POSTER NUMBER: **71**

SCAN TO GET A DIGITAL VERSION OF THE POSTER

Integration of RNA *in situ* hybridization and sequential immunofluorescence for same-slide fully automated multiomics analysis of the tumor microenvironment

Alice Comberlato[1], Arec Manoukian[1], Paula Juričić[1], Pino Bordignon[1], Alexander Kehren[1], Alix Faillétaz[1], Anushka Dikshit[2], Emerald Doolittle[2], Rose Delvillar[2], Steve Zhou[2], Ching-Wei Chang[2], Li-Chong Wang[2], Saska Brajkovic[1], Maithreyan Srinivasan[2], Diego G. Dupouy[1]

[1]Lunaphore Technologies, Tolochenaz, Switzerland, [2]Advanced Cell Diagnostics, Inc., Newark, California, USA

Background

Spatial biology has transformed our understanding of the tumor microenvironment (TME) by enabling the study of tissue composition and intercellular interactions at a single-cell level while preserving spatial context [1-3]. **Hyperplex immunofluorescence (IF)** techniques allow the simultaneous detection of multiple protein biomarkers, enabling immune cell profiling in the TME [4]. Similarly, **RNA *in situ* hybridization (ISH)** techniques have enabled the detection of RNA biomarkers, such as soluble factors with high sensitivity and specificity [5]. Combining the detection of key RNA and protein targets can provide valuable insights into unique **infiltrating immune cell populations** and their activation states.

Methods

In this study, we propose a novel approach that combines **RNAscope™** and sequential immunofluorescence (**seqIF™**) protocols for the detection of RNA and protein targets **on the same slide**. The integrated **multiomics protocol** is automated on the **COMET™ platform**, an advanced platform for tissue staining that uses precise temperature control and automation capabilities, ensuring reproducibility and efficiency in the workflow (**Figure 1**). To achieve high-quality same-slide detection, an **optimized target retrieval** and a **protease-free tissue pre-treatment** were developed.

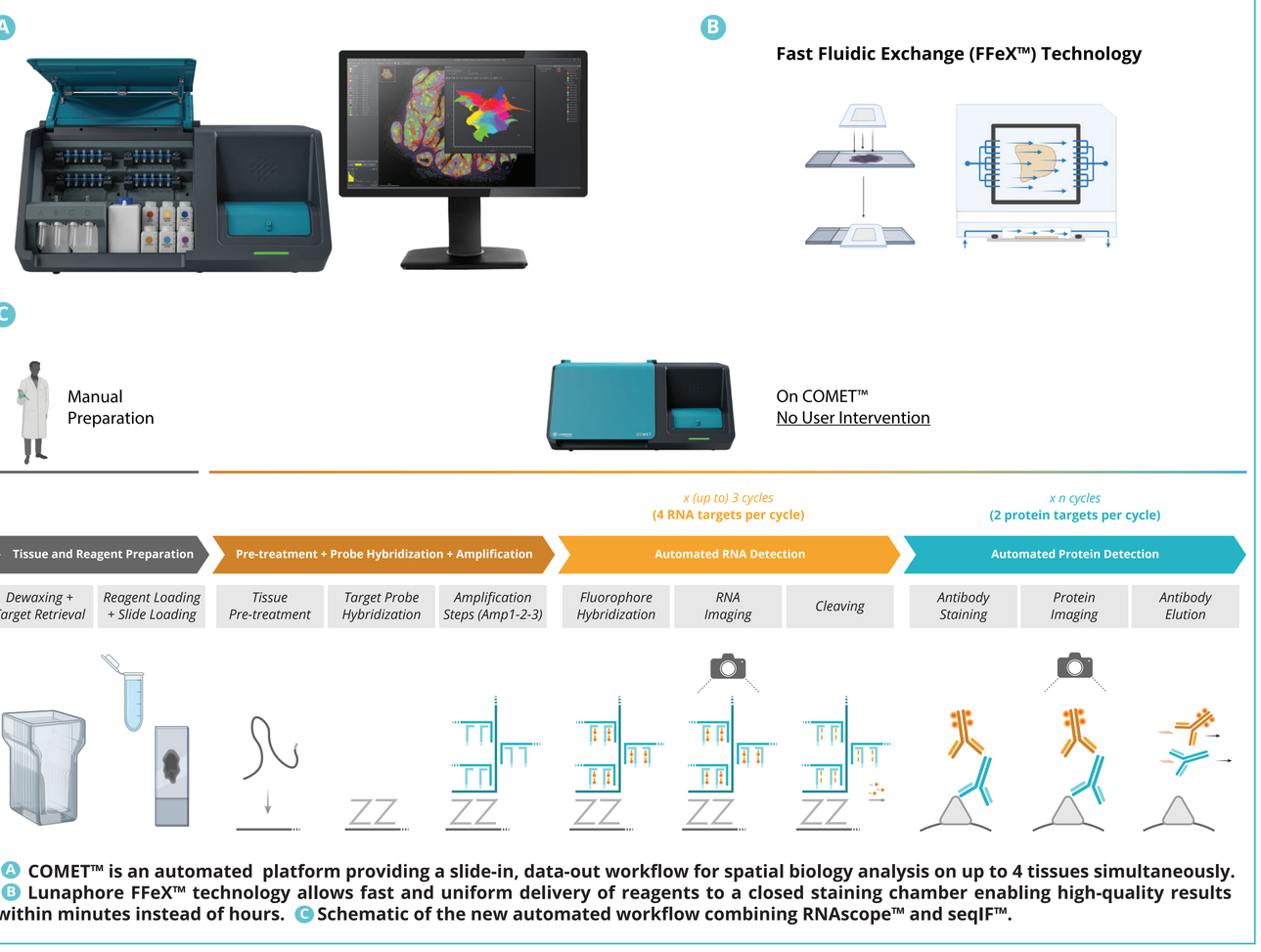
By **combining RNAscope™ HiPlex assay with seqIF™** we could sequentially detect multiple RNAs and proteins in the same tissue sections, preserving the spatial relationship between different molecular species.

Results

We developed an integrated protocol for the co-detection of RNA and protein targets, with three cycles of RNA detection (four fluorescent channels per cycle), enabling in total a 12-plex RNA panel detection (**Figure 2**), followed by 10 consecutive cycles of seqIF™ (with two protein markers detected per cycle), up to a 20-plex protein panel (**Figure 3**). We included antibodies to detect infiltration of T cells, B cells, macrophages, and other immune cells in combination with RNA probes for key biomarkers such as cytokines and granzymes. The automated process on COMET™ seamlessly synchronized all protocol steps, including imaging, and allowed multiomics analysis without any user intervention.

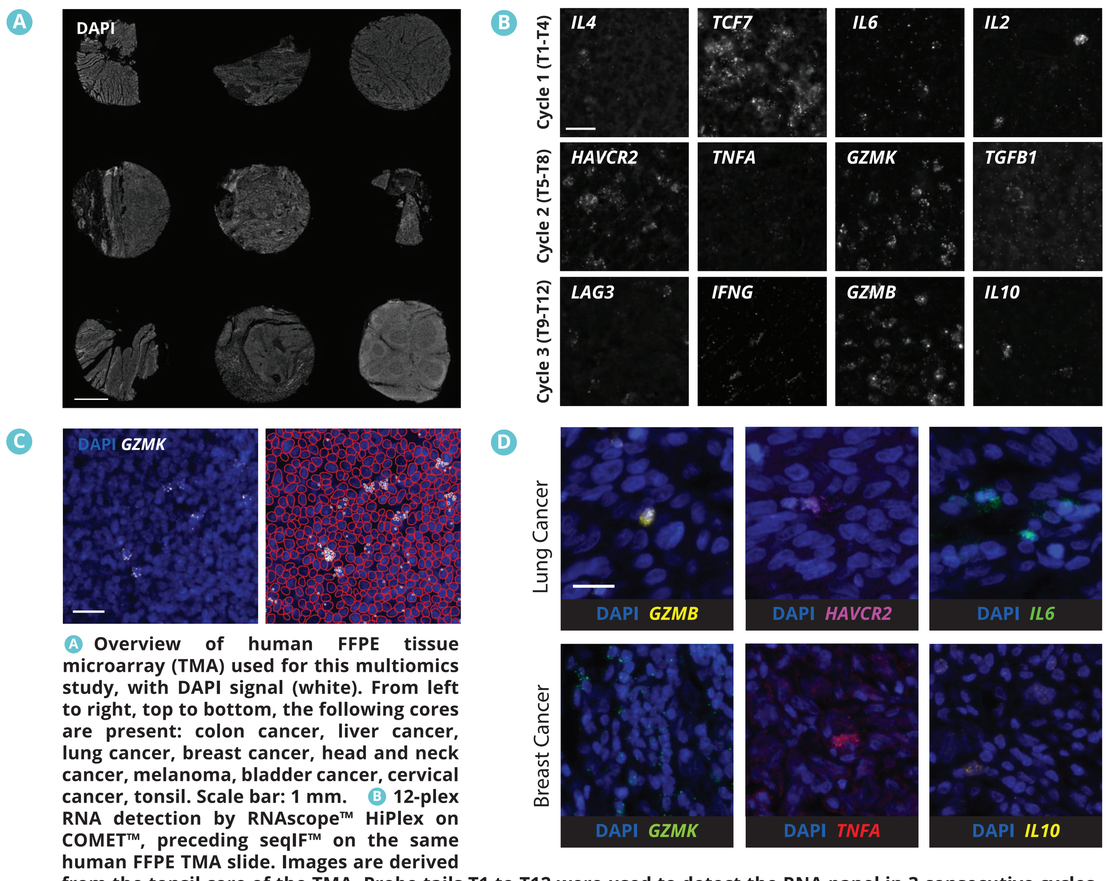
By combining RNA and protein detection, we gained extensive insights into the TME molecular landscape. In details, we showed that an in-depth analysis of tumor-infiltrating immune cell populations can be carried out, allowing for example the identification of multiple subclasses of cytokine-secreting CD3$^+$ T-cell lymphocytes and CD56$^+$ Natural Killer cells expressing *GZMB*, together with a characterization of tumor cells and their secretome (e.g. *IL6*$^+$ cancer cells in lung cancer) (**Figure 4**). Multiomics analyses like this one allow to uncover also co-expression patterns and relationships between RNAs and proteins within individual cells, as shown in **Figure 5**.

FIGURE 1. COMET™ - automated platform for multiomics approach



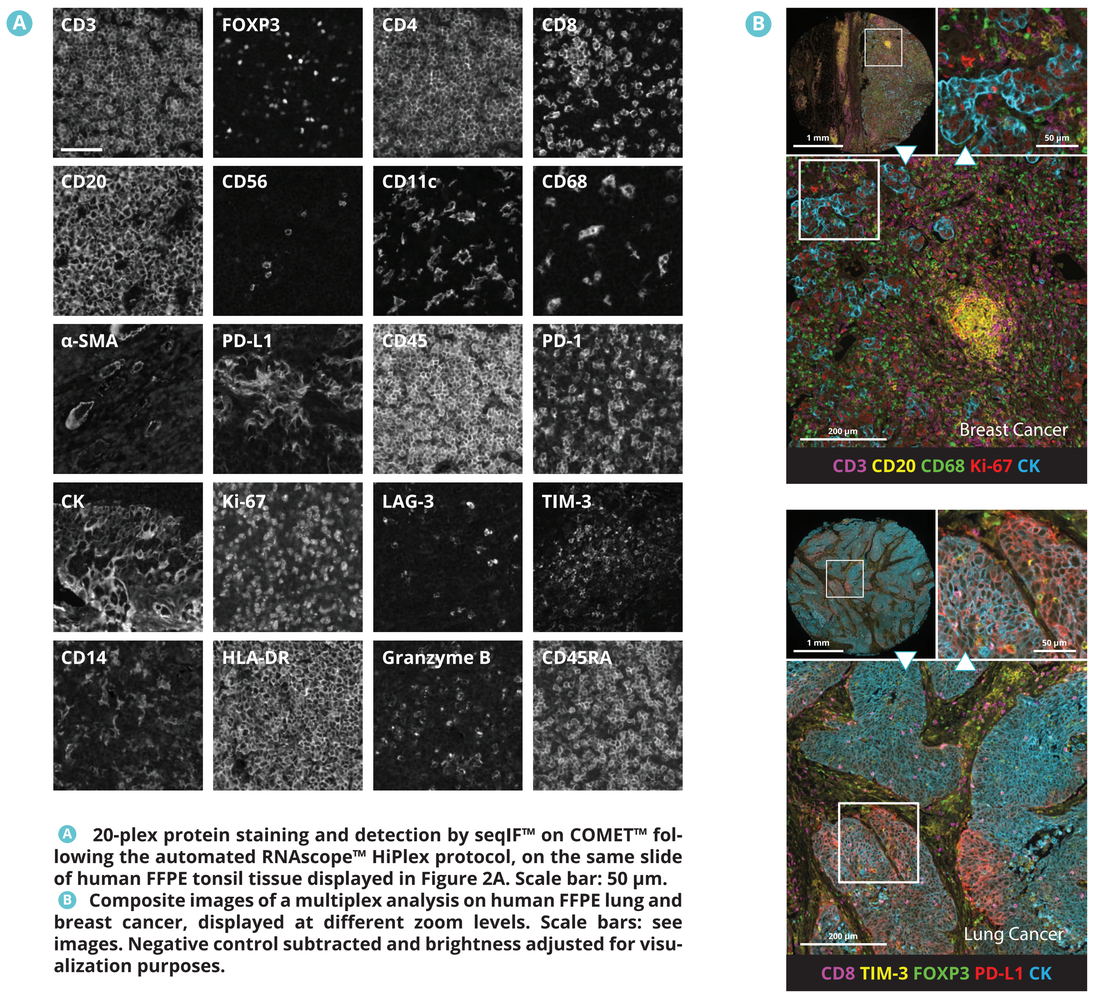
A COMET™ is an automated platform providing a slide-in, data-out workflow for spatial biology analysis on up to 4 tissues simultaneously. B Lunaphore FFeX™ technology allows fast and uniform delivery of reagents to a closed staining chamber enabling high-quality results within minutes instead of hours. C Schematic of the new automated workflow combining RNAscope™ and seqIF™.

FIGURE 2. RNAscope™ HiPlex automation on COMET™ for RNA marker detection



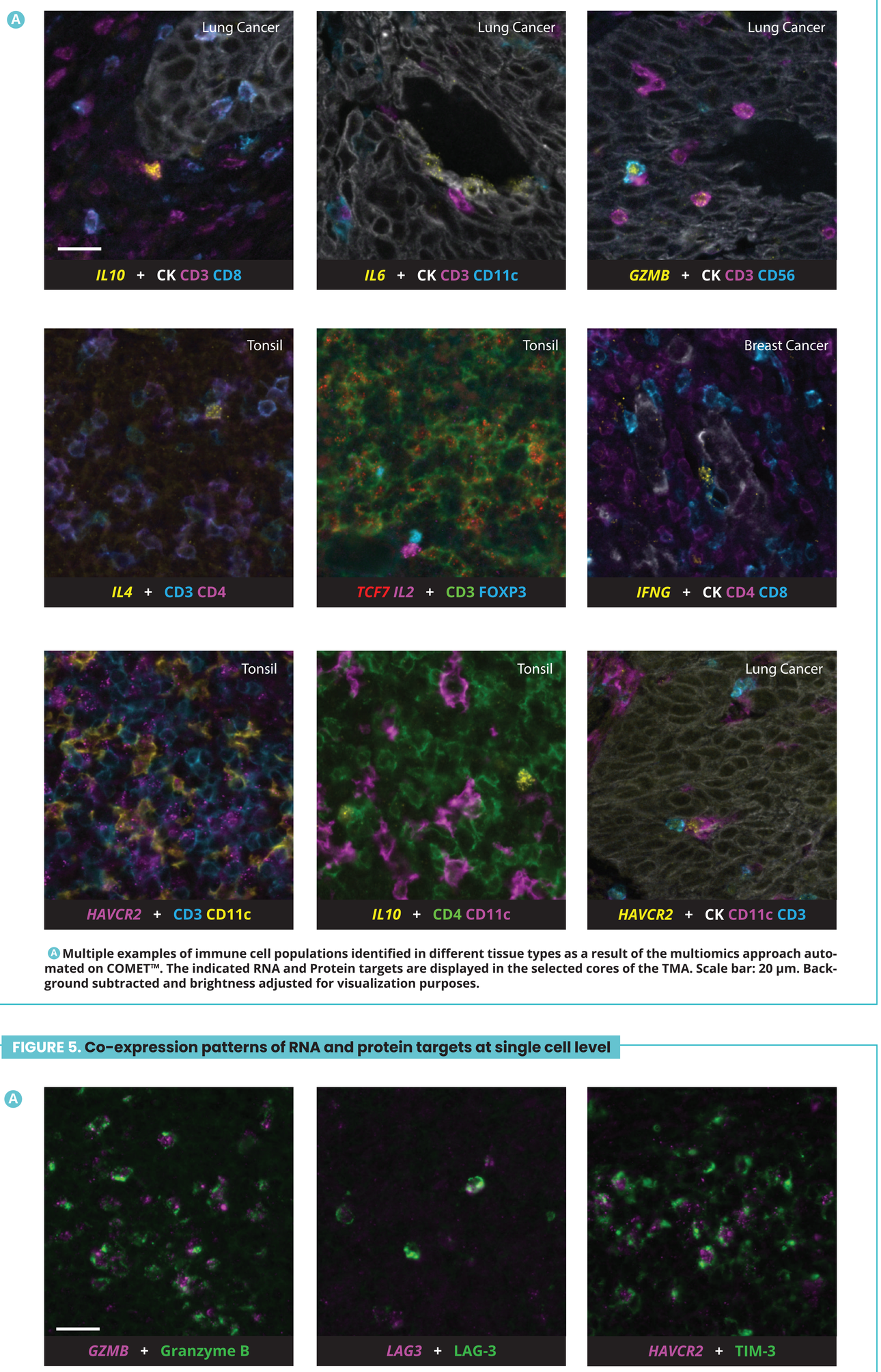
A Overview of human FFPE tissue microarray (TMA) used for this multiomics study, with DAPI signal (white). From left to right, top to bottom, the following cores are present: colon cancer, liver cancer, lung cancer, breast cancer, head and neck cancer, melanoma, bladder cancer, cervical cancer, tonsil. Scale bar: 1 mm. B 12-plex RNA detection by RNAscope™ HiPlex on COMET™, preceding seqIF™ on the same human FFPE TMA slide. Images are derived from the tonsil core of the TMA. Probe tails T1 to T12 were used to detect the RNA panel in 3 consecutive cycles, using 4 fluorescent channels in each (from left to right: FITC, TRITC, Cy5, Cy7). Scale bar: 20 µm. C Example of detection of RNAscope™ results using QuPath for quantification of dot counts per cell. DAPI (blue) and *GZMK* (white) in human FFPE tonsil. Scale bar: 20 µm. D Composite images of RNA detection in human FFPE breast and lung cancer. Scale bar: 20 µm. Background subtracted and brightness adjusted for visualization purposes.

FIGURE 3. Detection of protein targets by seqIF™ following RNAscope™ HiPlex



A 20-plex protein staining and detection by seqIF™ on COMET™ following the automated RNAscope™ HiPlex protocol, on the same slide of human FFPE tonsil tissue displayed in Figure 2A. Scale bar: 50 µm. B Composite images of a multiplex analysis on human FFPE lung and breast cancer, displayed at different zoom levels. Scale bars: see images. Negative control subtracted and brightness adjusted for visualization purposes.

FIGURE 4. Combined RNA and protein detection enables analysis of infiltrating immune cell populations



A Multiple examples of immune cell populations identified in different tissue types as a result of the multiomics approach automated on COMET™. The indicated RNA and Protein targets are displayed in the selected cores of the TMA. Scale bar: 20 µm. Background subtracted and brightness adjusted for visualization purposes.

FIGURE 5. Co-expression patterns of RNA and protein targets at single cell level

A Co-expression studies of specific RNA molecules and the proteins they code for at single-cell level. All images derived from human tonsil TMA core. Scale bar: 20 µm. Background subtracted and brightness adjusted for visualization purposes.

Conclusions

Our results demonstrate the successful implementation of the combined RNAscope™ and seqIF™ protocols on the COMET™ platform. Preserving spatial context and intercellular relationships, this approach offers a more holistic understanding of the TME molecular landscape and the complex cellular interactions exhibited by different cell populations.

Multiomics analysis on the same slide will allow a better comprehension of the interplay between transcriptomics and proteomics information, opening new perspectives for personalized medicine and the discovery of novel therapeutic targets.

References

[1] Bosisio FM, Van Herck Y, Messiaen J, Bolognesi MM, Marcelis L, Van Haele M, Cattoretti G, Antoranz A, De Smet F. Next-Generation Pathology Using Multiplexed Immunohistochemistry: Mapping Tissue Architecture at Single-Cell Level. Front Oncol. 2022 Jul 29;12:918900.

[2] Binnewies M, Roberts EW, Kersten K, Chan V, Fearon DF, Merad M, Coussens LM, Gabrilovich DI, Ostrand-Rosenberg S, Hedrick CC, Vonderheide RH, Pittet MJ, Jain RK, Zou W, Howcroft TK, Woodhouse EC, Weinberg RA, Krummel MF. Understanding the tumor immune microenvironment (TIME) for effective therapy. Nat Med. 2018 May;24(5):541-550.

[3] Vitale I, Shema E, Loi S, Galluzzi L. Intratumoral heterogeneity in cancer progression and response to immunotherapy. Nat Med. 2021 Feb;27(2):212-224.

[4] Hickey JW, Neumann EK, Radtke AJ, Camarillo JM, Beuschel RT, Albanese A, McDonough E, Hatler J, Wiblin AE, Fisher J, Croteau J, Small EC, Sood A, Caprioli RM, Angelo RM, Nolan GP, Chung K, Hewitt SM, Germain RN, Spraggins JM, Lundberg E, Snyder MP, Kelleher NL, Saka SK. Spatial mapping of protein composition and tissue organization: a primer for multiplexed antibody-based imaging. Nat Methods. 2022 Mar;19(3):284-295.

[5] Palla G, Fischer DS, Regev A, Theis FJ. Spatial components of molecular tissue biology. Nat Biotechnol. 2022 Mar;40(3):308-318.