Depicting the cellular architecture of the tumour microenvironment by integrating hyperplex immunofluorescence and automated image analysis

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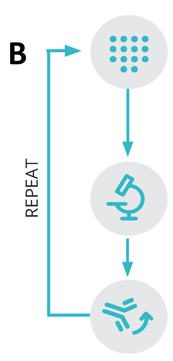
BACKGROUND

The tumour microenvironment (TME) is emerging as an important factor that shapes the dynamic of the tumour growth, its heterogeneity, and response to therapies(1). Thus, efforts are undertaken to understand better the biology of cells within the TME(2) and to provide spatial mapping of TME components and their interactions(3). In this study, we focus on the phenotyping of cells across different tumour types on a tissue microarray (TMA) with an immuno-oncology panel encompassing 20 biomarkers. We interrogated their TME with the use of the COMET™ automated staining and imaging instrument(4), and HALO® and HALO AI™ image analysis platforms.

20plex panel includes:FoxP3, CD68, aSMA, CD31, CD38, Ido-1, s100, CD11c, PD-L1, Ki67, CD8, PD-1, CD4, PanCK, CD3, CD20, CD16, HLA-DR, Vimentin, CD45

COMET™ WORKFLOW

A) Sequential IF (SeqIF™) protocol was executed in fully automated fashion on COMET™ device. FFPE slides were preprocessed with the use of PT module with dedicated reagents at pH9 (Epredia). Subsequently automated protocol was created by the user. Primary antibody and secondary antibodies were prepared offline and loaded on the machine together with proprietary buffers enabling all the steps of seqIF™ protocols: washing, imaging, quenching and elution buffers. At each cycle signal, from 2 markers and DAPI, was acquired by the integrated fluorescent microscope. As final results, single multichannel ome.tiff image is produced and delivered to the user.



1. STAIN Controlled by an automated protocol, primary and secondary antibodies are delivered onto the tissue. User can choose to deliver **1 antibody or a mix of 2 antibodies from different species.**

2. IMAGE The **integrated microscope** then acquires the image through the window of the imaging chip.

3. ELUTE The fluorescent signal is erased by removing the primary-secondary antibody complex with a **dedicated elution buffer and temperature-controlled cycle**.

B) COMET™ workflow applies sequential immunofluorescence principle, where tissues are subsequently undergoing cycles of staining, imaging and antibody elution in automated manner without the need of user intervention. COMET™ provides the possibility to perform up to 20 cycles on one single automated run, what yields in 40 plex immunostaining image. Final images are delivered ready to be analyzed and there is no need of data postprocessing from the user.

C

COMET™

Accessories:
PT module
Compressor

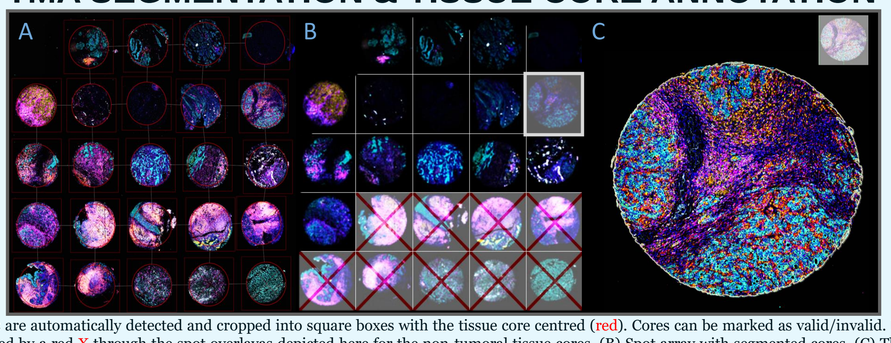
Software

COMET™ Explore Software – Instrument Control
COMET™ Viewer – Image Visualisation

C) COMET™ device is an automated platform providing slide in- data out workflow for spatial biology. Components of COMET™ includes PT module required for the preprocessing of FFPE slides, compressor, COMET™ instrument and COMET™ Explore Control Software. Images are delivered as multi-stack ome.tiff files, ensuring compatibility with different image visualization and analysis tools.

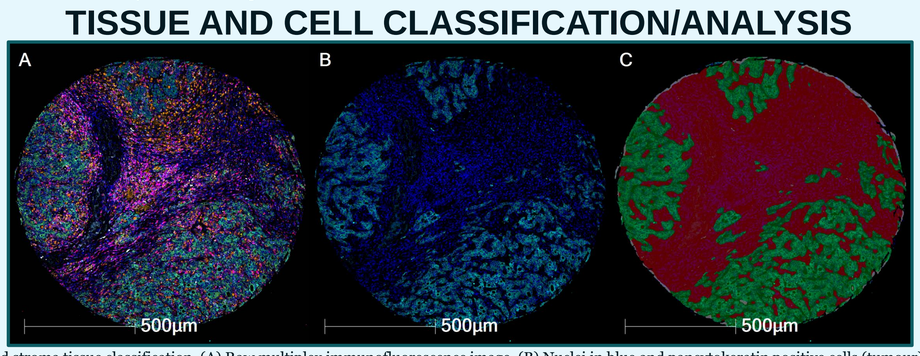
HALO® and HALO AI™ IMAGE ANALYSIS WORKFLOW

TMA SEGMENTATION & TISSUE CORE ANNOTATION

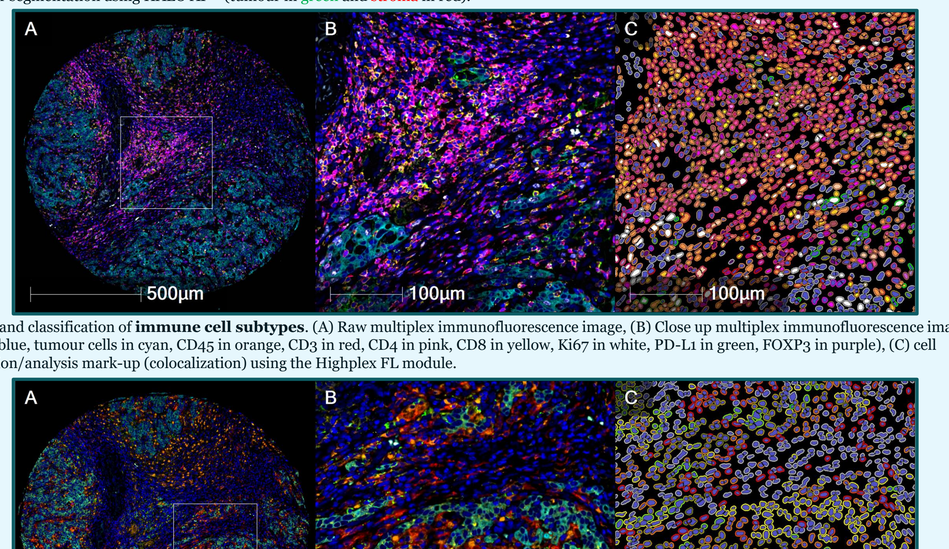


(A) The cores are automatically detected and cropped into square boxes with the tissue core centred (red). Cores can be marked as valid/invalid. Invalid cores are demarcated by a red X through the spot overlayas depicted here for the non-tumoral tissue cores. (B) Spot array with segmented cores. (C) The individual tissue cores can be automatically annotated using the flood tool. Manual edits as well as additional exclusion annotations can be made.

TISSUE AND CELL CLASSIFICATION/ANALYSIS



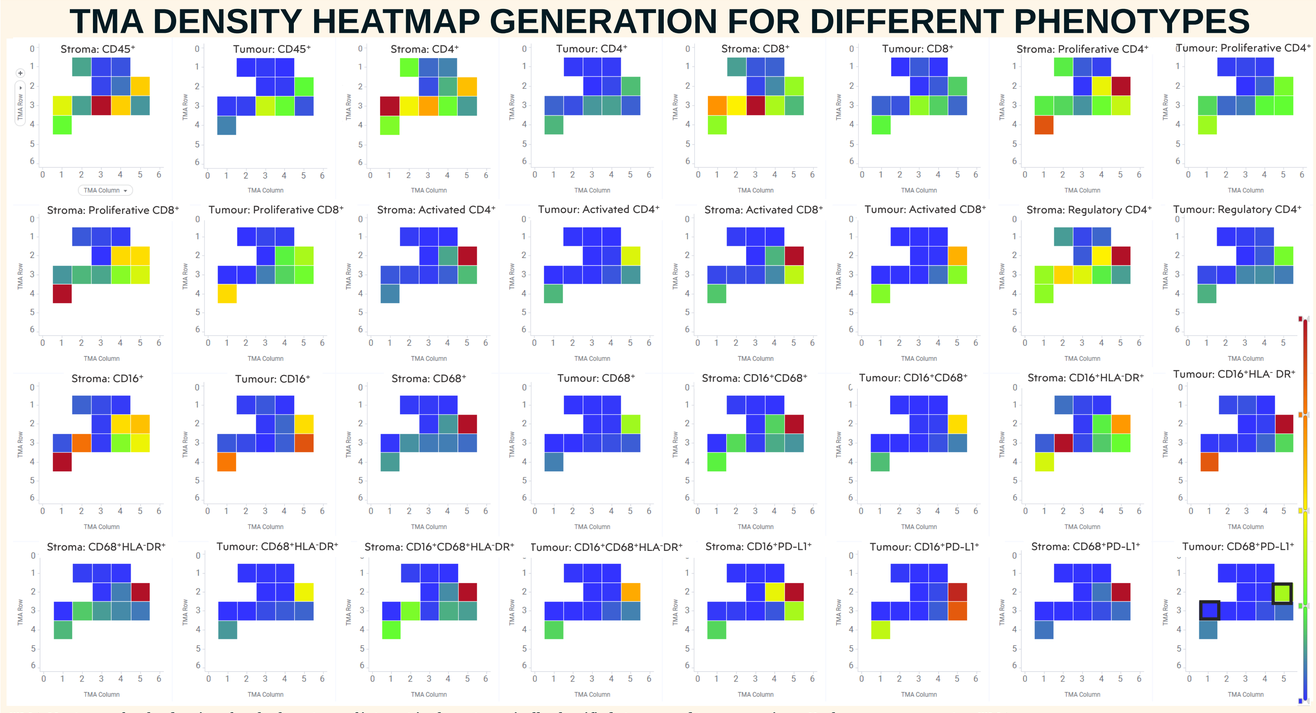
Tumour and stroma tissue classification. (A) Raw multiplex immunofluorescence image, (B) Nuclei in blue and pancytokeratin positive cells (tumour) in cyan, (C) tumour segmentation using HALO AI™ (tumour in green and stroma in red).



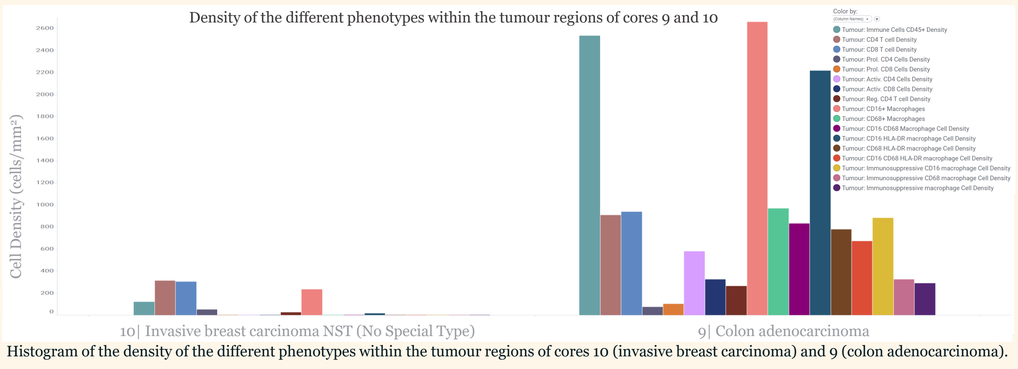
Detection and classification of **immune cell subtypes**. (A) Raw multiplex immunofluorescence image, (B) Close up multiplex immunofluorescence image (nuclei in blue, tumour cells in cyan, CD45 in orange, CD3 in red, CD4 in pink, CD8 in yellow, Ki67 in white, PD-L1 in green, FOXP3 in purple), (C) cell classification/analysis mark-up (colocalization) using the Highplex FL module.

Detection and classification of **macrophage subtypes**. (A) Raw multiplex immunofluorescence image, (B) Close up multiplex immunofluorescence image (nuclei in blue, tumour cells in cyan, CD68 in orange, CD16 in red, HLA-DR in yellow PD-L1 in green), (C) cell classification mark-up (colocalization) using the Highplex FL module.

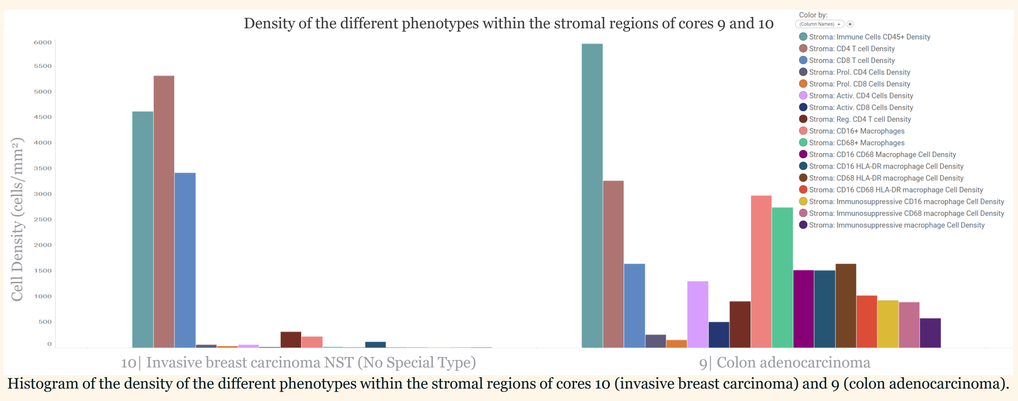
TMA DENSITY HEATMAP GENERATION FOR DIFFERENT PHENOTYPES



TMA Heatmaps for the density of each phenotype of interest in the automatically classified stroma and tumour regions. Each square represents a TMA core.

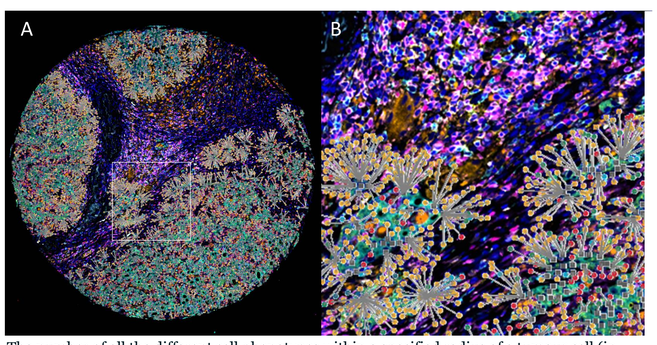


Histogram of the density of the different phenotypes within the tumour regions of cores 10 (invasive breast carcinoma) and 9 (colon adenocarcinoma).

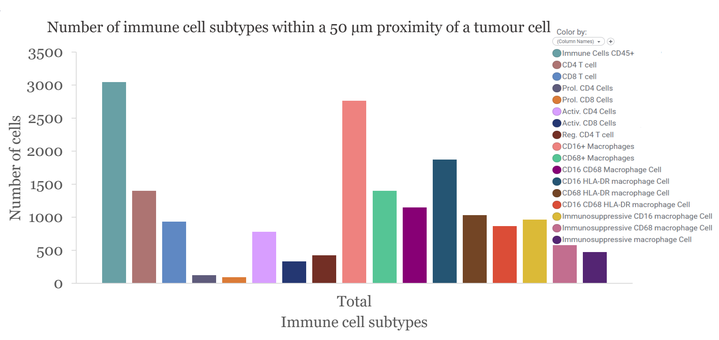


Histogram of the density of the different phenotypes within the stromal regions of cores 10 (invasive breast carcinoma) and 9 (colon adenocarcinoma).

PROXIMITY ANALYSIS

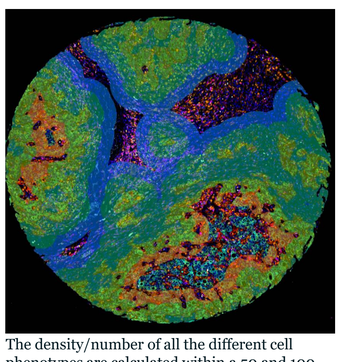


The number of all the different cell phenotypes within a specified radius of a tumour cell (in this case 50 µm) is calculated. Full core image (A) and zoom-in into tumour border area (B)

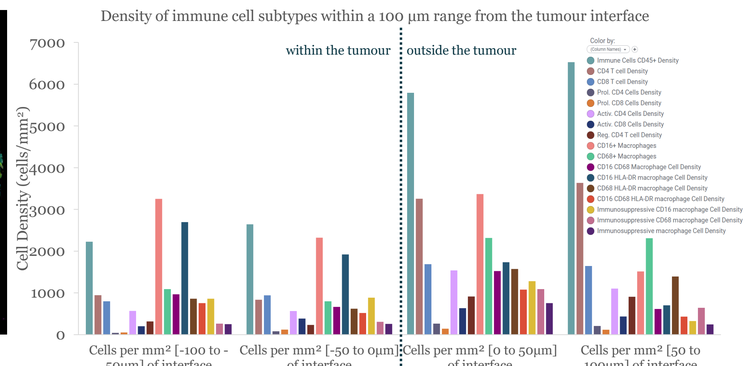


Histogram of the number of the different phenotypes within a 50 µm radius from any tumour cell of core 9 (colon adenocarcinoma).

INFILTRATION ANALYSIS



The density/number of all the different cell phenotypes are calculated within a 50 and 100 µm distance from the tumour boundary (inside and outside).



Histogram of the density of the different phenotypes within a 100µm range from the tumour interface of core 9 (colon adenocarcinoma).

CONCLUSIONS

Tumor microenvironment (TME) composition was depicted as important regulator of the response to treatment and patient prognosis (5). Significant efforts are continuously carried out to better understand how tumors affect their surrounding by recruiting healthy cells to their proximity and interfering with their functional states. Hyperplex immunofluorescence enables interrogating TME in the manner, where complex phenotypes can be identified within the spatial context of the tissue, empowering researchers to understand better intercellular interactions and tissue-intrinsic biology (3). Current state-of-art methodologies allowing questioning proteomic composition of TME at large scale are costly in terms of time and resources to execute both experiments and data analyses, limiting the adoption and day-to-day use of hyperplex immunofluorescence.

The workflow presented here highlights the easiness of adoption of seqIF™ protocol and a supervised image analysis pipeline. COMET™ platform ensures single-cell resolution and the simultaneous detection capability of multiple protein biomarkers with high reproducibility. COMET™ automates all steps of protocol execution and limits inter-experiments variability, while using standard reagents and delivering 20 20-plex images in 1 week. We demonstrate further how the combination of COMET™ hyperplex images with the HALO® and HALO AI™ image analysis platform from Indica Labs, results in an easy and straightforward workflow for interrogating heterogeneous TME and depicting tissue architecture on the single-cell level. HALO® and HALO AI™ enable guided and automated data extraction from hyperplex images with flexible workflow design. Our analysis of a 20-plex immuno-oncology panel on a TMA containing invasive breast carcinoma cores and colon adenocarcinoma cores yielded several insights into the cellular phenotypes and complex spatial relationships in the TME including T lymphocytes and macrophages. We were able to demonstrate distinct tumor infiltration patterns and characterize the accumulation of immune cells in the stroma and tumor compartment.

COMET™ images, together with HALO® and HALO AI™, can be directly used for quantitative analysis of the TME, enabling researchers the identification of the biomarkers across different tumors and at a single-cell level. The high throughput of COMET™ together with HALO® and HALO AI™ workflows stand out as tools that allow bringing the hyperplex immunofluorescence to every laboratory and streamlines the study of TME across basic and translational research.

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