Mapping the tumor microenvironment with sequential immunofluorescence, an automated image analysis pipeline, and spatial metrics

David Mason[2], Joanna Kowal[1], James Mansfield[2], Pedro Machado Almeida[1], Regan Baird[2], Benjamin Pelz[1], Fabian Schneider[2], Diego G. Dupouy[1]
1) Lunaphore Technologies S.A., Tolochenaz, Switzerland; 2) Visiopharm A/S, Hørsholm, Denmark

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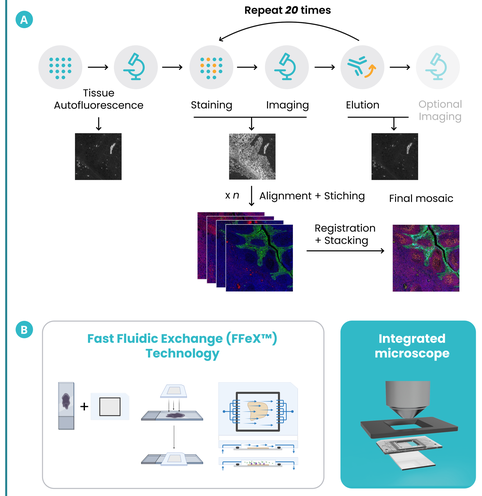
Introduction

Spatial biology enables the interrogation of tissue composition at a single cell level with the preservation of spatial context, which opens new avenues for tumor microenvironment (TME) studies [1]. Biomarkers' composition of the tissue can be interrogated with hyperplexed immunofluorescence, wherein an imaging detection is performed for each marker on the same slide. The COMET™ platform performs sequential immunofluorescence (seqIF™) and enables full automation of such workflow, where: up to 40 biomarkers can be detected with full automation from staining to data acquisition. The resulting hyperplex images are rich sources of data about the specimens. To extract information from such a dataset, Phenoplex™ (Oncotopix® Discovery, Visiopharm) has a dedicated workflow for image analysis that delivers single-cell phenotypic information and biodistribution, providing access to the spatial composition of the TME.

Methods

An FFPE lung tissue microarray underwent sequential cycles of staining and imaging with COMET™ platform seqIF™ assay. Iterative cycles of staining, imaging and antibody elution allowed the detection of 20 antigens spanning across epithelial tumor and immune markers (panCK, E-Cad, aSMA, CD31, CD3, CD4, CD8, FoxP3, CD20, HLA-DR, Ki67, Vimentin, CD16, CD68, CD11b, CD163, CD14, CD11c, PD1, PD-L1). The resulting image contains 23 channels: nuclear detection with DAPI, 2 channels of tissue autofluorescence (AF), and 20 marker channels. All layers were aligned and stitched into a single ome.tiff automatically by the COMET™ control software. Subsequent AF subtraction was performed in the Viewer by Lunaphore. The AF-subtracted image was analyzed using Oncotopix Discovery. The analysis pipeline consisted of a deep-learning (DL)-based tissue segmentation (tumor, stroma, necrosis, etc.), a pre-trained DL DAPI nuclear segmentation step, cellular phenotyping, and spatial metrics among the various cell types.

FIGURE 1. COMET™ – automated platform for hyperplex spatial biology

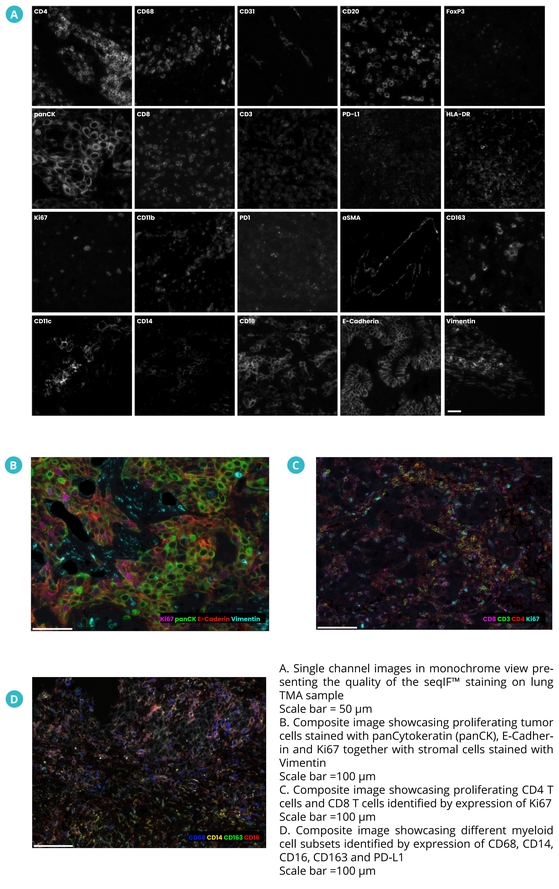


A seqIF™ protocol consists of an autofluorescent cycle to acquire tissue intrinsic fluorescence, subsequent cycles of staining-imaging-elution, where 2 biomarkers are detected at the time. COMET™ enables the detection of up to 40 biomarkers in 20 cycles on a single tissue slide. B FFeX™ technology allows fast and uniform delivery of reagents to a closed staining chamber. Precise control of all staining conditions allows to achieve of high-quality immunofluorescent stainings in the length of minutes instead of hours.

Multiplex Imaging

We interrogated tumor composition in Lung Tumor TMA using the COMET™ platform and Phenoplex™ Software from Visiopharm. Specific cellular phenotypes of interest are proliferating tumor cells, proliferating T cells, immunosuppressive macrophages and antigen-presenting cells and their interactions with a focus on the PD(-L)1 pathway.

FIGURE 2. Multiplex SeqIF™ Image data



A. Single channel images in monochrome view presenting the quality of the seqIF™ staining on lung TMA sample
Scale bar = 50 µm
B. Composite image showcasing proliferating tumor cells stained with panCytokeratin (panCK), E-Cadherin and Ki67 together with stromal cells stained with Vimentin
Scale bar =100 µm
C. Composite image showcasing proliferating CD4 T cells and CD8 T cells identified by expression of Ki67
Scale bar =100 µm
D. Composite image showcasing different myeloid cell subsets identified by expression of CD68, CD14, CD16, CD163 and PD-L1
Scale bar =100 µm

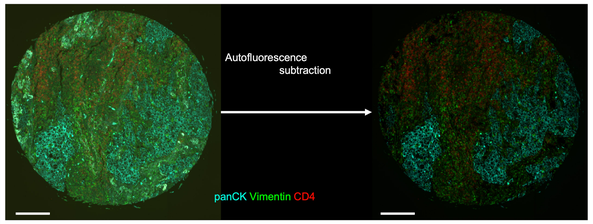
References

[1] Mund A, Brunner AD, Mann Unbiased spatial proteomics with single-cell resolution in tissues. Molecular Cell 2022; 82:2335-2349

Multiplex Image Analysis

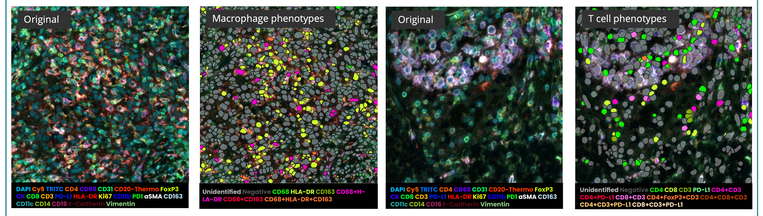
Image analysis was performed using Phenoplex™ by Visiopharm, based on its Oncotopix® Discovery platform. All Visiopharm products used in this poster are for Research Use Only (RUO).

FIGURE 3. Autofluorescence substraction



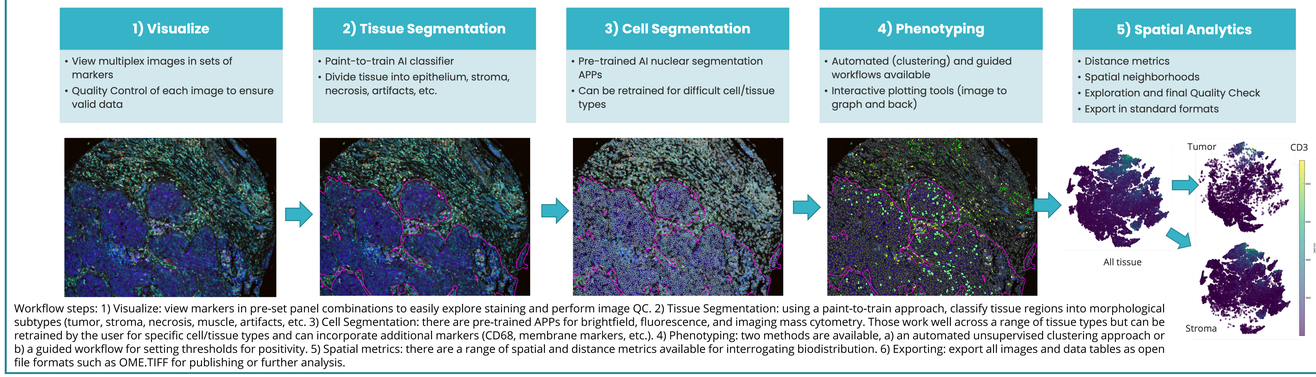
Lung tissue is highly autofluorescent. Before image analysis, a background subtraction has been performed using the Lunaphore Viewer software to discriminate the assay-specific fluorescent signal from the tissue-intrinsic autofluorescence. An image of the unstained tissue was acquired on COMET™ for subsequent wavelength-specific subtraction. PanCK (turquoise), vimentin (green) and CD4 (red) signal on a lung TMA core prior (left) and post (right) autofluorescence subtraction.

FIGURE 5. Exploring distribution of various cell phenotypes



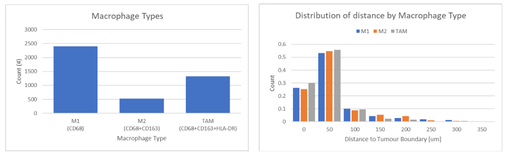
The automated unsupervised clustering method of phenotyping was used. Users are only required to select the input channels that are to be used for phenotyping. These figures show the original images next to the same image superimposed with colors representing the phenotyped cell. Left set: phenotyped for Macrophages (CD68, CD163, HLA-DR). Right set: phenotyped for T cells (CD3, CD4, CD8. FoxP3, PD-L1).

FIGURE 4. Overall analysis workflow



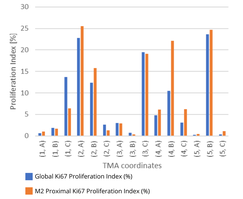
Workflow steps: 1) Visualize: view markers in pre-set panel combinations to easily explore staining and perform image QC. 2) Tissue Segmentation: using a paint-to-train approach, classify tissue regions into morphological subtypes (tumor, stroma, necrosis, muscle, artifacts, etc. 3) Cell Segmentation: there are pre-trained APPs for brightfield, fluorescence, and imaging mass cytometry. Those work well across a range of tissue types but can be retrained by the user for specific cell/tissue types and can incorporate additional markers (CD68, membrane markers, etc.). 4) Phenotyping: two methods are available, a) an automated unsupervised clustering approach or b) a guided workflow for setting thresholds for positivity. 5) Spatial metrics: there are a range of spatial and distance metrics available for interrogating biodistribution. 6) Exporting: export all images and data tables as open file formats such as OME.TIFF for publishing or further analysis.

FIGURE 6. Basic spatial analysis



Phenoplex™ gives the user a huge range of outputs including the ability to measure the distance between any number of phenotypes or from any phenotype to a boundary/border between tissue classes. The left panel shows data supporting counting macrophage types in the image, and histograms to the right show the distance to tumor for different macrophage types. Calculating both inter- and intra-phenotype cell distance statistics and cell neighborhood statistics are also possible.

FIGURE 7. Advanced spatial analytics



Cells are detected then classified according to their CD68 and CD163 status to detect M2 macrophages. Cells within 5µm of M2 cells (considered to be adjacent) are subsetted and then classified according to their Ki67 status (M2 cells are known to promote repair and cell proliferation). This proliferation index is show per TMA core in the sample.

Conclusions

The combination of hyperplex staining, cyclic fluorescence imaging, advanced image analysis, *in situ* cellular phenotyping, and spatial analytics allows a better interrogation of complex tumor microenvironment composition, which is a crucial step for understanding and harnessing tissue biology. Being able to analyze the spatial distribution of phenotyped cells in the TME enables the user to identify reliable biomarkers as predictive factors in response to therapies.

The COMET™ platform enables biomarker discovery by allowing the simultaneous detection of up to 40 biomarkers in a single tissue slide. Such hyperplex immunofluorescent panels set the stage for the detection of the wide range of immune cell phenotypes in the tissue within the spatial context.

The Phenoplex™ workflow provides all the image analysis steps required to fully appreciate the complexity of the hyperplex staining all in one software package, all without requiring programming skills. The COMET™ platform and Phenoplex™ workflow, when used together, provide a complete solution for tissue analysis with a slide-in, data-out approach that supports biomarker discovery in every laboratory.