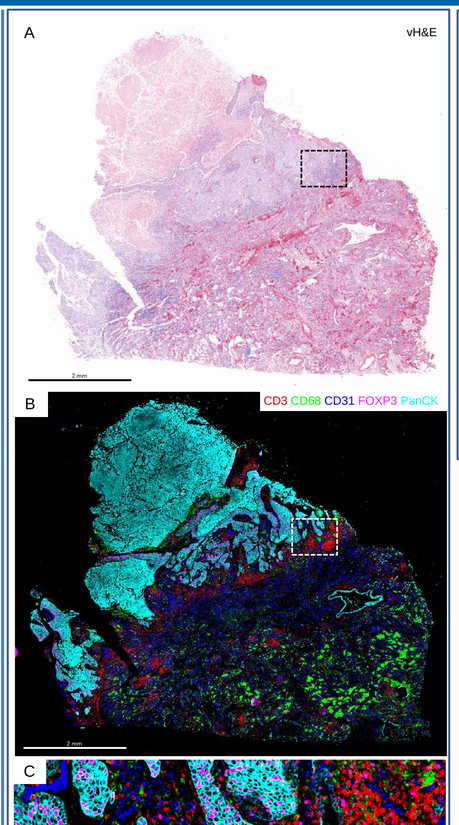


Figure 2: Whole slide imaging using MO 2.0. (A-B) Whole slide virtual H&E (vH&E) (A) and representative color overlay (B) showing distribution of CD3 (red), CD68 (green), CD31 (blue), FOXP3 (pink), and PanCK (cyan) in a NSCLC sample. (C) Magnified view corresponding to dotted box in A/B reveals detailed resolution of immune and proliferating cell expression within the NSCLC TME.

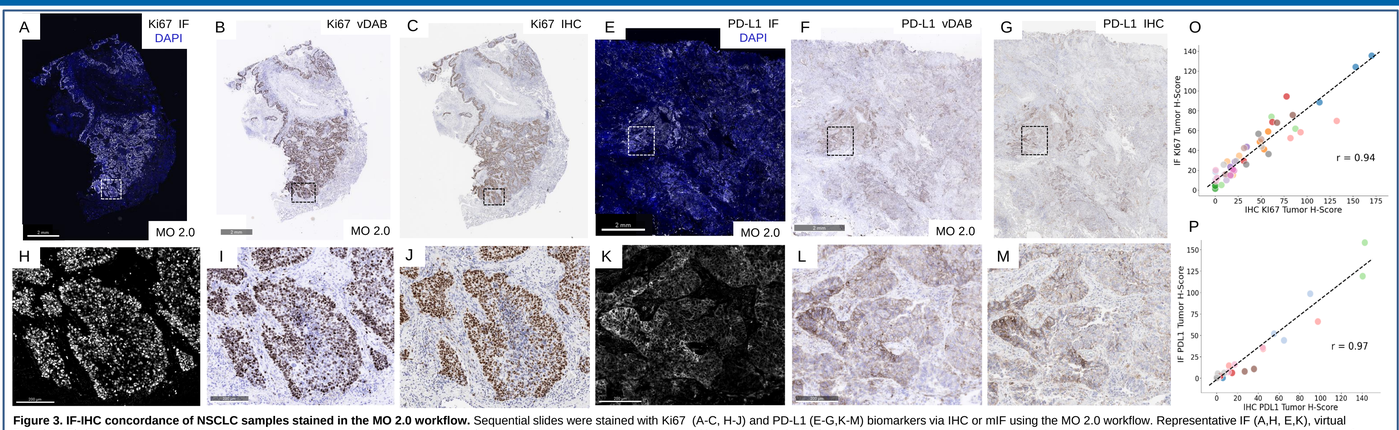


Figure 3. IF-IHC concordance of NSCLC samples stained in the MO 2.0 workflow. Sequential slides were stained with Ki67 (A-C, H-J) and PD-L1 (E-G,K-M) biomarkers via IHC or mIF using the MO 2.0 workflow. Representative IF (A,H, E,K), virtual DAB (vDAB) (B,I, F,L), and IHC (C,J,G,M) images are shown. Magnified views for Ki67 (H-J) and PD-L1 (K-L) correspond to dotted box, respectively. Stain pattern and appearance appears similar between IHC and MO 2.0 results. H-score of tumor+ PD-L1 and Ki67 was determined for all MO 2.0 slides and pathologist approved for all IHC slides. Comparison of Ki67 (O) and PD-L1 (P) tumor H-scores from IHC or MO 2.0 mIF slides show strong concordance (r=0.94-0.97).

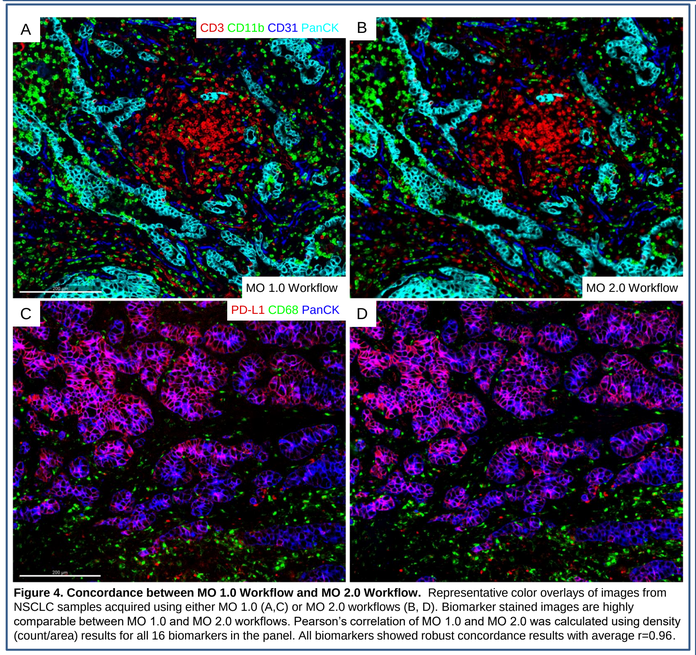


Figure 4. Concordance between MO 1.0 Workflow and MO 2.0 Workflow. Representative color overlays of images from NSCLC samples acquired using either MO 1.0 (A,C) or MO 2.0 workflows (B, D). Biomarker stained images are highly comparable between MO 1.0 and MO 2.0 workflows. Pearson's correlation of MO 1.0 and MO 2.0 was calculated using density (count/area) results for all 16 biomarkers in the panel. All biomarkers showed robust concordance results with average r=0.96.

Summary

- The MO 2.0 workflow shows strong concordance with both validated IHC and the MO 1.0 workflow.
- Precision analysis of the MO 2.0 workflow demonstrates robust assay performance.
- The MO 2.0 workflow allows for whole slide imaging, thereby providing greater histological context and unbiased spatial analysis of the TME. This approach can therefore facilitate greater insights into the immune response to therapeutic treatments.

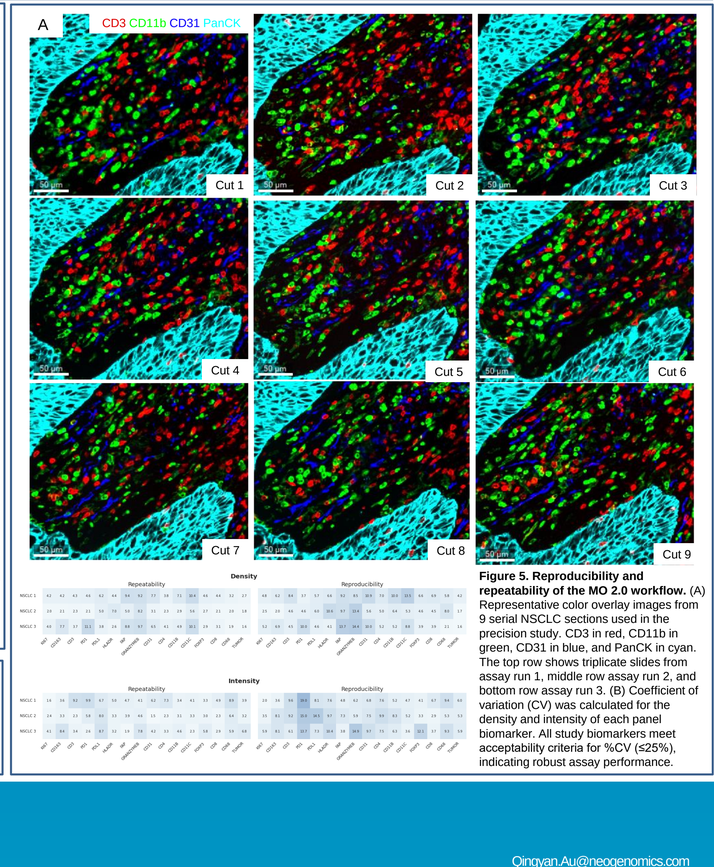


Figure 5. Reproducibility and repeatability of the MO 2.0 workflow. (A) Representative color overlay images from 9 serial NSCLC sections used in the precision study. CD3 in red, CD11b in green, CD31 in blue, and PanCK in cyan. The top row shows triplicate slides from assay run 1, middle row assay run 2, and bottom row assay run 3. (B) Coefficient of variation (CV) was calculated for the density and intensity of each panel biomarker. All study biomarkers meet acceptability criteria for %CV (≤25%), indicating robust assay performance.

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