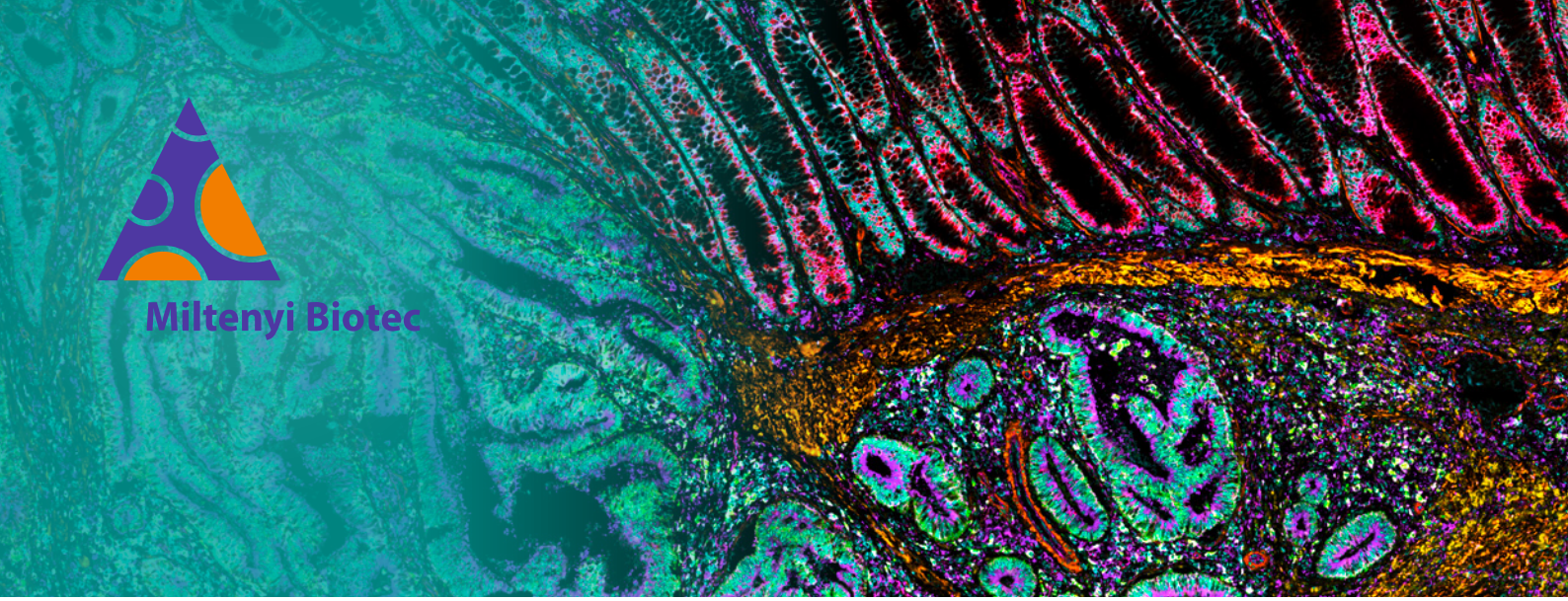

Miltenyi Biotec

REAscreen™ Immuno-oncology Panel

Predefined, plug-and-play antibody panel provides insight into spatial biology of the tumor microenvironment of solid tumors

Background

The tumor microenvironment (TME) is composed of a complex mixture of tumor, stromal, and immune cells, and non-cellular structural components. Its composition as well as the spatial relationship and localization of its components can dictate progression and indicate prognosis of solid tumors. Deep profiling of the TME will therefore improve the understanding of the complex interplay of cells within the tumor and aid the establishment of new treatment avenues.
However, a major challenge is the identification of relevant markers and development of assays that give insight into the TME and allow for the phenotyping of the different cell types that could be present. Along with immune phenotypes, additional structural and cell state markers are required to enable orientation within the tissue. Recent advancements in the field of spatial biology have led to various approaches to tackle these challenges – albeit with varying degrees of ease-of-use, standardization, and reproducibility.

Here we used Miltenyi Biotec's end-to-end solution for spatial biology – the MACSima™ Platform – to establish a comprehensive antibody panel for standardized and reproducible tumor analysis to minimize the time-to-data and thus the time required to get publication-ready results. With the novel predefined and ready-to-use 61-plex REAscreen Immuno-oncology Panel, users can identify immune cells, tumor stroma (including blood and lymphatic vessels), and malignant epithelial cell populations, as well as their proliferative or apoptotic state in a broad range of human FFPE solid tumor samples (fig. 1, table 1).

The panel enables the analysis of at least 11 potential immune cell subsets within the TME (table 2), as well as several activation/checkpoint statuses of those populations.
These cell subsets can be counted and the expression levels of their functional markers quantified with the integrated MACS® iQ View Software. In this application note, we present results from four exemplary tumor types: colorectal adenocarcinoma (CRC), pancreatic ductal adenocarcinoma (PDAC), head and neck squamous cell carcinoma (HNSCC), and melanoma.

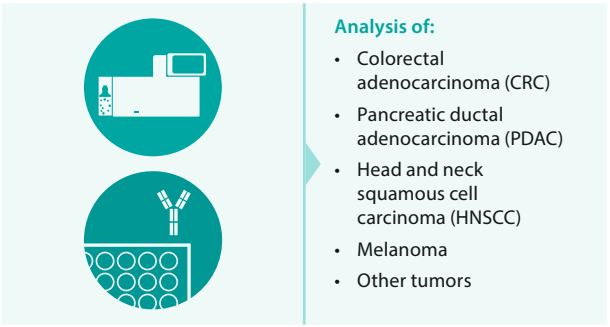

Figure 1: Versatility of the REAscreen Immuno-oncology Panel, human, FFPE. This predefined 61-plex panel for use within the MACSima Platform was tested on a variety of listed tumor types and can be applied to a broad range of other solid FFPE tumor samples.

Category	Antigen
Proliferation	Ki-67
	PCNA
Cell death	Bcl-2
	Cleaved PARP1
Structure	CD44
	β-Actin
Stroma	Actin (Smooth Muscle)
	Calponin
	CD31
	Collagen I
	Podoplanin
Immune cell profiling	CD45
Lymphoid cells	CD138
	CD20
	CD3
	CD38
	CD4
	CD56 (NCAM1)
	CD57
	CD79a
	CD8a
	FoxP3
Myeloid cells	CD11b
	CD11c
	CD14
	CD15
	CD16
	CD66b
	CD68
Immune activation/ function	CD134 (OX40)
	CD223
	CD274 (PDL1)
	CD279 (PD1)
	HLA-DR
Advanced profiling	CD107a (LAMP-1)
	CD107b
	CD163
	CD1c (BDCA-1)
	CD204
	CD209 (DC-SIGN)
	CD45RA
	CD45RB
	CD45RO
	HLA-ABC
	Mast Cell Tryptase
Tumor characterization	CD324 (E-Cadherin)
	CD326 (EpCAM)
	CD66 (CEA)
	Cytokeratin
	Cytokeratin 14
	Cytokeratin 19
	Cytokeratin 20
	Cytokeratin 5
	Cytokeratin 7
	Cytokeratin 8
	Cytokeratin HMW
	MART-1
	Melanocyte PMEL
	p53
	Vimentin
	β-Catenin

Table 1: Composition of the REAscreen Immuno-Oncology Panel, human, FFPE. The antibody panel targets diverse cellular and non-cellular structures. These 61 cellular markers are associated with ten functional categories.

Cell type / structure	Markers
Tregs	$CD4^+$ $FoxP3^+$
Mast cells	Mast Cell $Tryptase^+$
Neutrophils	$CD11b^-$ $CD66b^+$
M1 macrophages	$CD14^+$ $CD68^+$ $CD163^-$
M2 macrophages	$CD14^+$ $CD68^+$ $CD163^+$
Monocytes	$CD11b^+$
NK cells	$CD56^+$
Plasma cells	$CD79a^+$
B cells	$CD20^+$
Helper T cells	$CD4^+$
Cytotoxic T cells	$CD8^+$
Healthy epithelium	Manual image gate from pathologist annotation & HLA DR^- CD326 $(EpCAM)^+$
Immune cells	HLA DR^+ CD326 $(EpCAM)^-$
Stroma	HLA DR^- CD326 $(EpCAM)^-$
Tumor	HLA DR^+ CD326 $(EpCAM)^+$

Table 2: Markers used for the characterization of different cell types on the CRC sample.

LEARN MORE

For more information about single antibodies click on the respective antigen in the table. For the complete portfolio of Miltenyi Biotec antibodies pretested for MICS technology, visit our website.

▸ **miltenyibiotec.com/mics-antibodies**

Materials and methods

Antibody panel format

The REAscreen Immuno-oncology Panel is designed to easily integrate with Miltenyi Biotec's MACSima Imaging Cyclic Staining (MICS) workflow (fig. 2). This antibody panel contains 61 antibody conjugates pretested for MICS, securely conserved in a convenient, dry format. The dried antibodies are provided in a true plug-and-play plate setup and are dissolved in a fully automated fashion within the MACSima System during experiment execution. Therefore, no manual pipetting is needed. If required, the panel can be supplemented in the same experiment with any number of compatible antibody conjugates from Miltenyi Biotec or third parties.

Tissue samples and selection of ROI

In brief, FFPE tissue samples of various human cancer types, i.e., CRC, PDAC, HNSCC, and melanoma, were deparaffinized and antigen retrieval was performed using a heat-induced epitope retrieval protocol with TRIS/EDTA/citrate buffer at pH 9. Subsequently, tissue was stained with DAPI to identify nuclei. For each tissue, H&E staining was performed on an adjacent serial section for ROI selection by a pathologist. The prepared samples, the REAscreen Immuno-oncology Panel Plate including 61 markers, and supporting reagents were placed in the MACSima System. ROIs with the size of 2.6 mm × 1.7 mm were selected in tumor regions showing prominent immune cell infiltrates as determined by H&E staining. Tonsil tissue was used as internal control for each MICS experiment. After ROI selection, the MICS process was started.

MICS process

The cyclic process comprises three main steps: staining with fluorochrome-conjugated antibodies (1), image acquisition of the stained sample (2), and erasure of the fluorescent signal (3). All three steps are conducted fully automatically by the MACSima System.

Spatial mapping of immune cell populations

Upon completion of the experiment, image processing, segmentation, and single-cell analysis was performed using the MACS iQ View Software. Data of all tissue samples were analyzed for individual marker images and merged images of the indicated markers were generated (figs. 3 and 4).

For spatial mapping of immune cell populations, the CRC sample was analyzed for specific cell phenotypes based on immune and tumor markers. After visual assessment of the markers and ROI annotation by a pathologist to identify tissue regions, the samples were segmented using the advanced morphology for tissue algorithm of MACS iQ View for nuclei detection, followed by the constrained donut cell segmentation method using the majority of cell and cytoplasmic markers from the panel. In addition, a manual gate identifying healthy tissue was drawn based on the pathologist's annotation. Rough tissue phenotyping was performed using two markers, HLA-DR and CD326 (EpCAM), which allowed for a general quadrant gating to approximately distinguish the following categories: stroma (extracellular matrix, blood and lymphatic vessels, and fibroblasts), healthy epithelial tissue, tumor and immune cells (taking into account the annotation of the pathologist).

After defining the tumor cells, a distance map was created, allowing the gating of cells based on their distance to the tumor. Cells were divided into three groups: i) intratumoral, i.e., cells immediately adjacent to tumor cells (within 3 µm distance to center of a tumor cell); ii) peritumoral, i.e., cells within a distance of 3–50 µm to tumor cells; and iii) distant, i.e., cells more than 50 µm away from the tumor. Subsequently, immune cells were phenotyped based on the markers shown in table 2. Exclusion gates ensured cell allocation to single subtypes. This allowed for identification and quantification of 11 distinct immune subsets: regulatory T cells (Tregs), mast cells, neutrophils, M1-like macrophages, M2-like macrophages, monocytes, NK cells, plasma cells, B cells, helper T cells, and cytotoxic T cells. Combining the identified cell subsets with the distance map allowed us to extract the frequency of each cell population within each ROI/tumor region (intratumoral, peritumoral, and distant).

Results and discussion

Imaging of various solid tumors with the 61-plex REAscreen Immuno-oncology Panel

Different solid tumor types show varying degrees of immune infiltration and composition of infiltrates. Colorectal cancer (CRC) has a high prevalence in industrialized countries and therefore is a target for immune profiling and immunotherapies. Besides CRC, researchers also focus on the TME profile and targeted therapies for PDAC, HNSCC, and melanoma, for example.

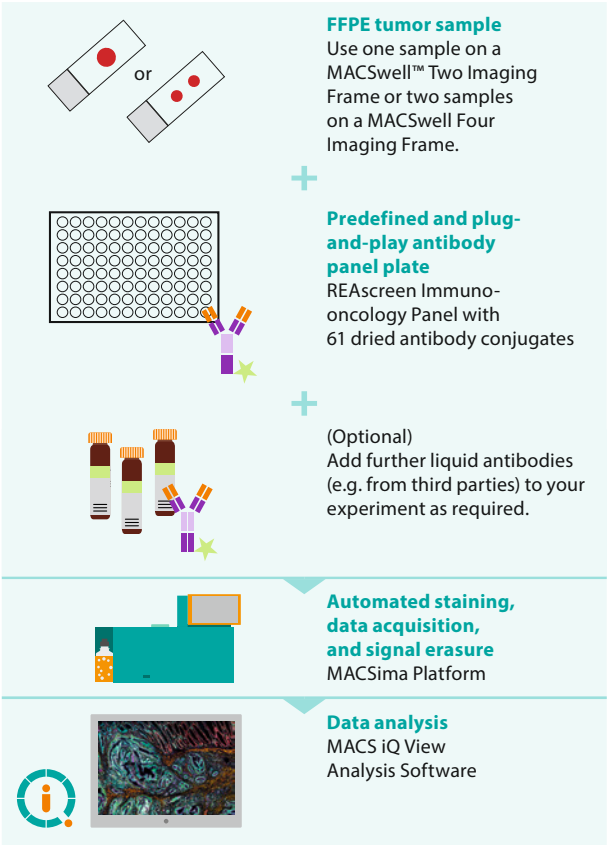

Figure 2: MICS workflow. Up to two tissue samples can be easily combined with the plug-and-play REAscreen Immuno-oncology Plate and run simultaneously in the MACSima System. Additional antibodies can be used on the same sample if required. Iterative cycles of fluorescent staining, imaging, and signal erasure are performed fully automatically by the MACSima System. The MACS iQ View Analysis Software enables straightforward analysis of intricate data.

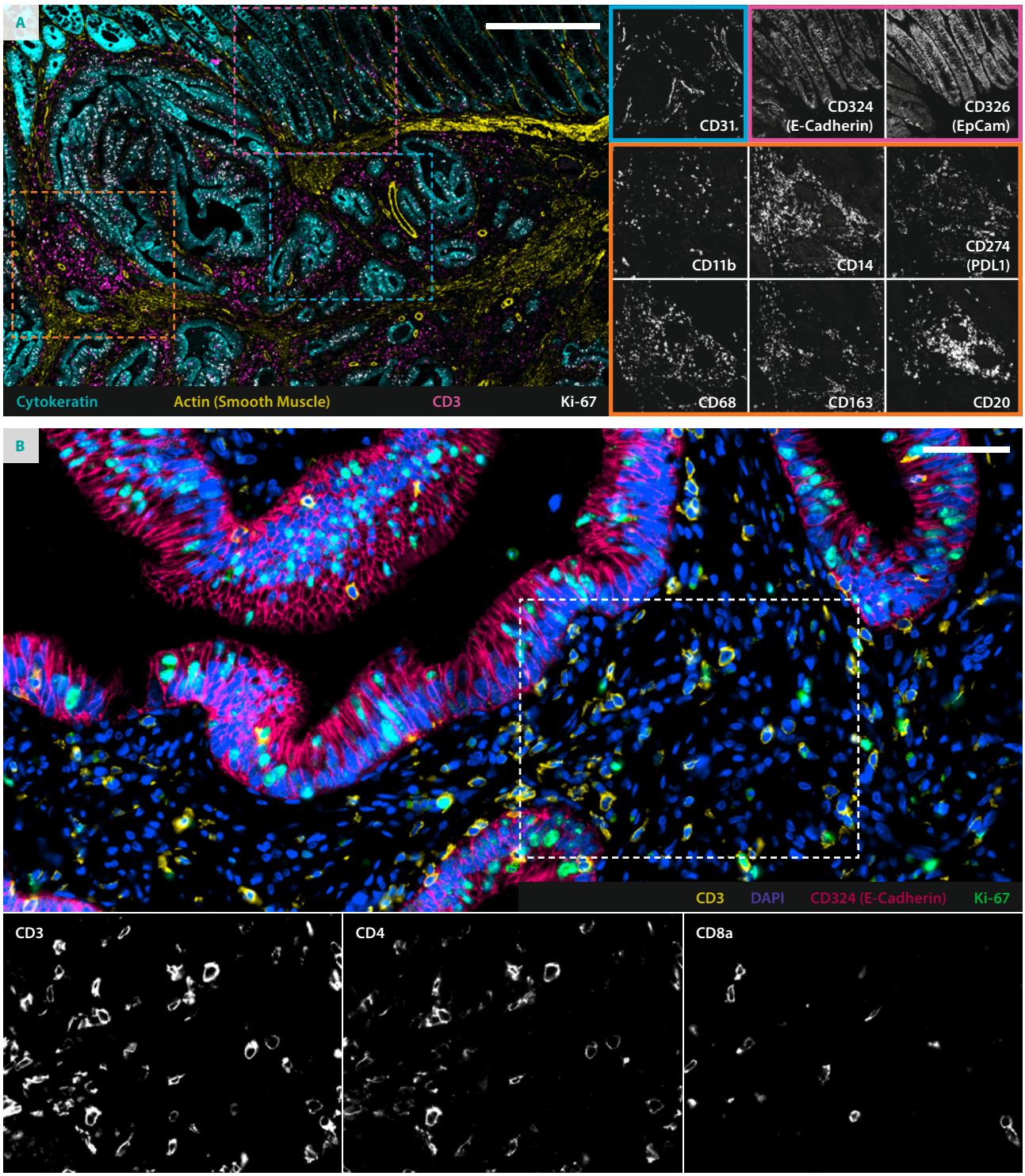

Figure 3: Colorectal adenocarcinoma section stained with 61-plex REAscreen Immuno-oncology Panel. (A) Overview of an ROI with a mixture of tumor, immune, and stromal components. Scale bar: 500 µm. Selected markers are displayed to the right; the position of the small images in the overview is indicated by the color-coded insets. (B) Overview image of a high-resolution, magnified area of a colorectal cancer sample. CD3, CD4, and CD8a are from the same location as indicated in the upper picture. Scale bar: 50 µm.

The REAscreen Immuno-oncology Panel covers 61 markers suitable to characterize the TME, which makes it an effective tool to investigate tumor-infiltrating lymphocytes in the context of a great variety of markers and their spatial relationship. In this application note, we used the REAscreen Immuno-oncology Panel to demonstrate its broad applicability across different tumor types and its efficiency as a standardized tool to examine all these sample types.

To demonstrate its power for TME analysis, MICS using the REAscreen Immuno-oncology Panel was performed on human colorectal adenocarcinoma tissue. The selected ROI shows significant immune infiltration (fig. 3A). Within the ROI, the tumor region/parenchyma is identified by pan-cytokeratin staining along with additional epithelial markers like CD324 (E-cadherin) and CD326 (EpCAM) (fig. 3A, pink inset). The immune infiltrate is composed of a mixture of lymphoid cells (identified by CD3 for T cells and CD20 for B cells) and myeloid cells (identified by CD11b, CD14, CD68, and CD163; checkpoint molecule CD274 (PDL1) for comparison; fig. 3A, orange inset). The image in figure 3B illustrates that the (co-)expression of lymphoid markers CD3, CD4, and CD8a can be determined at single-cell resolution. Table 2 shows the markers used to identify cell types or structures.

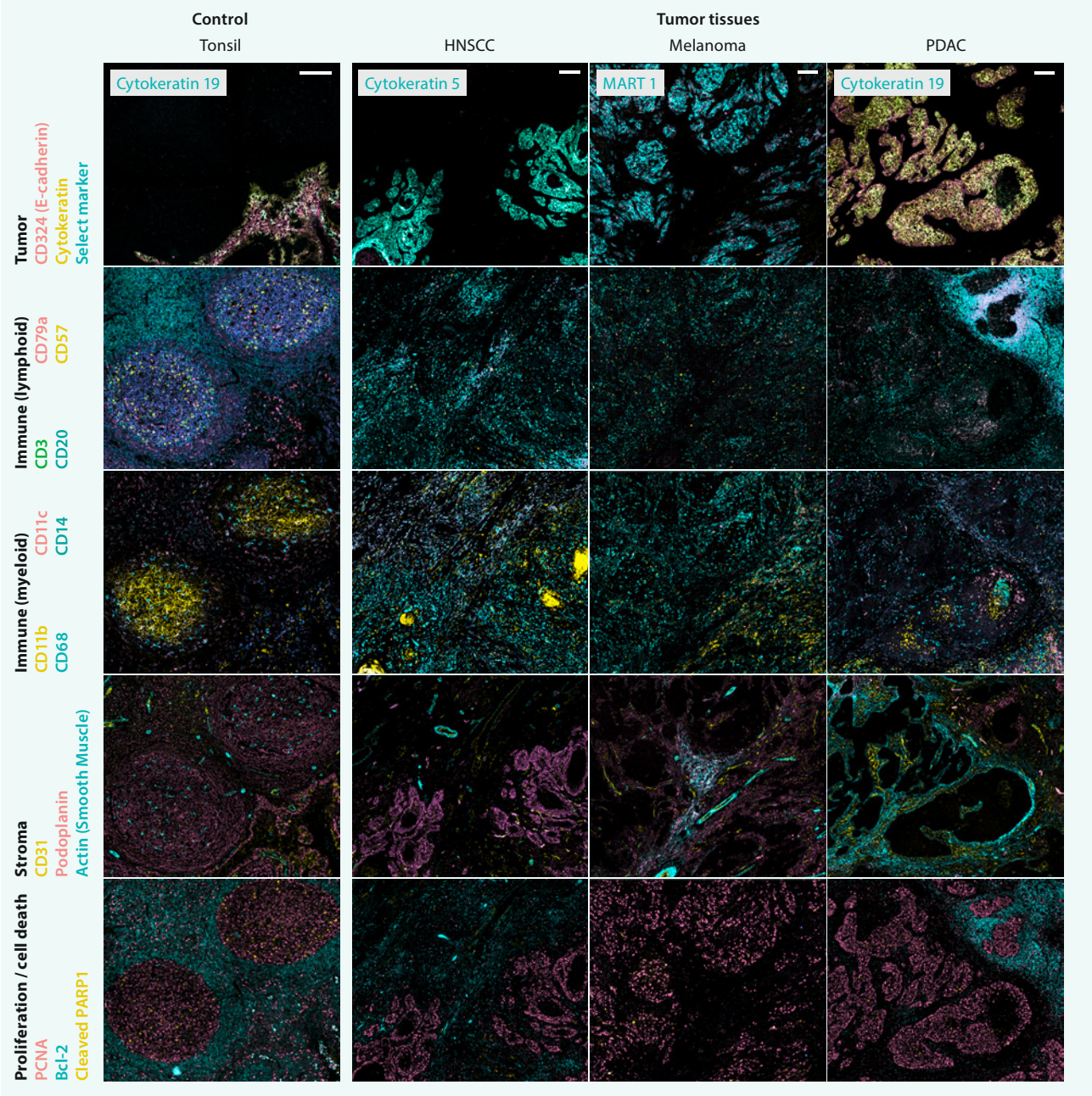

Figure 4: REAscreen Immuno-oncology Panel applied to multiple cancer types. Each panel row displays different marker groups representing the categories tumor, immune, stroma, and cell state. For the tumor category, different select markers (indicated in the respective images) were used dependent on the given tumor type. Tonsil was used as control sample. Scale bar: 150 µm.

In addition to CRC, samples of PDAC, HNSSC, and melanoma were stained. Figure 4 shows a selection of markers for each of these samples and tonsil tissue, which was used as internal control during each MICS run. The data show that the MACSima System in combination with the REAscreen Immuno-oncology Panel enables labeling and imaging of different tumor types and the detection of distinct distribution of several immune cell populations within the TME. However, visualization of the stained markers is only the first step to extract meaningful data that can help to overcome the challenges faced during the development of immunotherapies for solid tumors.

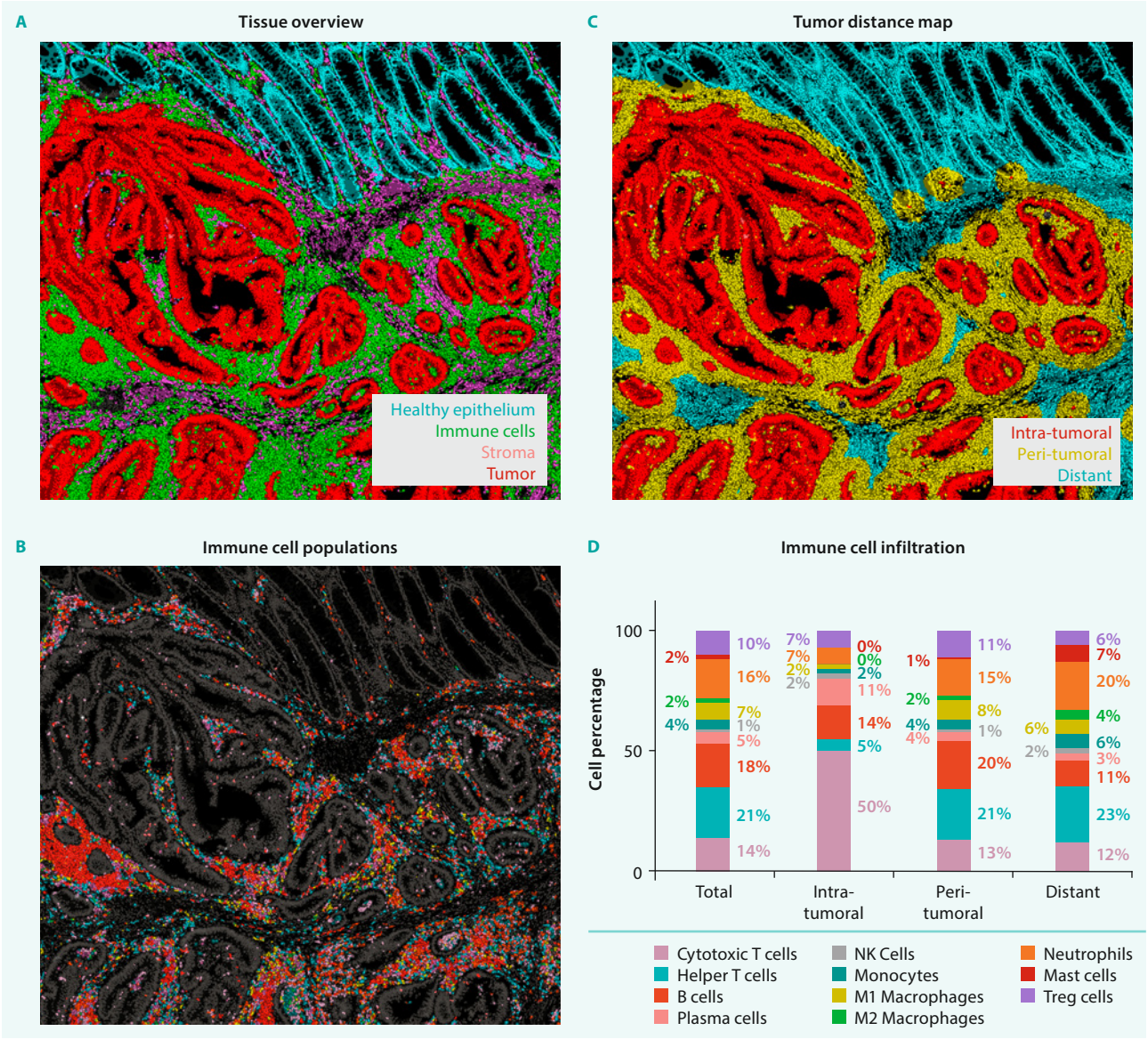

Figure 5: Spatial mapping of immune cell populations relative to tumor areas. (A) Sample area was categorized into healthy epithelium, immune cells, stroma, and tumor for overview. (B) Subsequently, distinct immune cell populations were depicted. See table 2 for the markers used for categorization. The color code is shown to the right. (C) Next, a tumor distance map was generated in which the intratumoral region was defined by the tumor cells and a surrounding distance of 3 µm, the peritumoral region included the adjacent area within a distance of 50 µm. Cells with a distance from tumor cells larger than 50 µm were considered distant. (D) The percentage of different cell types in the various regions are shown in the bar charts.

Spatial analysis of multiple immune cell populations within the TME of CRC

While the REAscreen Immuno-oncology Panel provides markers for many different functional categories of the TME, the task of single-cell identification and categorization based on the presence or absence of a fluorescence signal is another crucial step towards meaningful data analysis. The MACS iQ View Analysis Software enables users to assign cell types to individual cells based on common markers. An example analysis was performed on the CRC sample, combining marker-based and manual gating to differentiate between stroma (including extracellular matrix, blood and lymphatic vessels, and fibroblasts), healthy epithelial, tumor, and immune cells (fig. 5A).

Subsequent classification of specific cell subtypes based on marker expression (table 2) allowed overall quantification of key immune cell types across the ROI (fig. 5B and D, total ROI).

Spatial distribution of immune cell subtypes in relation to the tumor cells was analyzed and quantified by generating a distance map defining intratumoral and peritumoral regions and a region distant to the tumor (fig. 5C and D). Interestingly, while overall only few immune cells infiltrated the tumor parenchyma, half of the immune cells present within the intratumoral region were CD8⁺ cytotoxic T cells hinting at the functional capacity of these T cells to infiltrate the tumor tissue.

These results represent only a first example of a possible analysis workflow. Comprehensive phenotyping in combination with the spatial information will open new perspectives during analysis. The vast amount of conclusive results from a single dataset allows users to answer scientific questions that weren't even on their mind at the beginning of the experiment. Based on the individual research question, a plethora of downstream analyses can be conducted to further assess expression profiles and spatial correlation.

Conclusions

- The data shown in this application note demonstrate how standardized in-depth phenotyping of sample cohorts is unlocked by combining the predefined REAscreen Immuno-oncology Panel with sophisticated data analysis using the MACS iQ View Software.
- The great variety of immuno-oncology–related antibodies contained in the panel saves panel development time and allows analysis of a wide range of tumor types. However, if required, the panel can be expanded by adding desired third-party antibodies easily.
- Thus, the ready-to-use, plug-and-play REAscreen Immuno-oncology Antibody Panel will enable faster discovery and further development of predictive and prognostic biomarkers critical to patient stratification, clinical management, and response to immunotherapy.

Ordering information

Product	Order no.
REAscreen™ Immuno-oncology Kit, human, FFPE, version 01	130-132-525

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