Application of a Novel Multiplex Imaging-based Immunotherapy Panel and AI-powered Analysis Solution for Spatial Biomarker Identification on Immunotherapy-treated Melanoma Patients

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Introduction

Identifying biomarkers that predict patient response to immunotherapy is critical for optimizing treatment strategies and improving clinical outcomes. Despite the success of immunotherapy, a significant proportion of patients do not respond to treatment. Thus, there is an urgent need for more robust methods to differentiate responders from non-responders. Spatial biology, which examines the precise localization and interaction of cells within the tissue, plays a crucial role in understanding the complex tumor-immune microenvironment. In this study, we present a novel multiplex imaging-based immunotherapy panel and a comprehensive analysis pipeline to characterize the spatial distribution and function of immune cells and its application for spatial biomarker identification in a cohort of immunotherapy- and targeted therapy-treated melanoma patients.

Methods

We designed a 28-plex panel to perform sequential immunofluorescence (seqIF™) on the COMET™ platform (Figure 1A-C) [1] to target key biomarkers associated with tumor microenvironment composition (TME), immune cell infiltration, and immune checkpoint pathways (Figure 1D-F). Utilizing Nucleai's deep-learning-based multiplex imaging analysis pipeline (Figure 2A) [2], we identified 16 cell types, including 10 different immune cell populations, in addition to 10 cell state markers. Single channel and cell typing models' performance was validated vs. expert annotations. Following cell typing, cellular neighborhoods were designated as previously described [3], and were assigned to the tumor or stromal areas (Figure 2B-C). The tumor area was further divided into the tumor core (TC) and tumor invasive margin (TIM), while the stroma was divided into adjacent TME (aTME) and outer TME (oTME) based on the distance from the tumor-stroma border. We obtained pre-treatment biopsies from patients in arm B (Ipilimumab + Nivolumab until progression, followed by BRAF targeted therapy (Encorafenib + Binimetinib)) from the SECOMBIT Phase II Trial (NCT02631447) [4, 5] with known long-term response or rapid progression to immunotherapy combination treatment. These samples were profiled using the aforementioned panel and analysis solution. More than 1,000 spatial features were calculated using a combination of cell type, marker positivity and area assignments, as well as cell-cell interactions and cell state marker co-expression. Spatial features were compared between responders and non-responders to immunotherapy using Welch's t-test for the binary outcome and logrank test for survival analysis.

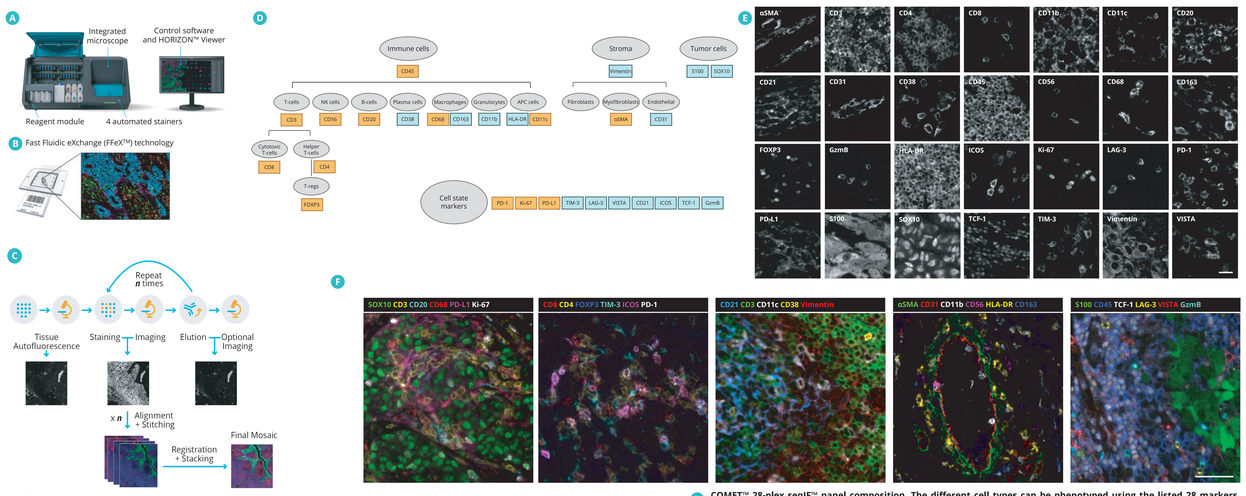
Results

Our novel multiplex imaging panel and analysis pipeline demonstrated high accuracy in both cell typing and quantification of protein expression as demonstrated by a balanced accuracy (> 0.9) in all markers and F1 scores (> 0.75) in >97% of markers, as well as F1 scores > 0.75 in >90% of cell types . Combination of cell type, cell phenotypic state and the spatial context of the cells enables the quantification of known biomarkers such as T cell activation states, T cell infiltration patterns, and TLS maturation, as well as discovery of novel biomarkers that capture biologically relevant cell states and tumor-immune cells and immune-immune cells interactions.

Comparison of calculated spatial features between long-term responders (n=6) and rapid progressors (progression free survival < 3 months, n=6), revealed distinct immune cell interactions and activation status across the tumor areas associated with immunotherapy response. Within the tumor area, the reciprocal interactions of tumor cells, cytotoxic CD8 T-cells and antigen presenting cells (APC) such as HLA-DR+ macrophages and dendritic cells were associated with better immunotherapy outcome. In contrast, a high percentage of proliferating regulatory T cells within the tumor invasive margin (TIM) was associated with a worse clinical outcome from combination immunotherapy. In the adjacent TME (aTME), endothelial cell interactions with T-cells and macrophage proliferation were associated with immunotherapy resistance, while in the outer TME (oTME), regulatory T-cell proliferation as well as regulatory T-cells – plasma cell interactions and TCF-1 positivity within CD4 T-cells were associated with lack of response. In contrast, the interaction between MHC class II macrophages and APC cells was associated with improved clinical outcome.

Taken together, we demonstrate that area-specific immune niches contribute to the success or failure of immunotherapy response and highlight the importance of spatial biology in predicting immunotherapy outcomes and tailoring personalized treatment strategies.

FIGURE 1. Panel design and optimization using seqIF™ approach on the Lunaphore's COMET™ platform



A **COMET™ is an automated platform providing a slide-in, data-out workflow for spatial biology on up to 4 tissues simultaneously.**

B **Lunaphore microfluidic FFeX™ technology allows fast and uniform delivery of reagents to a closed staining and imaging chamber. Precise control of all staining conditions allows for the achievement of high-quality immunofluorescent staining in minutes instead of hours.**

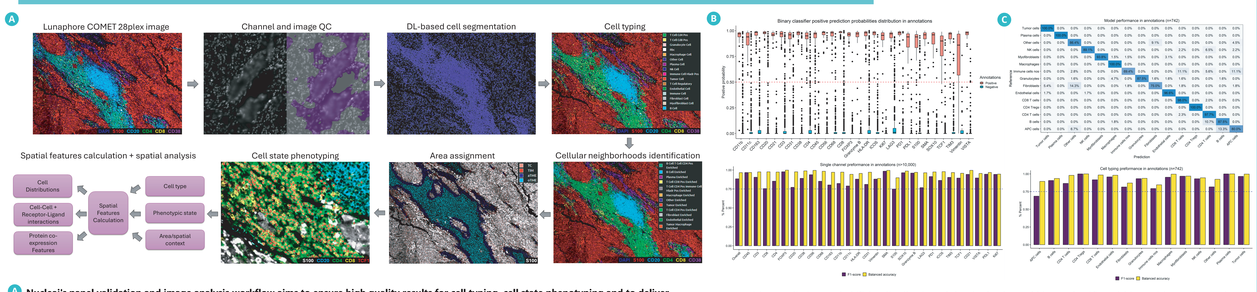
C **The seqIF™ protocol starts with acquiring of the tissue autofluorescence followed by sequential staining-imaging-elution cycles, where 2 biomarkers are detected at a time.**

D **COMET™ 28-plex seqIF™ panel composition. The different cell types can be phenotyped using the listed 28 markers. Markers labeled in orange are part of the Lunaphore SPYRE™ Immuno-Oncology Core Panel of antibodies.**

E **Representative seqIF™ ROIs images of each individual markers part of the 28-plex COMET™ panel on a single slide of melanoma FFPE sample. All images: autofluorescence subtracted and brightness adjusted for visualization, no contrast adjustment. Scale bar 20μm**

F **Composite images generated from the 28-plex seqIF™ panel shown in (D). All images: autofluorescence subtracted and brightness adjusted for visualization, no contrast adjustment. Scale bar 50μm**

FIGURE 2. Nucleai's panel validation and multiplex imaging analysis workflow performance evaluation in the 28-plex melanoma IO panel.

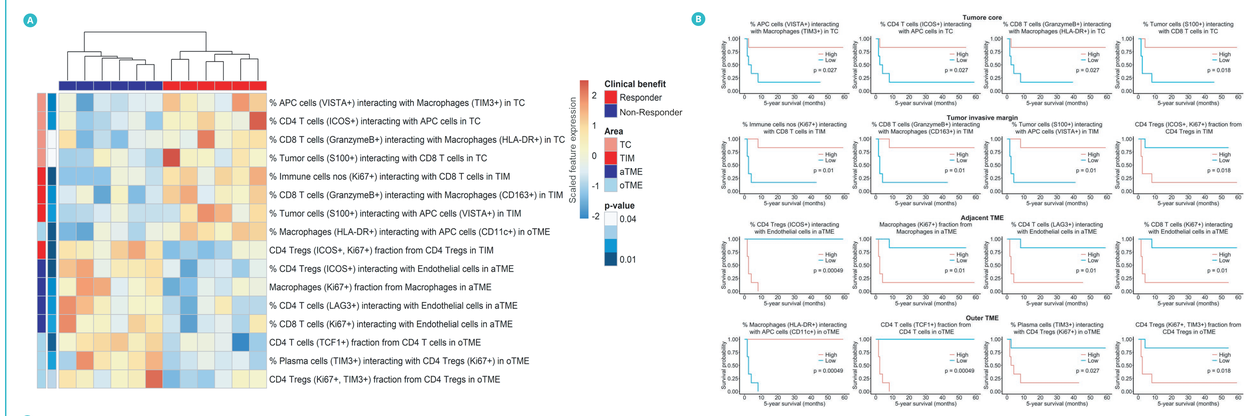


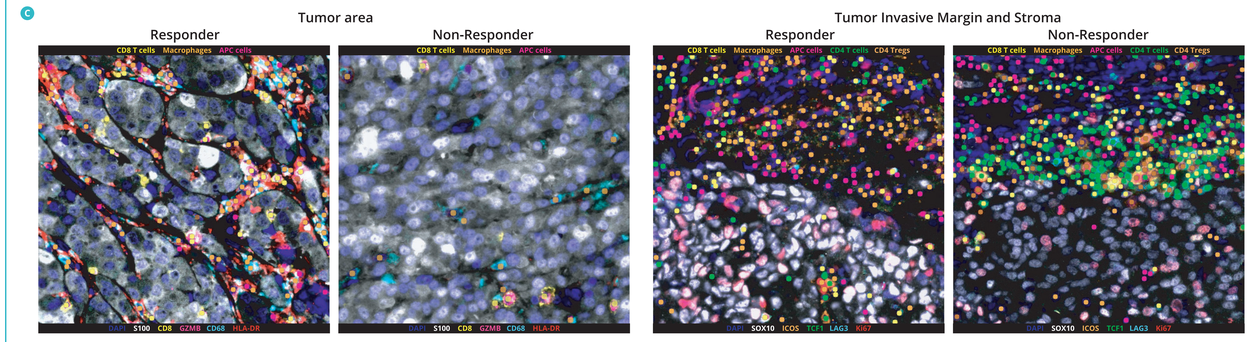
A **Nucleai's panel validation and image analysis workflow aims to ensure high quality results for cell typing, cell state phenotyping and to deliver actionable biological insights. Following channel and image QC, we perform cell segmentation followed by DL-based protein quantification for each marker within each cell. These outputs are used to determine the cell type of each cell, followed by spatial neighborhoods and area assignment to contextualize each cell within its spatial environment. Lastly, we combine the cell type, phenotypic state and area and neighborhoods assignments to calculate human-interpretable spatial features which can be used to derive actionable spatial insights**

B **Positive prediction probabilities distribution of the single-channel binary classifier within positive and negative annotations (top) and F1 scores and balanced accuracy for single marker positivity classification (bottom).**

C **Comparison between cell typing predictions and annotations (top). F1 scores and balanced accuracy for cell type detection (bottom).**

FIGURE 3. The top spatial features associated with clinical benefit to combination immunotherapy in advanced melanoma patients





A **Heatmap demonstrating the top differentially expressed spatial features between responders and non-responders to immunotherapy combination treatment within the tumor core (TC), tumor invasive margin (TIM), adjacent TME (aTME) and outer TME (oTME)**

B **Kaplan-Meier curves comparing between the progression free survival of the upper and lower medians of the top differentially expressed spatial features**

C **Representative images of the tumor and stromal areas of long-term responders and non-responders to immunotherapy demonstrating upregulation of HLA-DR+ macrophages and APC, and Granzyme B+ CD8 T-cells in the tumor area of responders and upregulation of TCF1+ CD4 T-cells and proliferative CD4 Tregs in the tumor invasive margin and stroma of non-responders.**

Conclusions

Enabling the precise identification of cells and cellular states in immunotherapy-treated patients is critical for guiding personalized treatment strategies. Here we present a validated immunotherapy specific 28plex panel and deep learning-based analysis pipeline that can be deployed on large-scale cohorts to enhance our understanding of treatment efficacy and resistance mechanisms, ultimately aiming to be used for treatment personalization and improve patient outcomes in clinical practice. This panel and analysis pipeline were deployed to identify tumor-immune interactions that are associated with better or worse prognosis to combination immunotherapy in subjects of the SECOMBIT trial. We demonstrated that distinct spatial features capturing immune cell interactions are crucial in predicting immunotherapy outcomes. Interactions involving tumor cells, cytotoxic CD8 T-cells, and antigen-presenting cells were linked to better responses while proliferating regulatory T cells in specific regions were associated with poorer outcomes. Although our findings are promising, they are based on a limited number of slides, underscoring the need for validation in a larger cohort. These insights emphasize the importance of spatial biology and multiplex imaging in tailoring personalized treatment plans, offering a promising avenue for advancing personalized medicine and improving clinical outcomes in immunotherapy and other treatment modalities.

References

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