Identifying a role for macrophages in predicting immunotherapy responses of non-small cell lung cancer (NSCLC)

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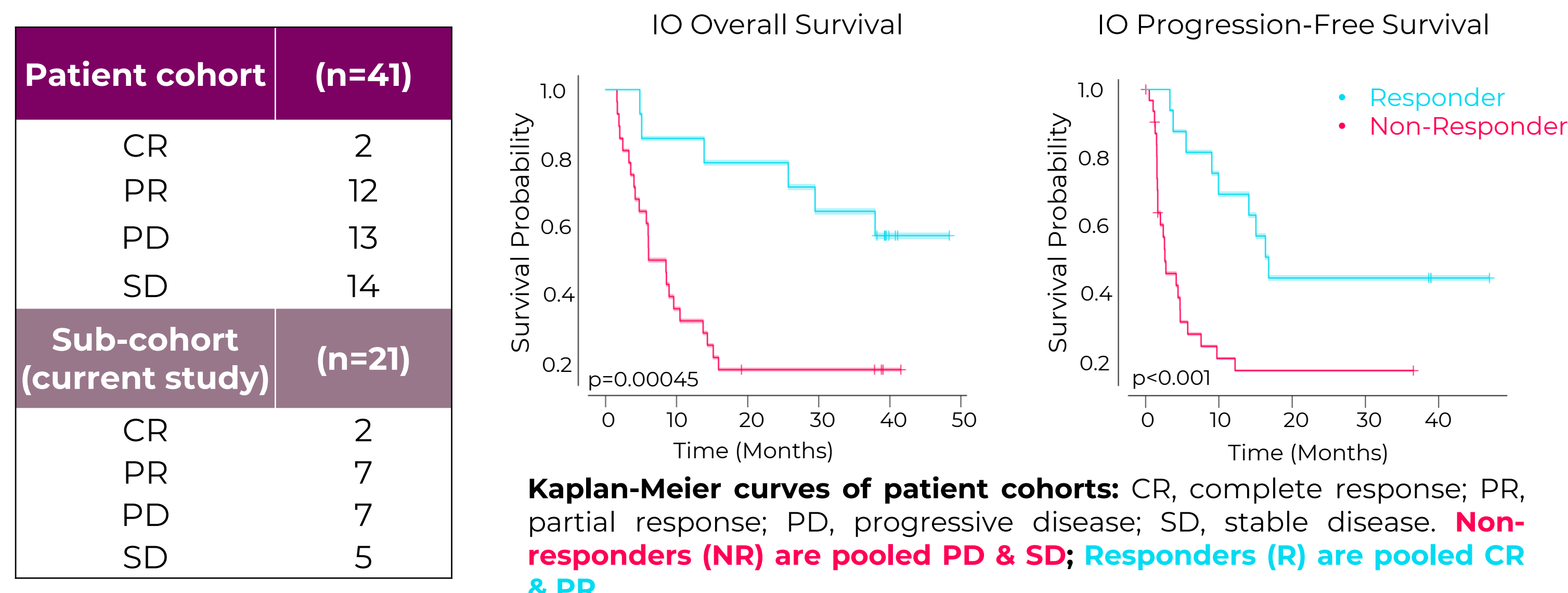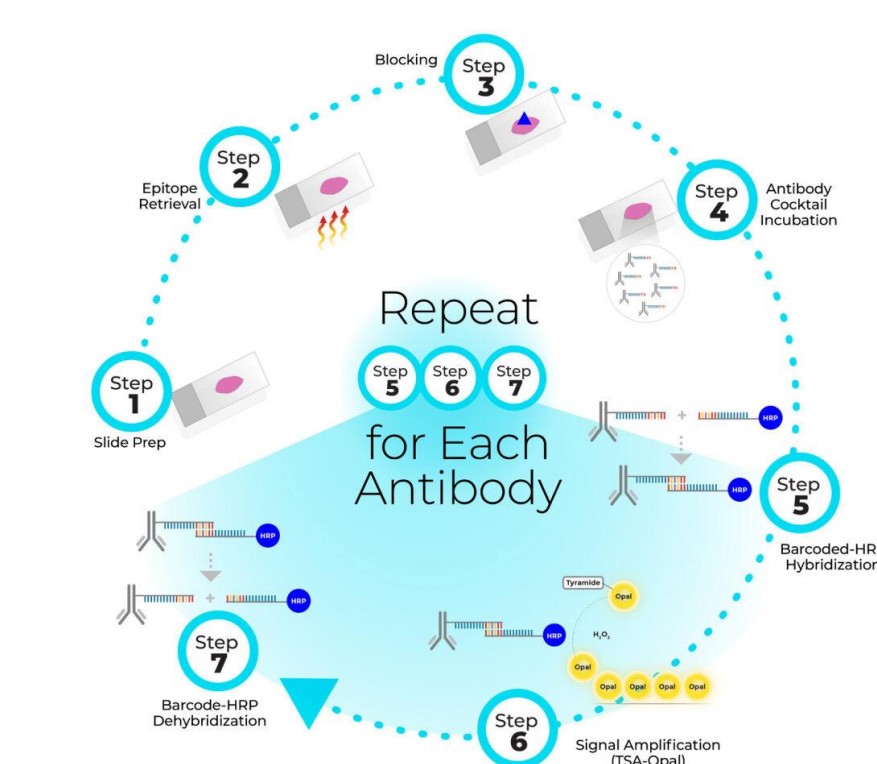
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NSCLC immunotherapy study (Prospective)

Lung cancers are the leading cause of cancer incidence and mortality, with a 5-year survival of ~20%. Treatment with immune checkpoint inhibitors has led to durable and prolonged survival, but only a subset of patients remains responsive. Additional biomarkers are needed to better predict which patients will respond to or develop resistance against immune checkpoint inhibitor (ICI) therapies. In this study, we phenotyped pretreatment biopsies from non-small cell lung cancer (NSCLC) patients enrolled in a single-agent Nivolumab clinical trial. We first deployed customizable PhenoCode™ Signature Panels (PSP) for high-throughput profiling of the tumor immune-contexture and macrophage (M1 & M2) polarization utilizing the PhenoImager® HT 2.0 platform. Our analyses revealed key differences between the proximity of M1 and M2 macrophages to tumor cells in the responder and non-responder groups. To further investigate, M1 and M2 functional states we performed deep spatial phenotyping using a 57-plex PhenoCode Discovery panel on the PhenoCycler®-Fusion 2.0 platform. Our study underscores the pivotal role of advanced spatial phenotyping in unraveling the complexities of macrophage polarization states and metabolism dynamics and their influence on treatment response.

Patient cohort	(n=41)
CR	2
PR	12
PD	13
SD	14
Sub-cohort (current study)	(n=21)
CR	2
PR	7
PD	7
SD	5

IO Overall Survival — p=0.00045
IO Progression-Free Survival — p<0.001

Responder
Non-Responder

Kaplan-Meier curves of patient cohorts: CR, complete response; PR, partial response; PD, progressive disease; SD, stable disease. **Non-responders (NR)** are pooled PD & SD; **Responders (R)** are pooled CR & PR.

Spatial 2.0 - High-throughput to High-plex exploration
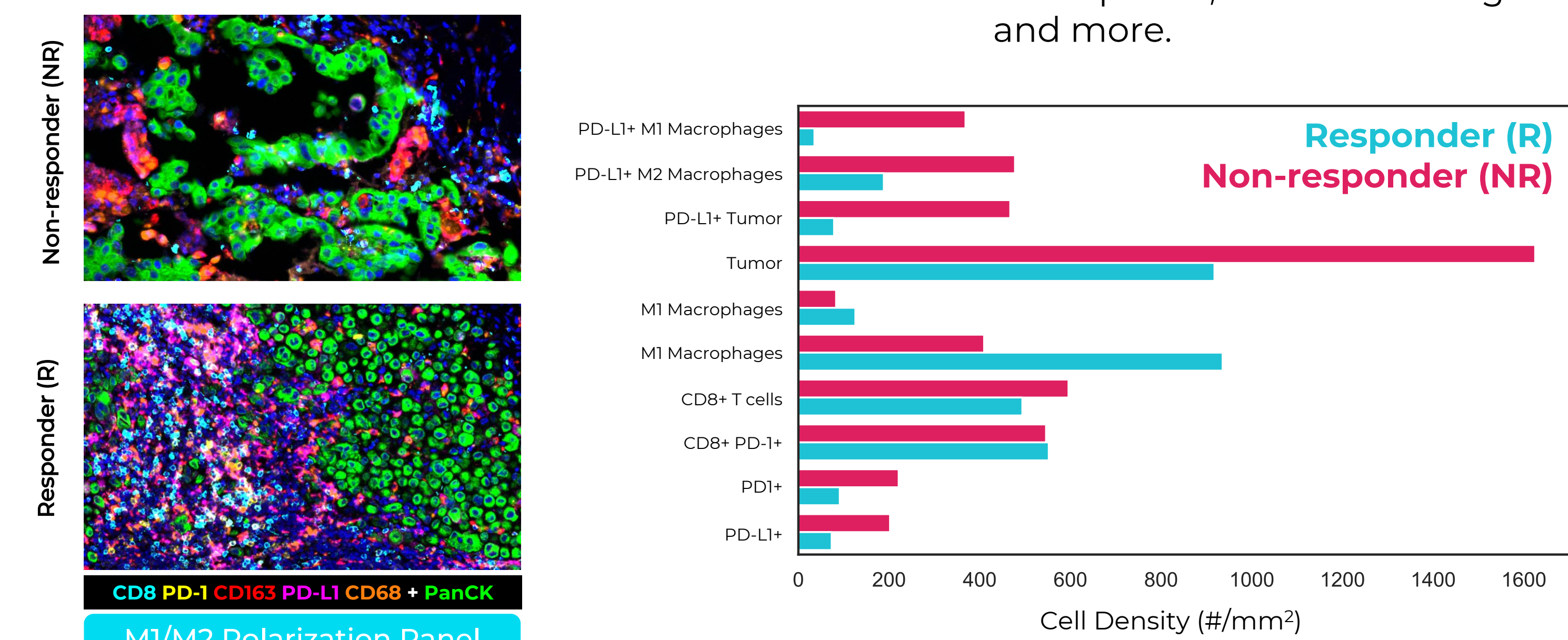
Repeat for Each Antibody

PhenoCode Signature Panels use Akoya's barcode-based antibody labeling chemistry and are validated for the **PhenoImager HT 2.0** workflow. Featuring a flexible design component that allows for the easy integration of a novel checkpoint or immune cell marker, these panels offer 3-fold faster assay development and optimization times when compared to other custom 6-plex panels.
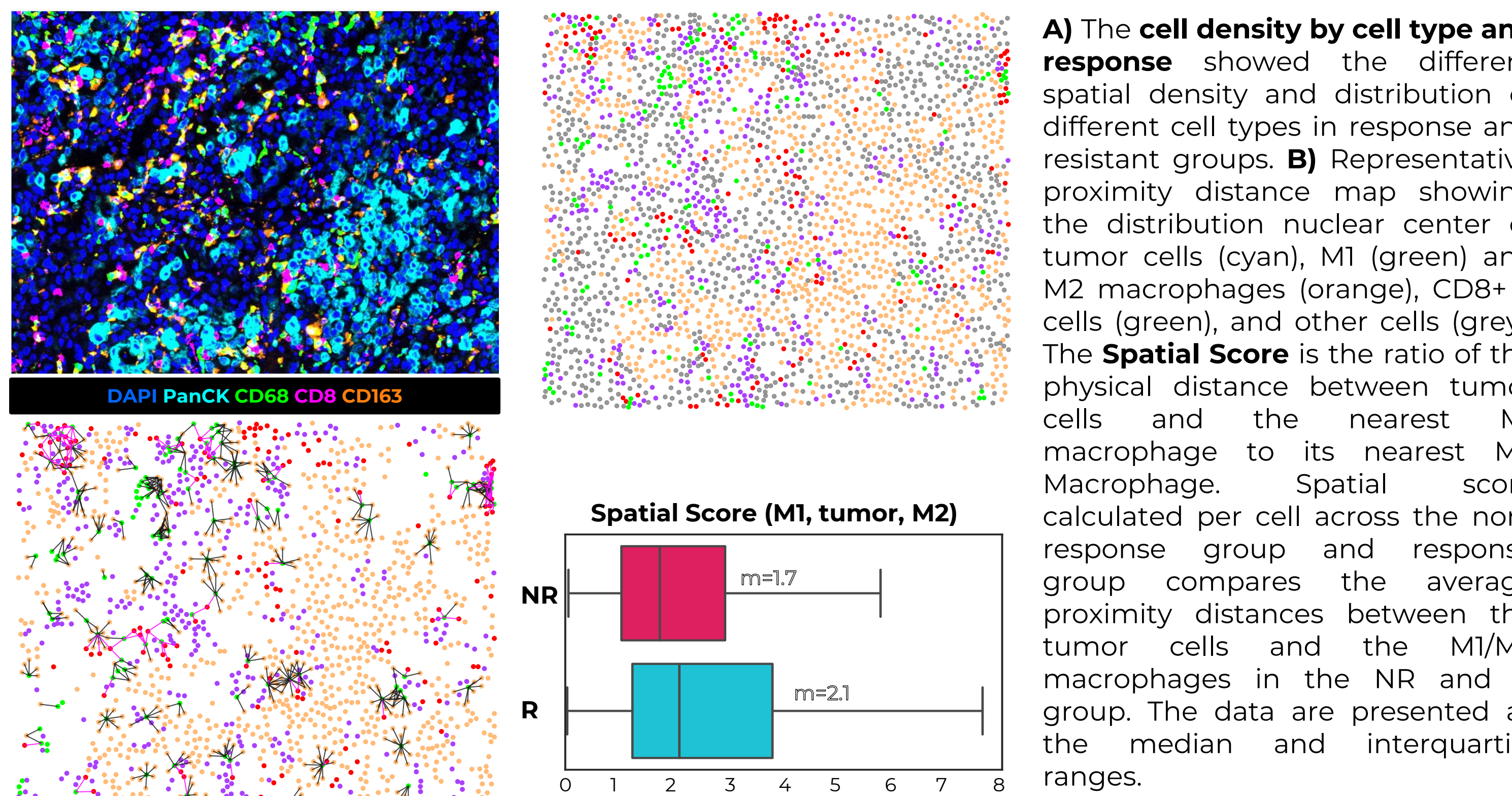
Translational to Ultrahigh-plex discovery Workflow: NSCLC core biopsies (FFPE) were phenotyped using **PhenoCode Signature M1/M2 Polarization Human Protein Panel** + PanCK in the open channel. These samples were imaged on the **PhenoImager HT 2.0 platform.** Serial sections were then stained with a 57-plex **PhenoCode Discovery panel on the PhenoCycler-Fusion 2.0 platform** using markers for cell lineage, immune activation, checkpoints, cellular energetics, and more.

PhenoImager HT 2.0 (Imaging + unmixing)

A) Cell Density by Cell Type and Response

Non-responder (NR)
Responder (R)
CD8 PD-1 CD163 PD-L1 CD68 PanCK
M1/M2 Polarization Panel

Responder (R)
Non-responder (NR)

PD-L1+ M1 Macrophages
PD-L1+ M2 Macrophages
PD-L1+ Tumor
Tumor
M1 Macrophages
M1 Macrophages
CD8+ T cells
CD8+ T cells
PD1+
PD-L1+

Cell Density (#/mm²) — 0, 200, 400, 600, 800, 1000, 1200, 1400, 1600

B) Spatial Score

DAPI PanCK CD68 CD8 CD163

Spatial Score (M1, tumor, M2)

NR — m=1.7
R — m=2.1

0 1 2 3 4 5 6 7 8

A) The **cell density by cell type and response** showed the different spatial density and distribution of different cell types in response and resistant groups. **B)** Representative proximity distance map showing the distribution nuclear center of tumor cells (cyan), M1 (green) and M2 macrophages (orange), CD8+ T cells (green), and other cells (grey). The **Spatial Score** is the ratio of the physical distance between tumor cells and the nearest M1 macrophage to its nearest M2 Macrophage. Spatial score calculated per cell across the non-response group and response group compares the average proximity distances between the tumor cells and the M1/M2 macrophages in the NR and R group. The data are presented as the median and interquartile ranges.
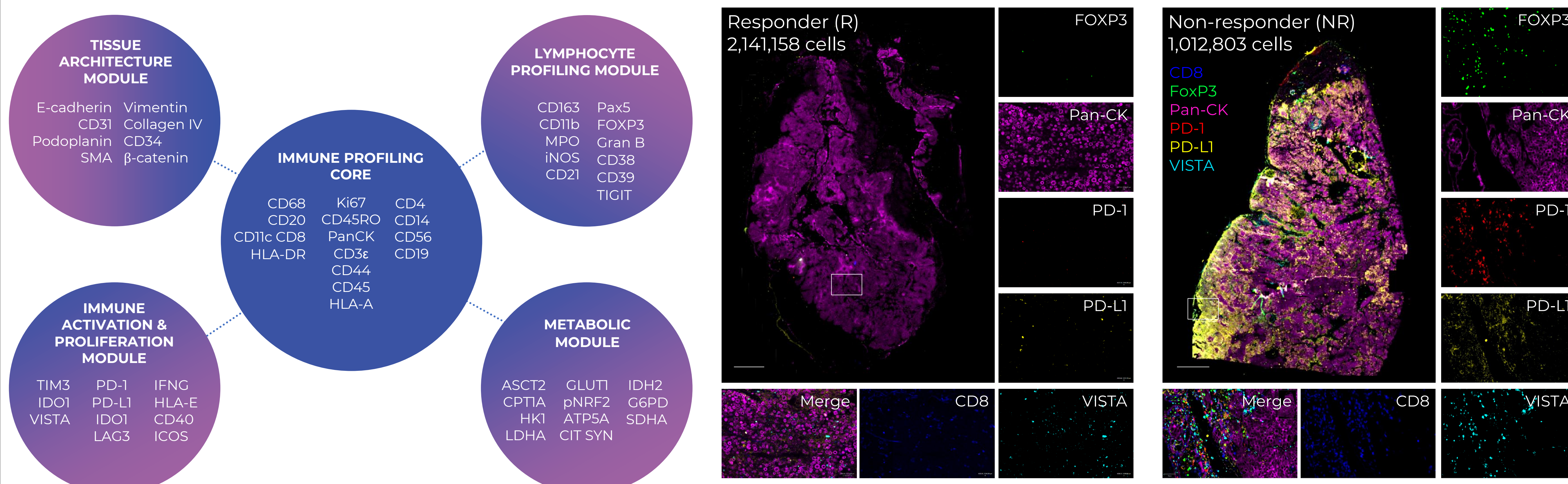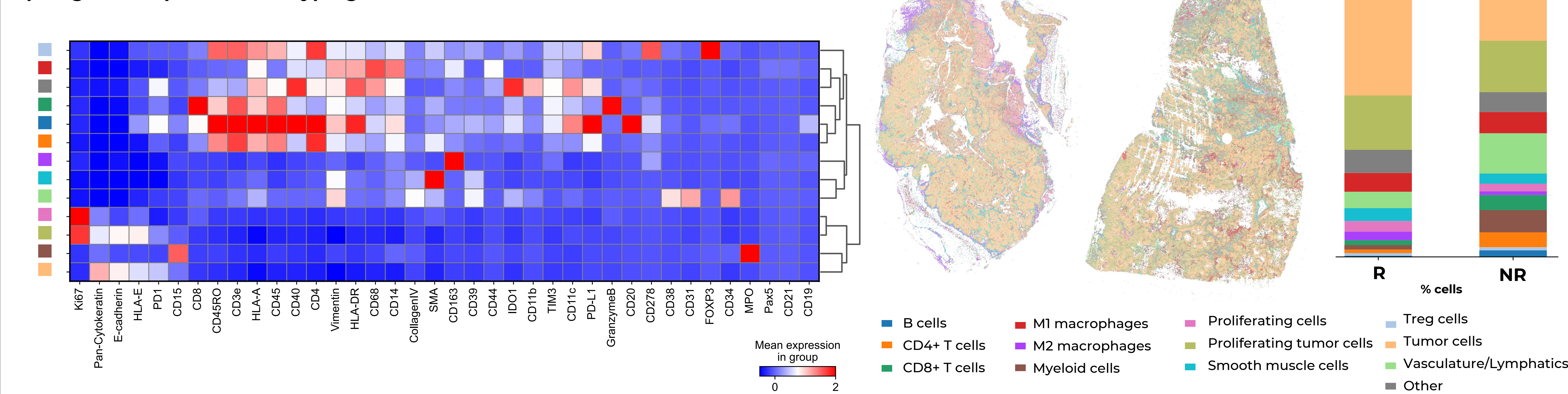
57-plex Spatial Phenotyping Identified 13 Immune Cell Populations

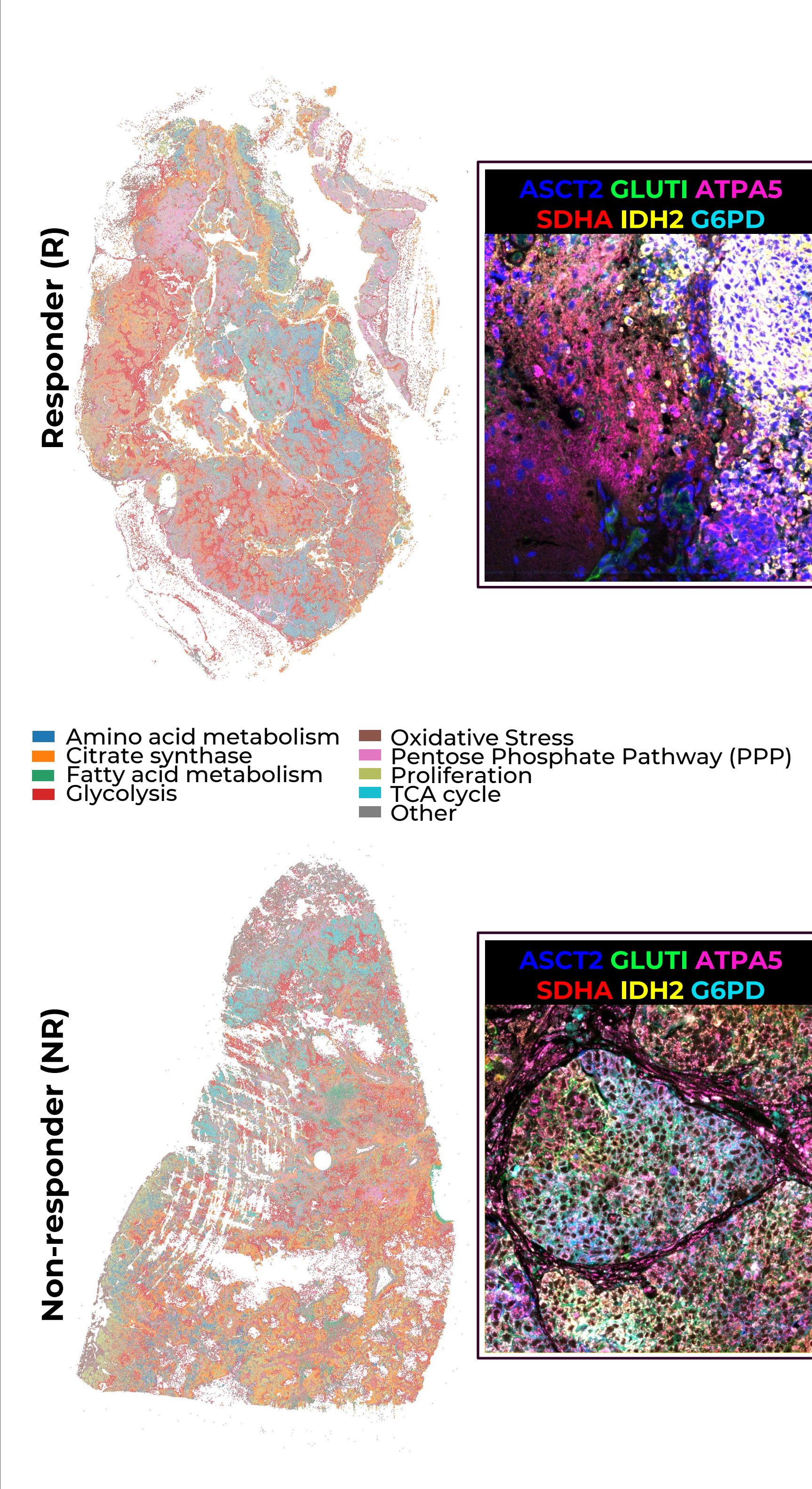### A) 57-plex Whole-slide Imaging and spatial phenotyping of 3.4 million cells of pretreatment biopsies

TISSUE ARCHITECTURE MODULE: E-cadherin, CD31, Podoplanin, SMA, Vimentin, Collagen IV, CD34, β-catenin

IMMUNE PROFILING CORE: CD68, Ki67, CD20, CD45RO, CD11c CD8, PanCK, HLA-DR, CD3ε, CD44, CD45, HLA-A, CD4, CD14, CD56, CD19

LYMPHOCYTE PROFILING MODULE: CD163, CD11b, MPO, iNOS, CD21, Pax5, FOXP3, Gran B, CD38, CD39, TIGIT

IMMUNE ACTIVATION & PROLIFERATION MODULE: TIM3, IDO1, VISTA, PD-1, PD-L1, IDO1, LAG3, IFNG, HLA-E, CD40, ICOS

METABOLIC MODULE: ASCT2, CPT1A, HK1, LDHA, GLUT1, pNRF2, ATP5A, CIT SYN, IDH2, G6PD, SDHA

Responder (R) 2,141,158 cells — FOXP3, Pan-CK, PD-1, PD-L1
Non-responder (NR) 1,012,803 cells — CD8, FoxP3, Pan-CK, PD-1, PD-L1, VISTA — FOXP3, Pan-CK, PD-1, PD-L1
Merge, CD8, VISTA

B) Single Cell Spatial Phenotyping

Mean expression in group — 0, 1, 2

% cells — R, NR

- B cells
- CD4+ T cells
- CD8+ T cells
- M1 macrophages
- M2 macrophages
- Myeloid cells
- Proliferating cells
- Proliferating tumor cells
- Smooth muscle cells
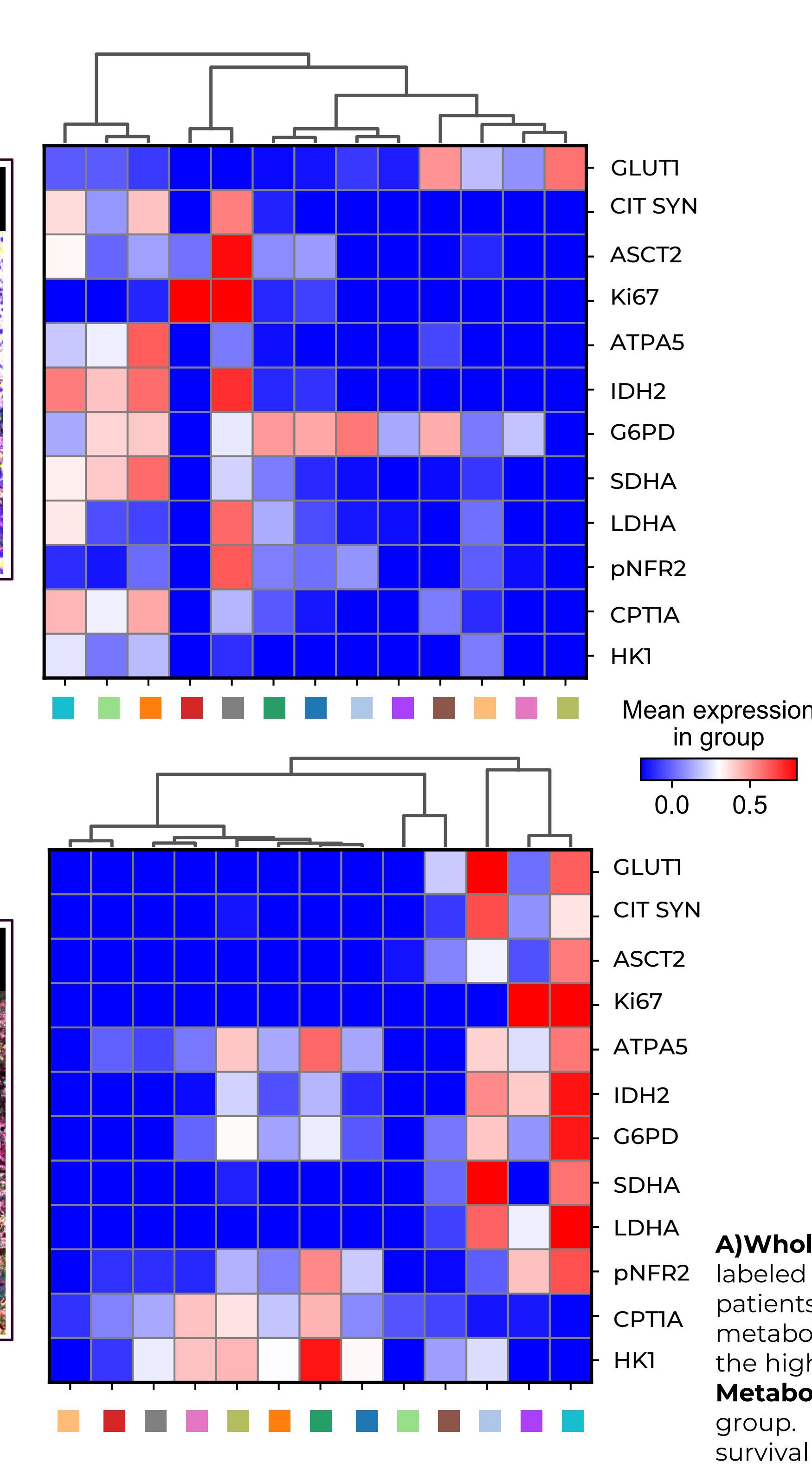- Treg cells
- Tumor cells
- Vasculature/Lymphatics
- Other

A) PhenoCycler-Fusion 2.0 (PCF 2.0) whole-slide 57-plex imaging of NSCLC tissue from different immunotherapy cohorts (biomarkers as indicated, scale bar, 2mm). The antibody panel on the left was designed for deep-phenotyping of immune cells and metabolic module to reveal cancer progression. **B)** Representative Single-cell Spatial Phenotyping Application of 3,153,961 cells in an NSCLC PR tissue. Cells are phenotyped via unsupervised clustering of biomarkers. The heatmap summarizes 13 immune cell populations. The abundance of each cell type is quantified in the accompanying stacked bar graph.

Spatial Metabolic Functional Mapping of Tumor-Associated Macrophages (M1/M2) Predicts immune response
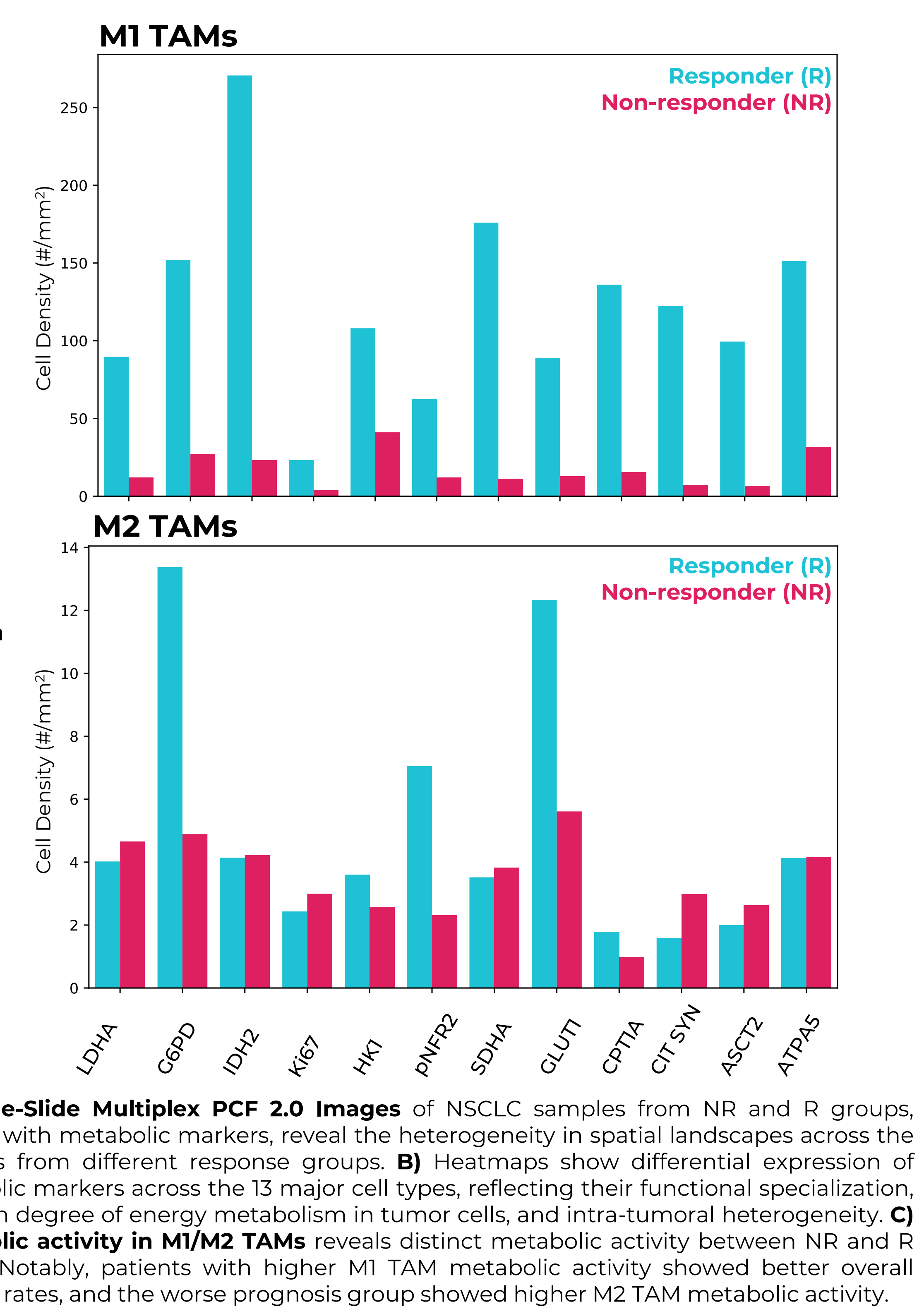
A) Metabolic Functional Phenotyping

Responder (R)
ASCT2 GLUT1 ATPA5 SDHA IDH2 G6PD

Non-responder (NR)
ASCT2 GLUT1 ATPA5 SDHA IDH2 G6PD

- Amino acid metabolism
- Citrate synthase
- Fatty acid metabolism
- Glycolysis
- Oxidative Stress
- Pentose Phosphate Pathway (PPP)
- Proliferation
- TCA cycle
- Other

B) Cell Type-Specific Metabolic Expression

GLUT1, CIT SYN, ASCT2, Ki67, ATPA5, IDH2, G6PD, SDHA, LDHA, pNFR2, CPT1A, HK1

Mean expression in group — 0.0, 0.5

GLUT1, CIT SYN, ASCT2, Ki67, ATPA5, IDH2, G6PD, SDHA, LDHA, pNFR2, CPT1A, HK1

C) Decoding Metabolic activity in Tumor-associated M1/M2s

M1 TAMs
Responder (R)
Non-responder (NR)
Cell Density (#/mm²) — 0, 50, 100, 150, 200, 250

M2 TAMs
Responder (R)
Non-responder (NR)
Cell Density (#/mm²) — 0, 2, 4, 6, 8, 10, 12, 14

LDHA, G6PD, IDH2, Ki67, HK1, pNFR2, SDHA, GLUT1, CPT1A, CIT SYN, ASCT2, ATPA5

A) Whole-Slide Multiplex PCF 2.0 Images of NSCLC samples from NR and R groups, labeled with metabolic markers, reveal the heterogeneity in spatial landscapes across the patients from different response groups. **B)** Heatmaps show differential expression of metabolic markers across the 13 major cell types, reflecting their functional specialization, the high degree of energy metabolism in tumor cells, and intra-tumoral heterogeneity. **C)** Metabolic activity in M1/M2 TAMs reveals distinct metabolic activity between NR and R group. Notably, patients with higher M1 TAM metabolic activity showed better overall survival rates, and the worse prognosis group showed higher M2 TAM metabolic activity.

Conclusions

- This study amounts to a uniquely comprehensive Single Cell Spatial Phenotyping analysis of pretreatment NSCLC biopsies from a single-agent Nivolumab clinical trial.
- **whole-slide spatial phenotyping of macrophage immune and metabolic status revealed unique spatial distribution and metabolic features within tumor-associated macrophages (TAMs). This discovery establishes a promising predictive marker for the immune response in NSCLC.**
- Our data illustrate that immune cell quantification alone may be insufficient to stratify patient cohorts with NSCLC. The identification of unique spatial signatures, such as a spatial score and the metabolic phenotyping of macrophages, may need to be performed to predict NSCLC patient response to immunotherapy better. Development of clinically relevant and highly predictive spatial signatures may require iterative cycles of discovery studies for deep spatial phenotyping and high-throughput translational studies for verification.
- **This study shows how Akoya's solutions are uniquely positioned to enable discovery to translational workflows thereby accelerating the development of clinically relevant and highly predictive spatial signatures.**

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