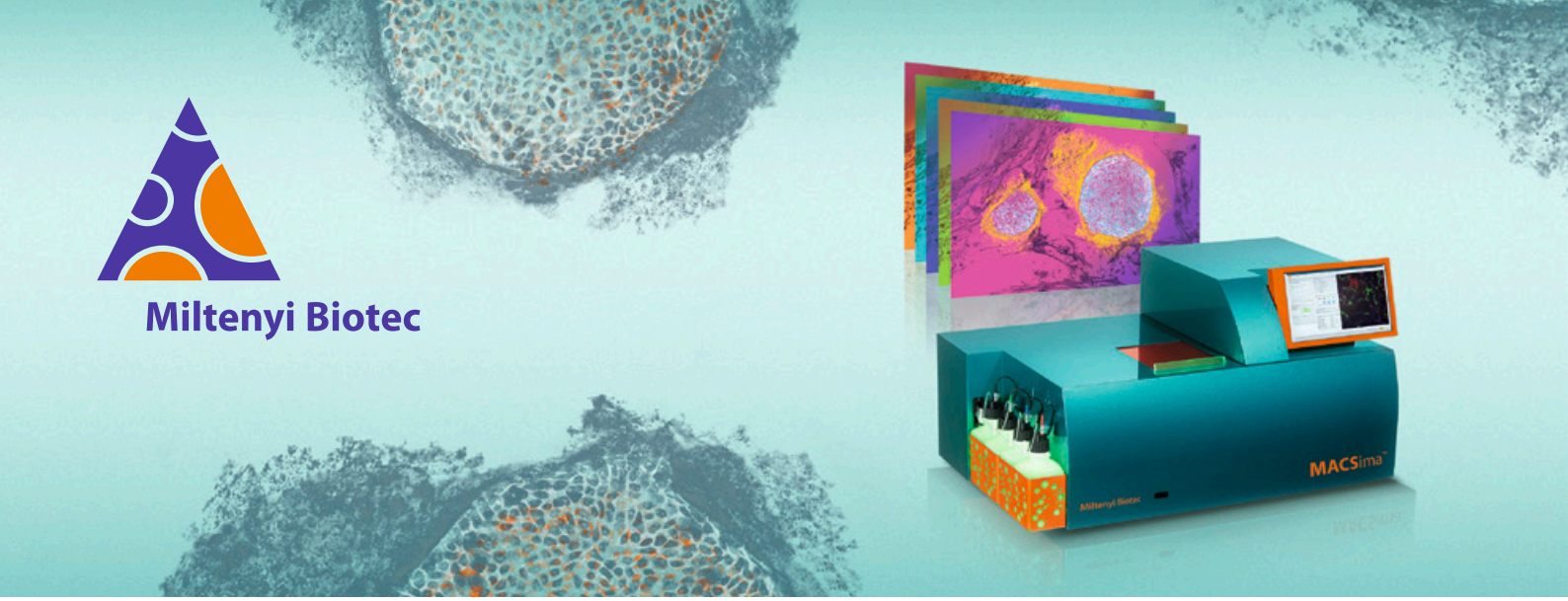


Spatial analysis of the tumor microenvironment in lung carcinoma using the MACSima™ Platform

Background

Tumors consist of a heterogeneous group of cells, including cancer cells, resident and infiltrating immune cells, and other tissue-resident cells such as fibroblasts, as well as secreted factors and extracellular matrix proteins. All these components together constitute the tumor microenvironment (TME). Continuous interaction of cancer cells with their environment has a tremendous effect on tumor progression. Processes and interactions within the TME can have a high impact on therapeutic responses and resistance[1]. Therefore, the development of cancer therapies that target certain components of the TME is currently of high interest, and comprehensive understanding of the spatial architecture of the TME is necessary. TME characterization traditionally relied on flow cytometry and immunohistochemical methods. However, these methods are restricted to a limited number of markers without providing contextual information of the tissue[2,3].

Miltenyi Biotec's MACSima Imaging Cycling Staining (MICS) technology (fig. 1) was developed to circumvent such limitations. MICS technology is based on an iterative cyclic staining process, which is composed of three steps that are conducted by the MACSima System in a fully automated manner. First, the sample is stained with multiple fluorochrome-conjugated antibodies produced and pre-tested by Miltenyi Biotec. This is followed by acquisition and subsequent processing of an image with a widefield microscope. To complete the cycle, the fluorescence signal is erased. This three-step process is repeated automatically for as many times as needed, allowing a single sample to be safely stained with hundreds of markers for spatial analysis. With the MACSima Platform various types of fixed samples, ranging from tissue sections to adherent cells, can be analyzed.

Methods

Sample preparation

FFPE samples of human lung adenocarcinoma and human squamous cell carcinoma were sectioned to a thickness of 3 µm using a microtome and carefully placed on a SuperFrost Plus™ microscope slide (Thermo Scientific). On the day of the experiment, slides were processed for (a) deparaffinization using xylol and (b) antigen retrieval using a heat-induced epitope retrieval protocol with TEC Buffer at pH 9. Subsequently, slides were mounted in a MACSwell™ Four Imaging Frame, and samples were stained with DAPI. Slides were then placed in the MACSima System to start the MICS run.

MACSima workflow

The antibody panel was defined by selecting antibodies with the specificities of interest and in accordance with fixation type (FFPE) and species (human). Antibody working solutions were prepared in MACSwell Deepwell Plates and sealed with MACSwell Sealing Foil. Samples and antibody plates were placed in the instrument and two regions of interest (ROIs) with 16 and 18 fields of view (FOV) were selected. The experiment was started and the MACSima System performed the entire process in an automated manner. Once a cycle was completed, the corresponding images were directly processed and saved in the common TIF format, which allowed on-the-fly data analysis while the experiment was still running.

Image analysis

Raw data from the ROI of the lung adenocarcinoma were processed for registration and background correction. The pre-processed dataset was then opened in MACS® iQ View Analysis Software, and boundaries of cells and nuclei were defined using the segmentation tool. After segmentation, several gating strategies were applied based on known markers such as, CD3 and CD4 for T helper cells. Using the workflow editor, it was ensured that cells would not be part of more than one identity group.

Also within the MACS iQ View Analysis Software, distance maps of the gated populations were created using the option "Calculate cell distance features" and plotted in a heatmap. Dimension reduction analysis was performed using the t-SNE approach on all expression features of all cells. The previously identified cell groups were then analyzed respect to their distribution in the t-SNE plot.

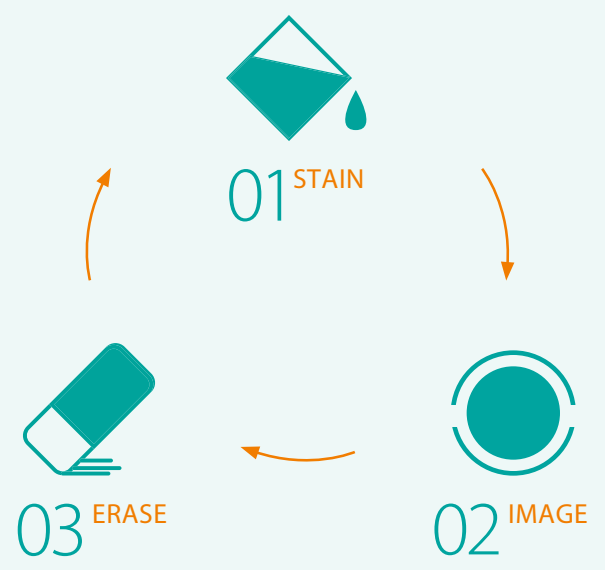


Figure 1: Principle of MICS technology. The MACSima System performs all three steps of MICS technology in a fully automated manner: (a) The sample is stained, (b) imaged, and (c) the signal is erased. After a cycle is completed, the instrument automatically continues with the next cycle, enabling the analysis of hundreds of markers on a single sample.

Results

To identify tumor-associated antigens for adenocarcinoma and squamous cell carcinoma of the lung, we investigated the expression of 155 markers on single tissue sections using the MACSima Platform. As an example, we present the data of the adenocarcinoma sample, where we identified 125 markers in the TME (fig. 2).

In detail, we detected the expression of markers that are specific for immune cell populations such as macrophages, granulocytes, T cells, B cells, plasma cells, and NK cells. Moreover, cell proliferation (Ki-67) and cell death regulation markers (Bcl-2) were also detected (fig. 3).

Using the MACS iQ View Analysis Software we gated different areas based on the expression of the different cellular markers that we have identified in Figure 3. Representative data from the t-SNE approach show a general overview of the cellular organization as well as the distribution of $CD4^+$, $CD8^+$, and $CD68^+$ cells in the TME of the adenocarcinoma sample (fig. 4B). Further analyses based on relative distance maps illustrate the distance between different immune cell populations and between immune cells and tumor cells. Among all cell populations identified, $CD4^+$, $CD8^+$, and $CD68^+$ cells were located the closest to tumor cells (fig. 4C).

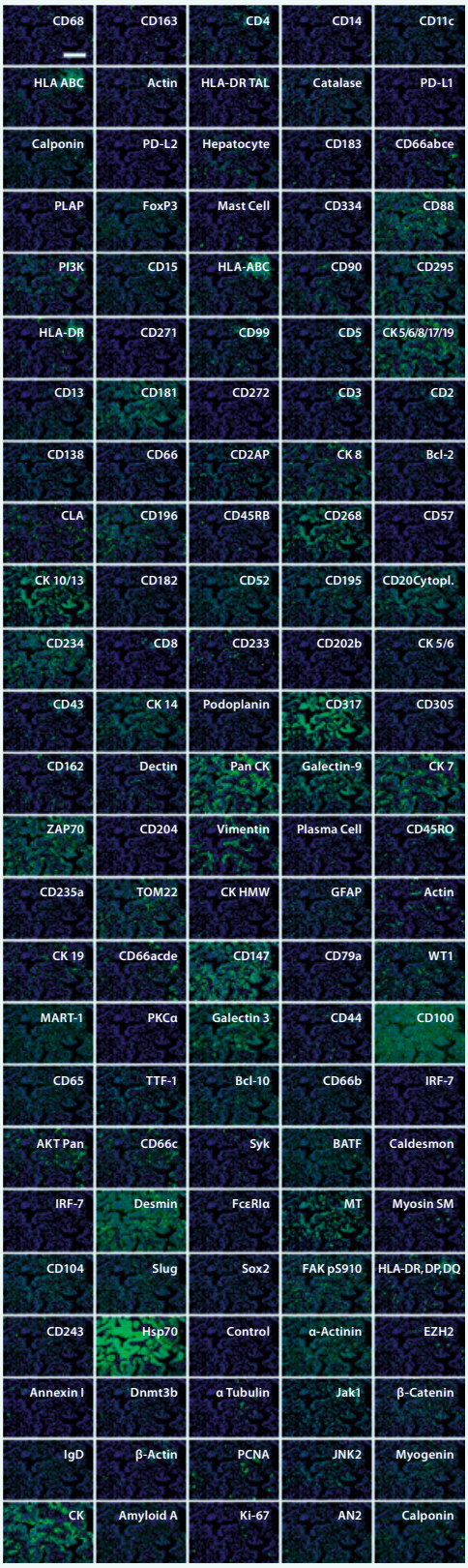


Figure 2: Screening of multiple markers in the TME of a single lung adenocarcinoma sample. Single-stain images of 125 markers (green), counterstained with the nuclear marker DAPI (blue), captured with the MACSima System are shown. Hepatocyte: Hepatocyte Antibody; Mast Cell: Mast Cell Tryptase Antibody; CK: Cytokeratin; CD20 Cytopl.: CD20 Cytoplasmic Antibody; Plasma Cell: Plasma Cell Antibody; IRF-7: IRF-7 pS477/pS479; MT: Metallothionein; Myosin SM: Myosin Smooth Muscle. Scale bar: 100 µm.

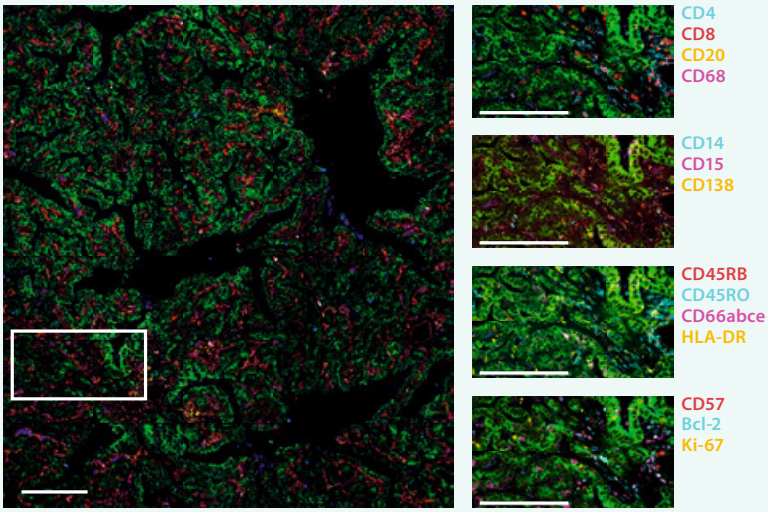


Figure 3: Identification of different cell populations in the TME of a lung adenocarcinoma sample, including CD4⁺ T helper cells, CD8⁺ cytotoxic T cells, CD20⁺ B cells, CD14⁺ or CD68⁺ monocytes/macrophages, CD15⁺ granulocytes, CD138⁺ plasma cells, CD45RB⁺ or CD45RO⁺ leukocyte subsets, CD66abce⁺ neutrophils, and CD57⁺ NK cells. Bcl-2 and Ki-67 represent cell death regulation and cell proliferation markers, respectively. Images to the right show a magnified view of the section marked in the large image. Scale bars: 200 µm.

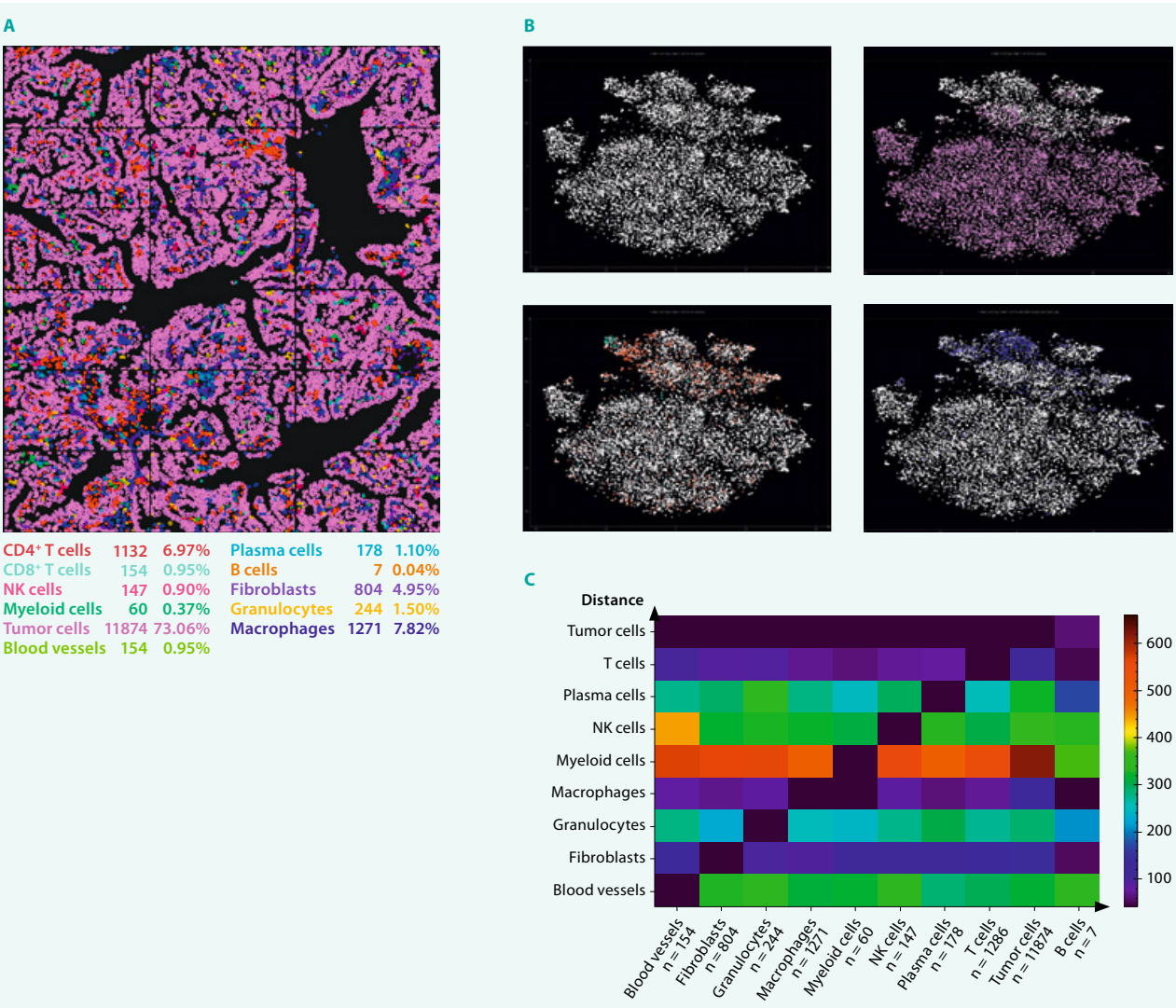


Figure 4: Spatial analysis of different cell populations in the TME of an adenocarcinoma sample. (A) Gating of markers specific for indicated cell populations. (B) t-SNE data for general cell distribution (white), distribution of tumor cells (violet), CD68⁺ cells (blue), CD4⁺ cells (red), and CD8⁺ cells (green), respectively. (C) Heatmap showing the distance between different immune cell populations and between immune cells and tumor cells. Blue: short distance; red: long distance.

This application note shows only a few examples out of a large range of data analyses that can be performed with images generated by the MACSima System. Please note that the images shown have been compressed and therefore do not reflect the original resolution obtained by the instrument.

Conclusion

- The MACSima System enables fully automated, spatial analysis of over 100 markers in the TME on a single sample of lung carcinoma tissue.
- Analysis of the data from lung adenocarcinoma using MACS iQ View Analysis Software allowed us to draw conclusions about the spatial distribution of different cell populations and their distance from tumor cells in the TME.
- Samples processed on the MACSima System can be further used for other downstream applications.

References

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