Application Note

Maxpar OnDemand Mouse Immuno-Oncology Panel Kit

Reveal the Spatial Landscape of the Tumor Microenvironment

Introduction

Mouse tumor models have greatly enhanced our understanding of tumorigenesis and are widely utilized as the preferred model organism for cancer studies and preclinical drug testing[1,2]. The ability to evaluate the multiparametric response in the tumor microenvironment (TME) is crucial to predict therapeutic drug efficacy[3]. Particularly, assessment of immunological and oncological processes that dictate tumor growth, metastasis, and immune response are essential for identifying candidates for further clinical evaluation. Imaging Mass Cytometry™ (IMC™) permits analysis of 40-plus distinct tissue and cellular markers simultaneously on tumor samples, providing a thorough evaluation of the spatial landscape of the TME. Application of IMC to study cancer has facilitated important discoveries regarding the interplay of tumor and immune cells in the TME[4-6]. Here, we introduce the Maxpar® OnDemand™ Mouse Immuno-Oncology IMC Panel Kit (Cat. No. 9100005) designed for high-parameter preclinical immuno-oncology studies.

Through systematic selection of mouse antibodies from both the Maxpar catalog and from Maxpar OnDemand, we have created panel kits that permit qualitative and quantitative evaluation of critical tumor pathophysiological parameters. This application note describes the design and application of an IMC panel consisting of 4 modular subpanels and defines their performance on multiple mouse tumor models. Furthermore, we present a consolidated single-cell analysis (SCA) pipeline by combining the panel kit with the IMC Cell Segmentation Kit (ICSK) and present quantitative evaluation of the cellular and structural landscape of mouse non-small cell lung cancer (NSCLC) TME. Overall, application of the Mouse Immuno-Oncology Panel successfully defines the tissue architecture of the TME, metastatic and growth potential of tumor cells, and immune cell phenotype and activation in mouse tumors.

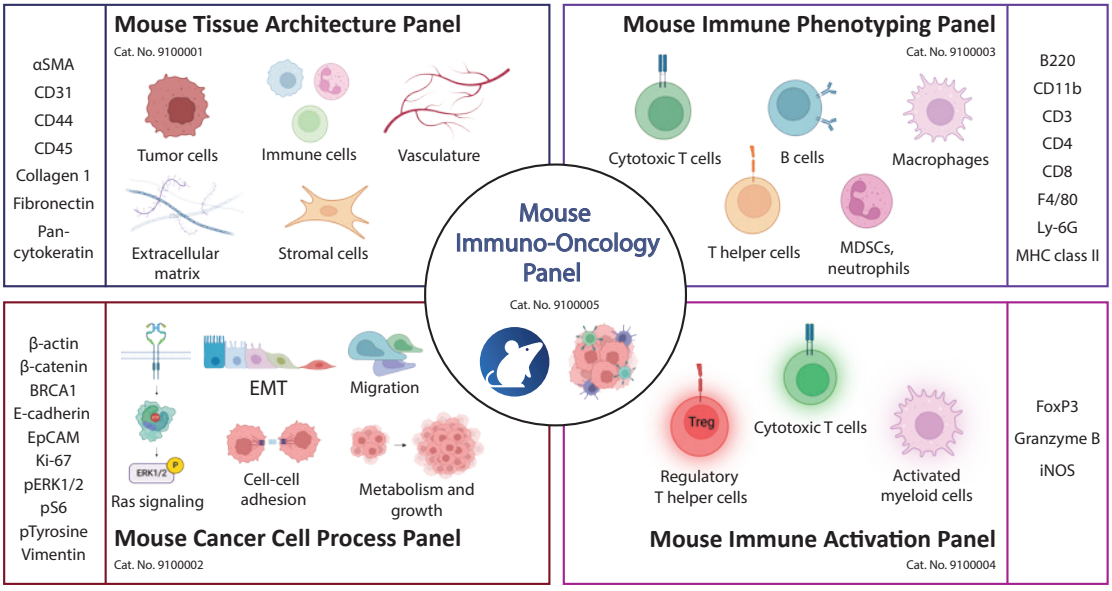

Figure 1. Mouse Immuno-Oncology Panel for IMC application. The subpanels are specifically designed to highlight the structure and cellular composition within the mouse TME. Additional details regarding the panel kits can be found in Table 1.

Objectives

- Showcase the strength of the Mouse Immuno-Oncology Panel to deliver relevant high-parameter images on a variety of mouse tumors.
- Illustrate the ability of the Mouse Immuno-Oncology Panel
  - to identify tissue architecture and pathophysiological processes of the TME;
  - to conduct SCA for the identification of tumor and immune cell phenotypes and activation states.

Study design

A 28-parameter antibody panel, designed to highlight central features of the mouse TME (Table 1), is presented in this application note. This panel consists of a combination of 4 inter-compatible mouse IMC panel kits, with each kit assembled to reveal critical insights about tumor biology (Figure 1, Table 1).

- Maxpar OnDemand Mouse Tissue Architecture IMC Panel Kit (Cat. No. 9100001) identifies the underlying cellular and structural markers of the tumor.
- Maxpar OnDemand Mouse Cancer Cell Process IMC Panel Kit (Cat. No. 9100002) identifies activation of signaling pathways, metabolism, growth, and metastatic potential in cancer cells.
- Maxpar OnDemand Mouse Immune Phenotyping IMC Panel Kit (Cat. No. 9100003) delineates lymphoid and myeloid cell subtypes of immune cell infiltrates in tumors.
- Maxpar OnDemand Mouse Immune Activation IMC Panel Kit (Cat. No. 9100004) assesses the functional state of immune cells in tumors.

The IMC Cell Segmentation Kit (TIS-00001) and Cell-ID™ Intercalator-Ir (Cat. No. 201192B) were applied to facilitate SCA. For Maxpar OnDemand Antibodies, metal tags were selected with precision to minimize potential signal overlap and optimize signal intensity.

The full panel was applied on mouse tumor tissue microarray (TMA) containing a wide variety of tumor types as well as on normal mouse TMA. Table 2 summarizes information regarding tissue used in this application note.

Normal and tumor TMA slides were prepared and stained using optimized antibody dilutions. All antibodies were titrated and tested on positive control tissue (spleen, colon, lung) alongside tumor samples. Tissue slides were ablated using the Hyperion™ Imaging System. Qualitative data analysis, multiplexed image rendering, and single-channel image extractions were performed using MCD™ Viewer. Quantitative analysis was performed for NSCLC using an analysis pipeline consisting of 3 major steps: A custom MATLAB® script was applied for pixel classification, CellProfiler™ was

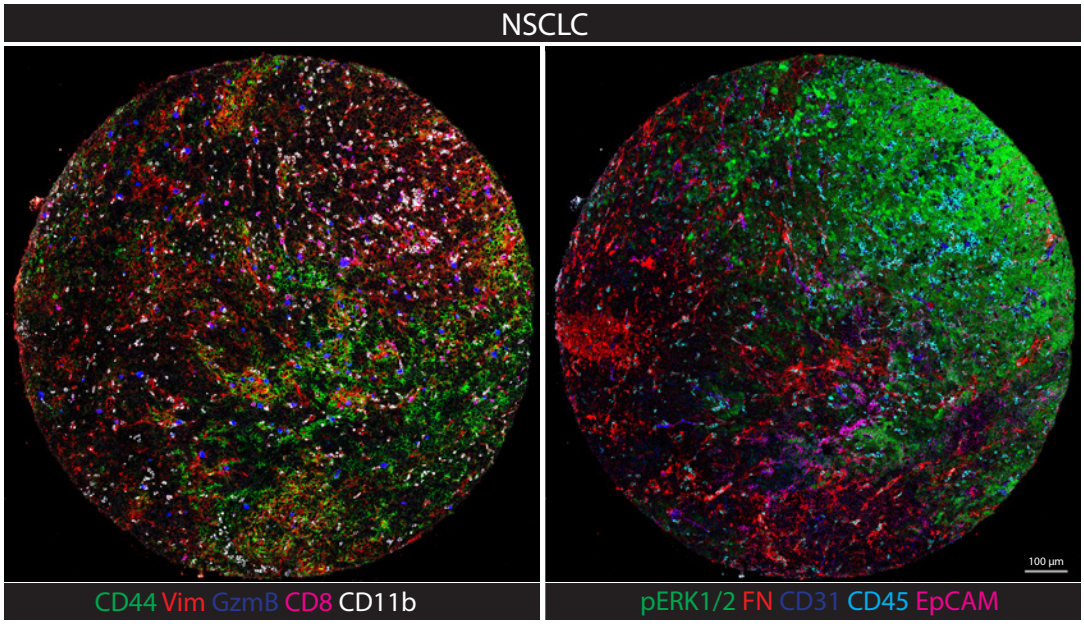

Figure 2. Multiplex images of mouse formalin-fixed, paraffin embedded (FFPE) NSCLC tissue. The Mouse Immuno-Oncology Panel embellishes the structure and cellular composition of the mouse TME. Selected markers from a single tumor core are shown. Scale bar = 100 µm

used for cell segmentation, and histoCAT™ was used for t-distributed stochastic neighbor embedding (t-SNE) and PhenoGraph clustering-based SCA.

See Methods for additional experimental details regarding staining, ablation, and data analysis.

Results

Mouse Tissue Architecture Panel highlights the structure and cellular composition of the TME

Defining the heterogenous spatial landscape of the TME is necessary for predicting disease progression. Presence of cancer cells, cancer stem cells, immune infiltrates, stromal cells, and extracellular matrix (ECM) composition can serve as valuable prognostic markers. The Mouse Tissue Architecture Panel offers markers for tumor cells, immune cells, vascular cells, stromal cells, and the ECM.

Altogether, this panel kit provides the necessary tools to create the cellular atlas of the mouse TME (Figure 3, Figure S1).

Tumors consist of a wide variety of cancer cells with distinct molecular signatures. Inclusion of diverse markers for tumor cell identification is required for their panoptic detection. The Mouse Tissue Architecture Panel contains pan-cytokeratin (pan-CK) and CD44 for identification of tumor cell populations. Pan-cytokeratin detects tumor epithelial cells and CD44 detects tumor cells with stem cell-like features[7-11]. In NSCLC, pan-cytokeratin and CD44 are expressed in tumor cells (Figure 3, A1). In colon adenocarcinoma, all tumor cells express CD44, which is indicative of high tumor stemness[12,13] (Figure 3, B1).

Assessing immune cell composition and positioning in the TME is an important factor in predicting disease progression. The vasculature provides a gateway for immune cell infiltration and nutrients and access for metastatic cells to escape the primary tumor site. The Mouse Tissue Architecture Panel includes CD45 for immune cell detection and CD31, also known as PECAM-1, for endothelial cell detection. In NSCLC, colon adenocarcinoma, B cell lymphoma, and glioblastoma, both immune cells and vascular cells can be readily identified (Figure 3, A2 and B2; Figure S1).

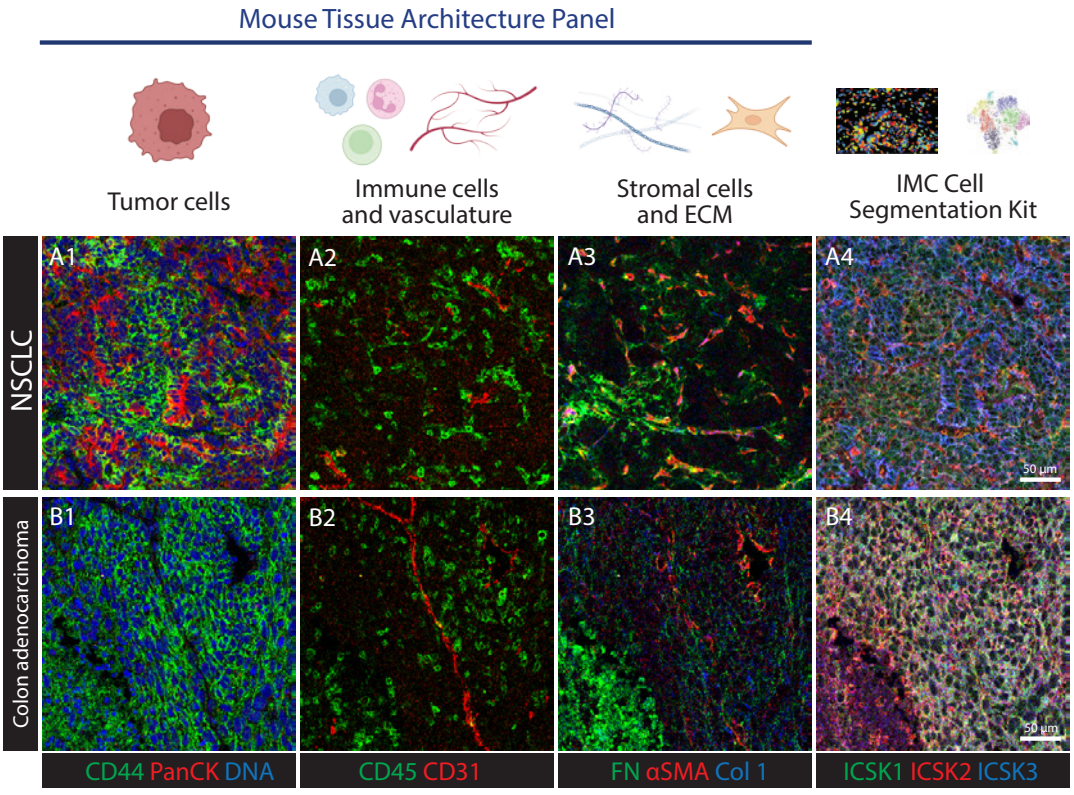

Figure 3. Mouse Tissue Architecture Panel identifies major cellular and structural components of mouse tumors. Expression of pan-CK and CD44 identify the tumor cells in NSCLC (A1) and colon adenocarcinoma (B1). Immune cells are marked by CD45, and vascular cells are marked by CD31 (A2, B2). ECM components, fibronectin (FN) and collagen 1 (Col 1), and αSMA-expressing stromal cells are detected (A3, B3). ICSK markers demarcate the cellular boundaries of all cells within the TME (A4, B4). The DNA marker and ICSK are not part of the Tissue Architecture Panel Kit. Scale bar = 50 µm

The ECM is an essential component of the TME and biochemical and biophysical signals from the ECM influence tumor development and progression. Fibronectin (FN) and collagen 1 are primary components of the ECM and serve as attachment sites for various cells found in the TME[14]. Stromal cells such as cancer-associated fibroblasts, myofibroblasts, and endothelial cell-associated pericytes reside adjacent to the ECM and contribute to its deposition. The Mouse Tissue Architecture Panel offers αSMA, fibronectin, and collagen 1 expression for identification of ECM and stromal cells. In NSCLC, stromal cells are abundant in the TME and contribute to deposition of fibronectin and collagen 1 (Figure 3, A3). In colon adenocarcinoma, pericytes expressing αSMA can be observed lining the CD31-expressing vasculature, and regions of high fibronectin and collagen 1 accumulation can be observed (Figure 3, B3; Figure S1).

Mouse Cancer Cell Process Panel enables assessment of tumor cell signaling, growth, and metastatic potential

Tumors exhibit heterogenous phenotypes due to clonal evolution of cancer cells during tumorigenesis[15,16]. Each cancer stem cell-derived population has a unique molecular signature that fine-tunes cellular processes. The Mouse Cancer Cell Process Panel reveals signaling pathway activation, proliferation, metabolism, and epithelial to mesenchymal transition (EMT), thus highlighting the variety of cancerous cell processes in the mouse TME.

Ras signaling modulates growth rate and metastasis of tumors and initiates upon activation of receptor tyrosine kinases (RTKs), which phosphorylate tyrosine residues (pTyr) on signaling receptors[18]. Subsequent activation of the phospho-relay pathway results in activation of extracellular signal-regulated kinases, ERK1 and ERK2 (pERK1/2)[19,20]. The Mouse Cancer Cell Process Panel offers pTyr and pERK1/2 markers for assessment of canonical and non-canonical Ras signaling. In normal lung tissue and NSCLC, elevated levels of pTyr and pERK1/2 in cells indicates activation of Ras signaling (Figure 4, A1 and B1).

Tumors rely on a constant supply of nutrients for sustained growth, made possible by constitutive activation of glycolytic metabolism[21]. Phosphorylation of ribosomal protein S6 (pS6) is a step in the mammalian target of rapamycin (mTOR) signaling cascade that regulates metabolic activity in cells[22]. Ki-67 is an exclusive nuclear marker for proliferating cells that is present at all active stages of the cell cycle[23]. The Mouse Cancer Cell Process Panel offers both pS6 and Ki-67 markers for the assessment of metabolic activity and tumor growth. In normal lung tissue and NSCLC, cells with individual and concomitant expression of pS6 and Ki-67 can be detected, indicating metabolic and growth capacity of tissues (Figure 4, A2 and B2).

EMT is linked to promoting cancer morbidity by contributing to tumor initiation, invasion, and resistance to therapeutic interventions[24]. During EMT, tumor cells disrupt cell-cell adhesions and activate pro-migratory cellular machinery. The Mouse Cancer Cell Process Panel includes 6 markers for assessing cellular processes associated with EMT. In normal lung tissue and NSCLC, high overlapping expression of cell-cell adhesion molecules, E-cadherin (E-cad), β-catenin (β-cat), and EpCAM highlight epithelial cells with suppressed EMT activation (Figure 4, A3 and B3). Expression of cytoskeletal components vimentin (Vim) and β-actin (β-act) identifies cells with activated migratory capabilities in normal lung and NSCLC tissue (Figure 4, A4 and B4). Expression of the tumor suppressor marker BRCA1 indicates cells with low capacity for EMT in normal lung and NSCLC tissue (Figure 4, B4).

Mouse Immune Phenotyping Panel facilitates identification of lymphoid and myeloid cell populations

Composition of the immune cells in the tumor immune microenvironment (TIME) is of vital importance for predicting treatment response and overall clinical outcomes[25]. Delineation of specific immune cell populations and their spatial organization in the TIME is required for discovering promising immunomodulatory cancer therapeutics. The Mouse Immune Phenotyping Panel offers 8 distinct markers that enable identification of lymphoid and myeloid immune cell infiltrates in tumors. Cytotoxic T cells (CD8) and helper T cells (CD4) can be detected amongst the total immune cell infiltrates in NSCLC and B cell lymphoma (Figure 5; A1, A2, B1, and B2). B cells were not observed in NSCLC. However, B cell expression identifies tumor cells in B cell lymphoma (Figure 5, A3 and B3). MHC class II expression delineates antigen-presenting cells in NSCLC and B cell lymphoma (Figure 5, A3 and B3). Macrophages, myeloid-derived suppressor cells (MDSCs), and neutrophils can be detected by expression of F4/80, CD11b, and Ly-6G in NSCLC and B cell lymphoma (Figure 5, A4 and B4).

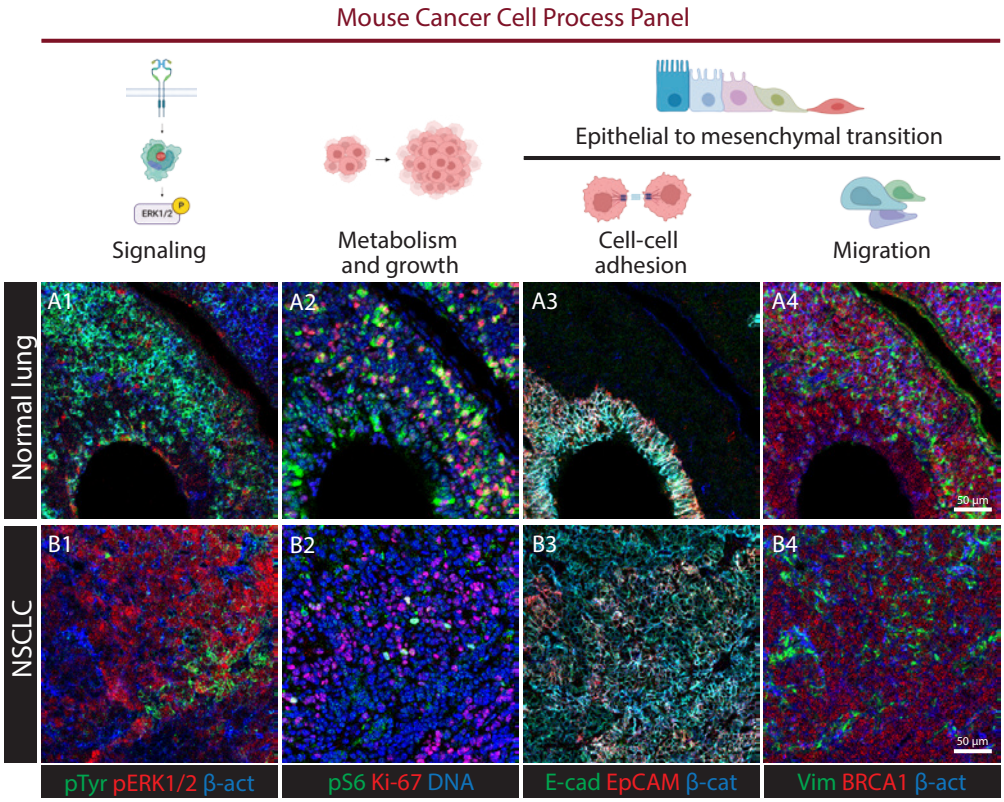

Figure 4. Mouse Cancer Cell Process Panel identifies growth and metastatic potential of mouse tumors. pTyr and pERK1/2 identify cells with activated signaling in normal (A1) and cancerous lungs (B1). Metabolically active and replicating cells are marked by pS6 and Ki-67, respectively (A2, B2). Cells with varying levels of epithelial cell-cell adhesion markers, E-cadherin, EpCAM, and β-catenin are observed (A3, B3). Cells with mesenchymal features are identified by expression of vimentin and β-actin, and expression of tumor suppressor transcription factor BRCA1 indicates low potential for EMT (A4, B4). The DNA marker is not part of the Maxpar OnDemand Cancer Cell Process IMC Panel Kit. Scale bar = 50 µm

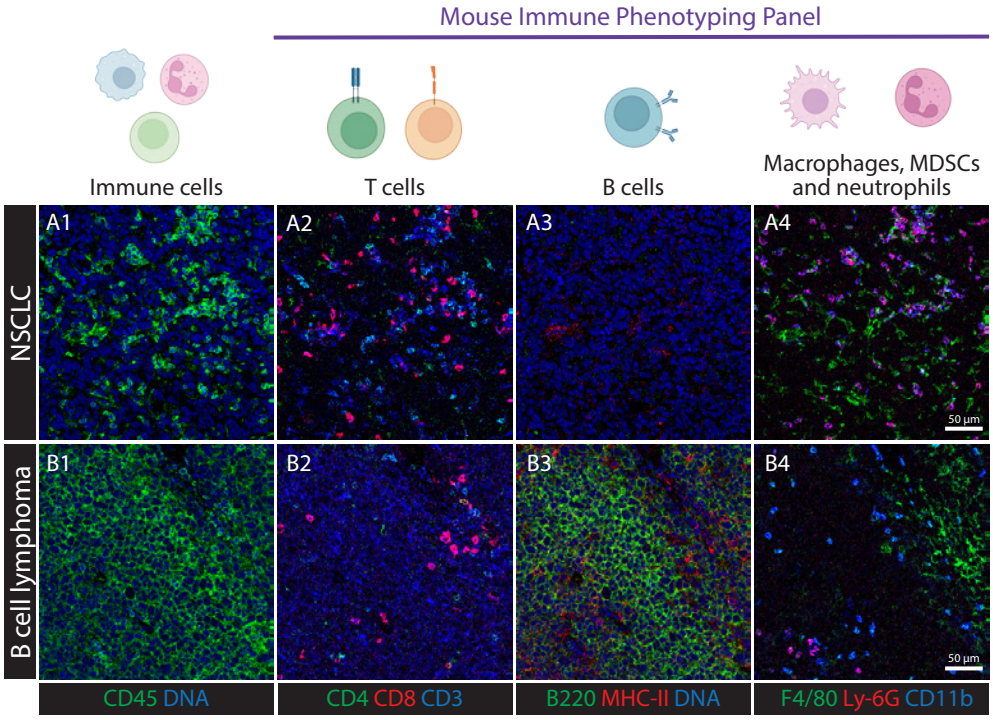

Figure 5. Mouse immune phenotyping panel identifies myeloid and lymphoid immune cell populations in mouse tumors. The CD45 marker identifies the total population of immune cells in NSCLC (A1) and B cell lymphoma (B1). Combined CD3, CD4, and CD8 markers highlight localization of T cell subtypes (A2, B2). The B220 marker identifies B cells while MHC-II marks antigen-presenting cells (A3, B3). Macrophages, MDSCs, and neutrophils are identified by expression of F4/80, CD11b, and Ly-6G markers (A4, B4). CD45 and DNA markers are not part of the Maxpar OnDemand Mouse Immune Phenotyping IMC Panel Kit. Scale bar = 50 µm

Mouse Immune Activation Panel identifies activation state of lymphoid and myeloid cells

Controlling the activation of immune cells in the TIME is at the forefront of cancer immunotherapy research[28]. The presence of pro-inflammatory immune cells or cytotoxic T cells in the TIME is generally considered a positive indicator for clinical outcomes, whereas the presence of regulatory T cells (Treg) specifies an inhibitory TIME with poor clinical prognosis. The Mouse Immune Activation Panel contains 3 distinct markers that enable identification of relevant activated immune cells in tumors. In NSCLC and B cell lymphoma, proliferation of immune cells can be assessed by expression of Ki-67 (Figure 6, A1 and B1). The expression of granzyme B (GzmB) assesses the cytotoxic activation of CD8 T cells (Figure 6, A2 and B2). FoxP3 expression identifies Tregs in NSCLC and B cell lymphoma (Figure 6, A3 and B3). Activated macrophages expressing granzyme B are identified in NSCLC and B cell lymphoma (Figure 6, A4 and B4). Additionally, iNOS-expressing activated macrophages can be identified in B cell lymphoma (Figure 6, B4).

Mouse Immuno-Oncology Panel enables single-cell analysis of mouse non-small cell lung cancer

The Mouse Immuno-Oncology Panel in combination with the ICSK enables the SCA of the mouse TME and identification of critical parameters for evaluation of tumor progression. The ICSK contains 3 platinum-conjugated markers that label all cells to facilitate SCA. Ubiquitous labeling of all cells within the TME was observed in these experiments (Figure 3, A4 and B4; Figure S1).

SCA of NSCLC identified a total of 47,816 cells from 4 tumor cores. Dimensionality reduction via t-SNE and PhenoGraph grouped the cells into 18 distinct clusters, where 9 clusters were designated as tumor cell-specific and 5 as immune cell-specific (Figure 7 and Figure 8). Each cluster phenotype was defined by the expression of a combination of markers from the Mouse Immuno-Oncology Panel. For tumor cell classification, epithelial markers E-cadherin and β-catenin were used in addition to pan-cytokeratin and CD44. The following sections describe the performance of the panel kits in identifying IO-related processes in mouse NSCLC.

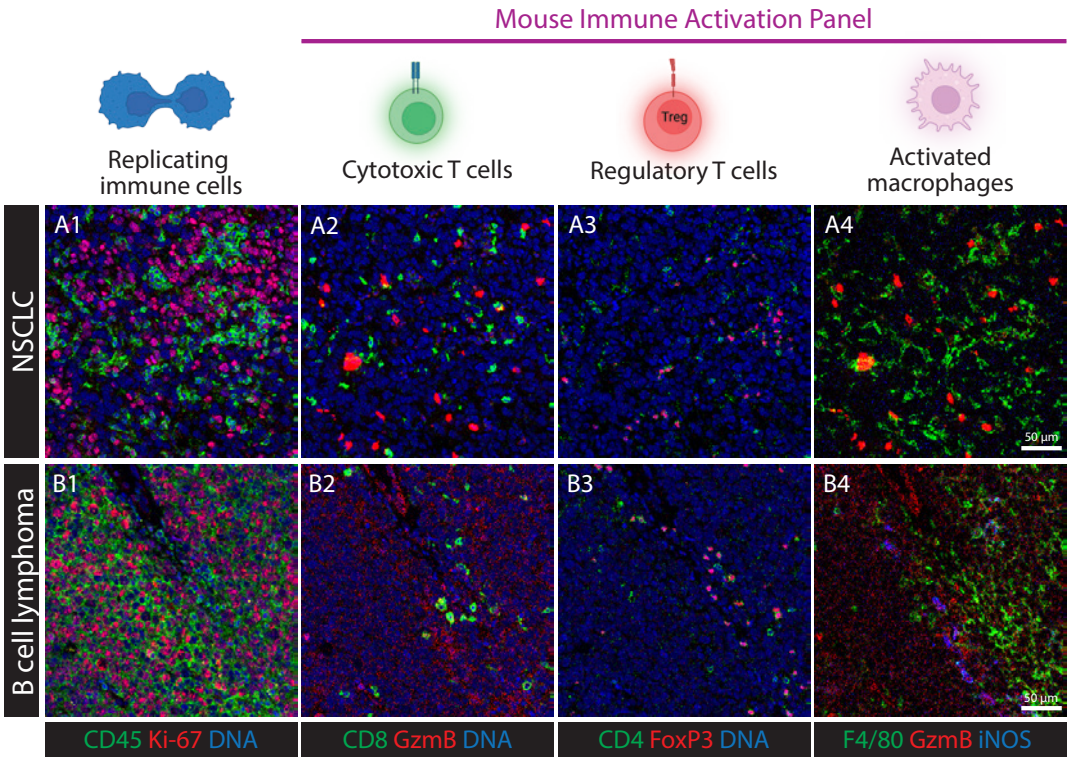

Figure 6. Mouse Immune Activation Panel identifies the activation state of immune cell subtypes in mouse tumors. Ki-67 highlights replicating immune cells in NSCLC (A1) and B cell lymphoma (B1). Granzyme B expression in CD8 T cells identifies activated cytotoxic T cells (A2, B2). FoxP3 expression in CD4 T cells identifies exhausted T regulatory cells (A3, B3). Granzyme B and iNOS expression in combination with macrophage marker F4/80 identifies inflammatory macrophages (A4, B4). CD45, Ki-67, and DNA markers are not part of the Maxpar OnDemand Mouse Immune Activation Panel Kit. Scale bars = 50 µm

Epithelial to mesenchymal transition

Clusters 1, 5, and 9 highlighted cells with differing levels of EMT activation. Tumor cells in cluster 1 had low levels of cell-cell adhesion molecules (E-cadherin, β-catenin, and EpCAM), suggesting an increase in capacity to undergo EMT. Cluster 5 contained tumor cells with the highest levels of cell-cell adhesion molecules and expression of BRCA1, suggestive of a strong epithelial tumor suppressive phenotype with low capacity for EMT. Cluster 9 represented cells with a high level of the mesenchymal marker vimentin, highlighting cells that have undergone EMT (Figure 7A and Figure S2).

Ras signaling

Clusters 2, 10, and 13 contained cells with activation of the Ras signaling pathway. Cluster 2 contained tumor cells that have enriched levels of pERK1/2 and low levels of pTyr at the plasma membrane, suggestive of receptor-independent Ras signaling pathway activation[18]. Clusters 10 and 13 contained tumor cells with enrichment of pTyr and pERK1/2 signal, suggestive of activation of the RTK-dependent canonical Ras signaling pathway. Interestingly, cluster 13 also displayed enrichment of fibronectin and collagen 1, indicative of ECM-dependent Ras signaling activation[29] (Figure 7A and Figure S2).

Metabolism and growth

Tumor cell-specific clusters 3, 4, 10, and 13 exhibited elevated levels of pS6, whereas cells in clusters 4, 10, and 13 also exhibited elevated levels of pERK1/2, suggestive of Ras-dependent metabolic regulation[26]. Highly replicative tumor cells with elevated levels of Ki-67 are detected in cluster 4 and represent 8.5% of total cells (Figure 7A and Figure S2).

Immune cell infiltration

Immune cell infiltrates represented 14.9% of the total TME population with myeloid cells being the predominant type at 85%. Cluster 8 contained macrophages with elevated F4/80 expression. Cluster 11 contained MDSCs with reported expression of CD11b and Ly-6G. Clusters 8 and 11 also exhibited elevated levels of β-actin, suggestive of high migratory capacity. Cluster 17 contained activated macrophages with enriched expression of granzyme B. Lymphoid cells represented 15% of the total immune cell population, with CD8 T cells present in cluster 16 and CD4 T cells present in cluster 18 (Figure 7B and Figure S2). In CD8 T cells, a slight elevation of Ki-67 and pS6 was detected, suggestive of proliferative and metabolic activity. In cluster 18, all CD4 T cells were classified as Tregs due to high levels of FoxP3. Additionally, increased levels of pS6 in Tregs were also detected, indicating high metabolic activity[27].

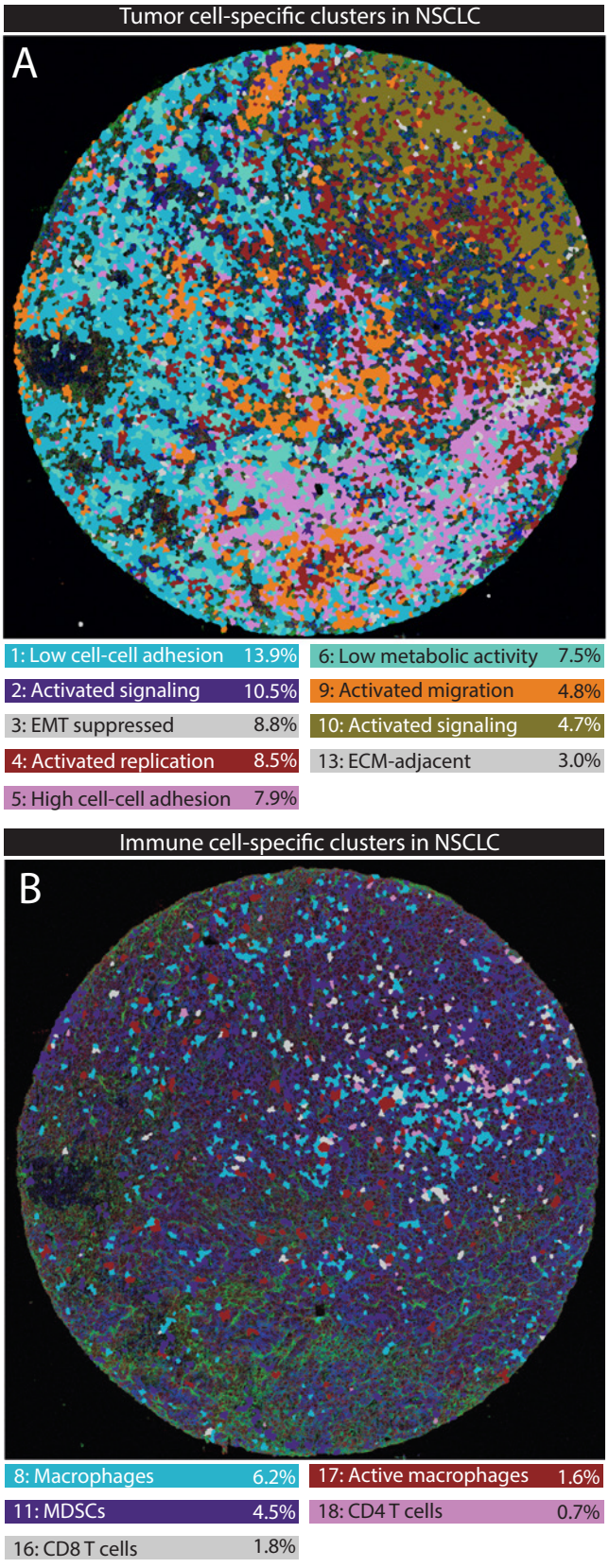

Figure 7. SCA identifies tumor and immune cell populations in NSCLC. Cell masks representing tumor-specific (A) and immune-specific (B) clusters demonstrate spatial position of distinct cells.

Vascular and stromal cells

Vascular cells, identified by CD31 signal and represented by cluster 15, made up 2% of the total cell population. High expression of vimentin was detected in the vascular cells, which suggests activation of vascular remodeling[28]. Stromal cells, identified by αSMA signal and represented by cluster 12, made up 3.7% of the total cell population. High expression of vimentin and ECM components, fibronectin and collagen 1, is detected in stromal cells (Figure S2).

Summary of SCA

The data presented in this section demonstrates the successful validation of the Mouse Immuno-Oncology Panel for conducting multiplexed IMC analysis and provides evidence for its utilization for SCA of mouse tumors. Quantitative analysis of tumor composition revealed critical insights regarding prognostic parameters such as metastatic and growth potential of tumors, identification and activation of immune cell infiltrates, and overall cellular and molecular composition of the mouse NSCLC TME.

Conclusions

Preclinical cancer therapeutics often fail in the clinic due to unavailability of holistic data about their precise effect on tumors[29]. As described in this application note, the use of the Mouse Immuno-Oncology Panel in conjunction with state-of-the-art molecular tools can enable mouse tumor studies that reveal significant details of tumor development, progression, and treatment. In addition, the panel kits and tools can be used to accelerate the creation of comprehensive cellular tumor atlases. By unleashing the power of IMC technology, it is possible to generate high-quality high-plex quantitative data with respect to key tumor parameters, which enables researchers to make high-confidence decisions about therapeutic drug targets for further clinical evaluation.

The Mouse Tissue Architecture Panel in combination with the Mouse Cancer Cell Process Panel unveiled the tumor heterogeneity of the cancer cells and provided detailed assessment of cancer stemness, mitogenic signaling activation, metabolic activation, ECM composition and vascular network structure. Additionally, the Mouse Immune Phenotyping Panel in combination with the Mouse Immune Activation Panel revealed the organization of the TIME and provided a comprehensive breakdown of the lymphoid and myeloid immune cell infiltrates and further classified their activation states. The panel kits are modular to allow the mixing and matching of kits for optimal study design. The panel is compatible with the Hyperion+™ Imaging System and can be further expanded to incorporate an additional 7 catalog and custom antibodies to further address individual research aims.

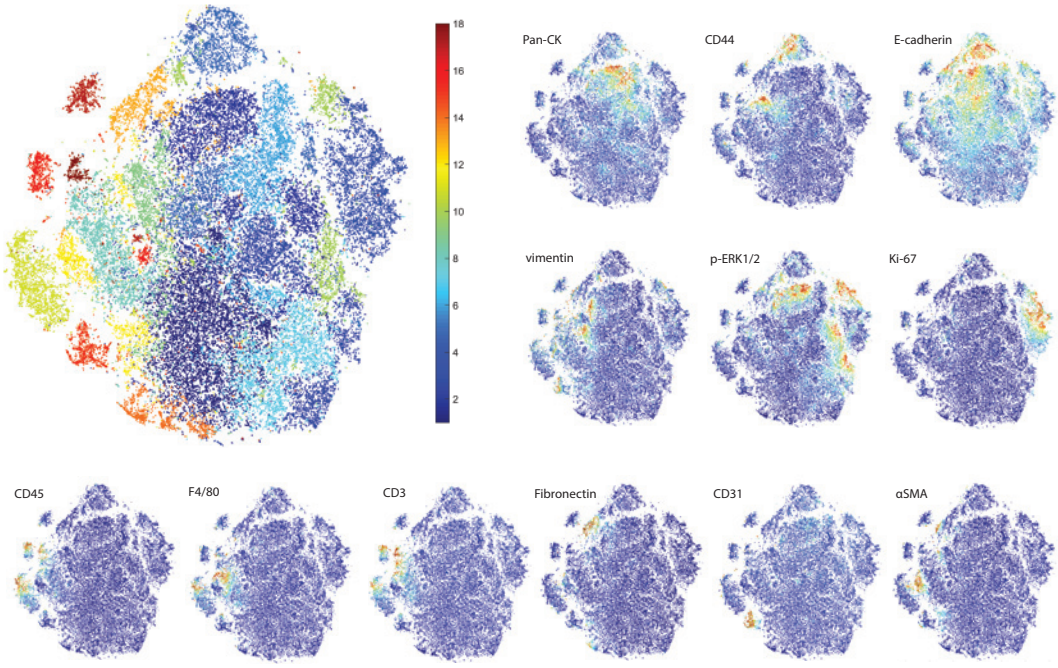

Figure 8. t-SNE and heat map analysis of mouse NSCLC tumor tissue microenvironment. PhenoGraph analysis subdivided the TME into 18 cellular clusters based on marker expression. Heat maps of selected individual markers overlaid on the t-SNE plot demonstrate expression in cellular clusters and showcase the accuracy of cluster classification. Additional information can be found in Figure S2. Autoscaling was applied for individual marker heat maps

Tips for Success

- For best results, use freshly cut FFPE tissue sections when possible.
- Perform a 3-point titration and include positive control tissue for all antibodies when optimizing working concentration on tumor tissue. Recommended dilution ranges for each antibody can be found in the Technical Data Sheet (TDS-00712).
- Due to the heterogenous nature of tumors with various tissue origins, carefully consider markers for tumor cell detection during study design and include additional markers. When adding additional markers to the existing Mouse Immuno-Oncology Panel (Cat. No. 9100005), design panel to minimize signal spillover.
- After staining, samples should be stored at room temperature in slide holders inside a sealed bag in a non-humid environment.
- Customers should reach out to their local Field Applications Specialist for assistance during experiment design and data analysis. To be connected to a FAS, contact **technical support**.

Methods

Panel kit design

Antibodies were selected based on the best fit for the immuno-oncology application on tumor tissues and metals were assigned to maximize the signal quality.

Tissue

Mouse normal TMA was obtained from US Biomax (MO541e) and tumor TMA was obtained from Charles River (Freiburg TMA1.0) and stored according to the manufacturer's recommendation before use. Tumor TMA contained a variety of duplicate cores from multiple types of tumors. Tumor types presented in this application note were selected based on biological and clinical relevance.

Staining

Slide preparation and staining was conducted based on Standard BioTools™ Imaging Mass Cytometry Staining Protocol for FFPE Sections (PN 400322). Briefly, slides were baked at 60 °C for 2 hr followed by dewaxing in xylene and stepwise rehydration in descending grades of ethanol. Slides were washed in Maxpar Water and inserted into preheated Antigen Retrieval Solution for 30 min. Subsequently, slides were washed in 1X Maxpar PBS and blocked with 3% bovine serum albumin in 1X PBS for 45 min at room temperature (RT). Antibody cocktails were prepared at tested dilutions, added to slides, and incubated overnight at 4 °C. The following day, slides were washed and stained with Cell-ID Intercalator-Ir in 1X PBS at RT for 30 min for DNA labeling. The slides were washed, air-dried, and prepped for ablation.

Imaging

Imaging was performed using the Hyperion Imaging System with CyTOF® Software v7.0. Before ablation, instrument tuning was performed using a tuning slide. For normal and tumor tissue imaging, each region of interest (ROI) of either 1 mm^2 or 1.56 mm^2 was selected and ablated at 200 Hz with 1 µm resolution. Data from 3–4 ROIs from 2 independent experiments were collected. MCD files were exported and used for subsequent analysis.

Data analysis

MCD Viewer v1.0.560.6 was used to render multiplexed and single-channel 16-bit TIFF images. For qualitative verification of staining, images for each channel were rendered and verified to ensure absence of non-specific and background staining. For NSCLC, single-channel OME-TIFF files were exported for further analysis. Graphics were created using biorender.com.

Pixel classification and cell segmentation

The ICSK and Cell-ID Intercalator-Ir were used to label the cell membrane and nuclei of all cells present in the tumor TME, respectively. A custom MATLAB script was used to conduct preprocessing of images and pixel classification to define 3 regions of the ROI: cell membrane (ICSK), nuclei (Ir), and background (no signal). Probability masks were generated and imported into CellProfiler v4.2.1 for cell segmentation. A basic pipeline for cell segmentation was assembled, which included primary (nuclei) and secondary (cell membrane) object identification modules. Images containing individual cell masks were generated and extracted for SCA.

Single cell analysis

Single-channel OME-TIFF files and cell masks for NSCLC ROIs were loaded into histoCAT v1.76. t-SNE analysis and PhenoGraph clustering was performed. The B220 channel was excluded due to absence of B cells in the tissue. Masks representing specific clusters were plotted onto ROIs rendered with ICSK channels (Figure 7) and cell quantities for each cluster were extracted and documented. All clusters as well as individual heat maps of marker expression were plotted on the t-SNE graphs (Figure 8).

References

1. Ireson, C.R. et al. “The role of mouse tumour models in the discovery and development of anticancer drugs.” *British Journal of Cancer* 121 (2019): 101–108.
2. Cheon, D-J. and Orsulic, S. “Mouse models of cancer.” Annual Review of Pathology: *Mechanisms of Disease* 6 (2011): 95–119.
3. Giraldo, N.A. et al. “The clinical role of the TME in solid cancer.” *British Journal of Cancer* 120 (2019): 45–53.
4. Van Maldegem, F. et al. “Characterisation of tumour microenvironment remodelling following oncogene inhibition in preclinical studies with Imaging Mass Cytometry.” *Nature Communications* 12 (2021): 5906.
5. Jackson, H.W. et al. “The single-cell pathology landscape of breast cancer.” *Nature* 578 (2020): 615–620.
6. Ijsselsteijn, M.E. et al. « A 40-marker panel for high dimensional characterization of cancer immune microenvironments by Imaging Mass Cytometry.” *Frontiers in Immunology* 10 (2019): 2534.
7. Senbanjo, L.T. and Chellaiah, M.A. “CD44: A multifunctional cell surface adhesion receptor is a regulator of progression and metastasis of cancer cells.” *Frontiers in Cell & Developmental Biology* 5 (2017): 18.
8. Barak, V. et al. “Clinical utility of cytokeratins as tumor markers.” *Clinical Biochemistry* 37 (2004): 529–540.
9. Moll, R. et al. “The human keratins: biology and pathology.” *Histochemistry and Cell Biology* 129 (2008): 705–733.
10. Szczepanik, A. et al. “CD44+ cytokeratin-positive tumor cells in blood and bone marrow are associated with poor prognosis of patients with gastric cancer.” *Gastric Cancer* 22 (2019): 264–272.
11. Yan, Y. et al. “Concise review: emerging role of CD44 in cancer stem cells: a promising biomarker and therapeutic target.” *Stem Cells Translational Medicine* 4 (2015): 1,033–1,043.
12. Lopes, L.F. and Bacchi, C.E. “Cytokeratin expression in gastrointestinal stromal tumor: a clinicopathologic and immunohistochemical study of 687 cases.” *Applied Immunohistochemistry & Molecular Morphology* 20 (2012): 8–12.
13. Todaro, M. et al. “CD44v6 Is a marker of constitutive and reprogrammed cancer stem cells driving colon cancer metastasis.” *Cell Stem Cell* 14 (2014): 342–356.
14. Efthymiou, G. et al. “Shaping up the tumor microenvironment with cellular fibronectin.” *Frontiers in Oncology* 10 (2020): 641.
15. Greaves, M. and Maley C.C. “Clonal evolution in cancer.” *Nature* 481 (2012): 306–313.
16. Bedard, P.L. et al. “Tumor heterogeneity in the clinic.” *Nature* 501 (2013): 355–364.
17. Hanahan, D. and Weinberg, R.A. “Hallmarks of cancer: the next generation.” *Cell* 144 (2011): 646–674.
18. McKay, M.M. and Morrison, D.K. “Integrating signals from RTKs to ERK/MAPK.” *Oncogene* 26 (2007): 3,113–3,121.
19. Lavoie, H. et al. “ERK signalling: a master regulator of cell behaviour, life and fate.” *Nature Reviews Molecular Cell Biology* 21 (2020): 607–632.
20. Dhillon, A.S. et al. “MAP kinase signalling pathways in cancer.” *Oncogene* 26 (2007): 3,279–3,290.
21. Jang, M. et al. “Cancer cell metabolism: implications for therapeutic targets.” *Experimental & Molecular Medicine* 45 (2013): e45.
22. Zoncu, R. et al. “mTOR: from growth signal integration to cancer, diabetes and ageing.” *Nature Reviews Molecular Cell Biology* 12 (2011): 21–35.
23. Yang, C. et al. “Ki67 targeted strategies for cancer therapy.” *Clinical and Translational Oncology* 20 (2018): 570–575.
24. Pastushenko, L. and Blanpain, C. “EMT transition states during tumor progression and metastasis.” *Trends in Cell Biology* 29 (2019): 212–226.
25. Binnewies, M. et al. “Understanding the tumor immune microenvironment (TIME) for effective therapy.” *Nature Medicine* 24 (2018): 541–550.
26. Anjum, R. and Blenis, J. “The RSK family of kinases: emerging roles in cellular signaling.” *Nature Reviews Molecular Cell Biology* 9 (2008): 747–758.
27. Zeng, H. et al. “mTORC1 couples immune signals and metabolic programming to establish Treg-cell function.” *Nature* 499 (2013): 485–490.
28. Dave, J.M. and Bayless, K.J. “Vimentin as an integral regulator of cell adhesion and endothelial sprouting.” *Microcirculation* 21 (2014): 333–344.
29. Ludwig, J.A. and Weinstein, J.N. “Biomarkers in cancer staging, prognosis and treatment selection.” *Nature Reviews Cancer* 5 (2005): 845–856.

Required reagents

Source	Product Name	Cat. No.
Standard BioTools™	Maxpar® PBS	201058
	Cell-ID™ Intercalator-Ir	201192A
	Maxpar Water	201069
	IMC™ Cell Segmentation Kit	TIS-00001
Sigma-Aldrich®	M-xylene ReagentPlus®	185566-1L
Commercial Alcohols	Anhydrous ethyl alcohol	P006EAAN
Agilent®	Antigen Retrieval Solution pH 9 (10x)	S236798-2
Thermo Scientific™	Triton™ X-100	85111
Sigma-Aldrich®	10% Bovine Serum Albumin	A3059
Charles River	Mouse FFPE syngeneic tumor TMA	Freiburg TMA 1.0
US Biomax	Mouse FFPE normal TMA	MO541e

Products		Metal	Marker	Clone	Target/Cellular Process
Maxpar® OnDemand™ Mouse Immuno-Oncology IMC™ Panel Kit (Cat. No. 9100005)	Maxpar OnDemand Mouse Tissue Architecture IMC Panel Kit (Cat. No. 9100001)	^{141}Pr	Alpha-smooth muscle actin	1A4	Smooth muscle/stromal cells
		^{171}Yb	CD31	EPR17259	Vascular cell
		^{153}Eu	CD44	IM7	Tumor cell/immune cell
		^{151}Eu	CD45	D3F8Q	Immune cell
		^{173}Yb	Collagen 1	Goat polyclonal	Extracellular matrix/stromal cells
		^{152}Sm	Fibronectin	EPR19241-46	Extracellular matrix/stromal cells
		^{174}Yb	Pan-cytokeratin	AE-1/AE-3	Tumor cell
	Maxpar OnDemand Mouse Cancer Cell Process IMC Panel Kit (Cat. No. 9100002)	^{154}Sm	β-actin	2F1-1	Cytoskeletal microfilament
		^{169}Tm	β-catenin	5H10	Ca^{2+} dependent cell adhesion
		^{172}Yb	BRCA1	MS110	Tumor suppressor
		^{158}Gd	E-cadherin	24E10	Ca^{2+} dependent cell adhesion
		^{147}Sm	EpCAM	EPR20532-222	Ca^{2+} independent cell adhesion
		^{150}Nd	Ki-67	B56	Proliferating cells
		^{164}Dy	pERK1/2	D13.14.4E	RAS signaling pathway activation
		^{175}Lu	pS6[S235/S236]	N7-548	mTOR pathway activation
		^{144}Nd	pTyrosine	P-Tyr-100	Receptor tyrosine kinase activation
		^{149}Sm	Vimentin	D21H3	Mesenchymal cells
	Maxpar OnDemand Mouse Immune Phenotyping IMC Panel Kit (Cat. No. 9100003)	^{176}Yb	B220	RA36B2	B cells
		^{163}Dy	CD11b	EPR1344	MDSCs, M1 macrophages
		^{170}Er	CD3	Polyclonal (C-terminal)	Pan T cell
		^{159}Tb	CD4	BLR16J	Helper T cells
		^{162}Dy	CD8	EPR21769	Killer T cells
		^{156}Gd	F4/80	D2S9R	Macrophages
		^{166}Er	Ly-6G	1A8	MDSCs, neutrophils
		^{161}Dy	MHC class II	M5/114.15.2	Antigen presenting cells
	Maxpar OnDemand Mouse Immune Activation IMC Panel Kit (Cat. No. 9100004)	^{165}Ho	FoxP3	FJK-16s	Regulatory T cells
		^{155}Gd	Granzyme B	EPR22645-206	Cytotoxic immune cell activation
		^{160}Gd	iNOS	SP126	Activated macrophages
Cell-ID™ Intercalator-Ir* (Cat. No. 201192A)		^{191}Ir	DNA1		DNA
		^{193}Ir	DNA2		DNA
IMC Cell Segmentation Kit*† (Cat. No. TIS-0001)		^{195}Pt	ICSK1		Cell membrane
		^{196}Pt	ICSK2		Cell membrane
		^{198}Pt	ICSK3		Cell membrane

Table 1. Mouse Immuno-Oncology Panel (Cat. No. 9100005) for mouse FFPE tumor tissue application

* Cell-ID™ Intercalator-Ir and the IMC Cell Segmentation Kit are not part of the Mouse Immuno-Oncology Panel.

† The IMC Cell Segmentation Kit is part of the Innovative Solutions menu of custom-made reagents and workflows developed and tested by Standard BioTools scientists to give faster access to new cutting-edge solutions for high-multiplex SCA. Innovative Solutions are not part of the Maxpar or Maxpar OnDemand catalog.

Tissue Type	Cancer Model	Cell Line*	Genetic Background†
Lung	Normal		C57Bl/6
Lung	NSCLC	KP4	C57Bl/6
Colon	Colon adenocarcinoma	MC38	C57Bl/6
2° lymphoid organ	B cell lymphoma	A20	BALB/c
Brain	Glioblastoma	GL261	C57Bl/6

Table 2. FFPE mouse tissue used for testing Mouse Immuno-Oncology Panel

* Original tumor cell line used for tumor modeling
† Syngeneic background of inoculated mice

Learn more at **fluidigm.com/hyperion-plus**

Or contact: **fluidigm.com/tech-support**

Scan for more information.

CORPORATE HEADQUARTERS
2 Tower Place, Suite 2000
South San Francisco, CA 94080 USA
Toll-free: 866 359 4354 in the US and Canada
Fax: 650 871 7152
standardbiotools.com

SALES
North America | +1 650 266 6170 | info-us@standardbio.com
Europe/EMEA | +33 1 60 92 42 40 | info-europe@standardbio.com
Latin America | +1 650 266 6170 | info-latinamerica@standardbio.com
Japan | +81 3 3662 2150 | info-japan@standardbio.com
China (excluding Hong Kong/Macau) | +86 21 3255 8368 | info-china@standardbio.com
All other Asia-Pacific countries | +1 650 266 6170 | info-asia@standardbio.com

For Research Use Only. Not for use in diagnostic procedures.

FLDM-01012 Rev 01