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Uncovering the spatial dynamics of the tumor microenvironment: integrated RNA and protein profiling on the same section through automated spatial multiomics analysis

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Background

Spatial biology techniques have revolutionized our approach toward the study of the tumor microenvironment (TME) and its complex cellular interplay. By preserving the spatial information and allowing analyses at single-cell resolution, an unprecedented understanding of the cellular composition of cancer tissues has been made possible. On the one hand, multiplex immunofluorescence (mIF) methods have enabled precise profiling of immune cells and other key cellular players of the TME while uncovering their spatial distribution and interactions. On the other hand, *in situ* hybridization (ISH) technologies have been shown to provide complementary information to protein profiling, such as the mapping of cytokine- and chemokine-expressing cells, which are essential to comprehend signaling networks and immune activation statuses.

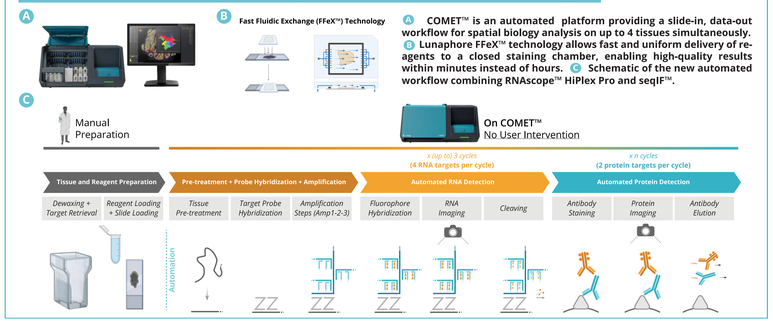
Methods

We made use of a novel multiomics approach that combines these two biological inputs by integrating **RNAscope™ HiPlex Pro Assay** (1) and **sequential immunofluorescence (seqIF™)** (2) protocols to achieve **same-section co-detection of RNA and protein targets**. The combined workflow is **fully automated** on COMET™, an advanced tissue staining and imaging platform (Figure 1). Through precise control over temperature and reagent distribution, the instrument ensures each assay's maximum efficiency and reproducibility. The integrated multiomics protocol presented here allows for up to three RNAscope™ detection cycles combined with twelve seqIF™ cycles for a total of **12-plex RNA and 24-plex protein panel in less than a day**.

Results

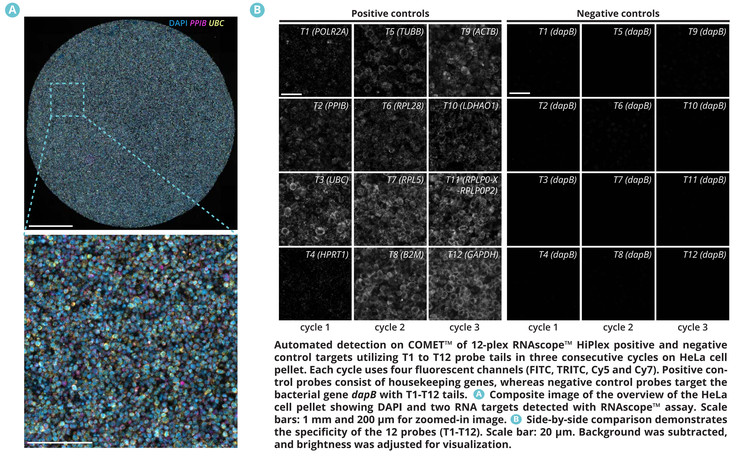
We first showed the capacity of the COMET™ platform to fully automate the **RNAscope™ HiPlex Pro** protocol and demonstrated its sensitivity and specificity with the analysis of positive (housekeeping) and negative (bacterial) control genes on HeLa cell pellets, demonstrating as well the flexibility of sample selection (Figure 2). Through Lunaphore's proprietary **Fast Fluidic Exchange (FFeX™)** technology (Figure 1B), which allows fast and optimal reagent distribution in parallel with controlled temperature and incubation times, highly reproducible and specific results were obtained from different stainers, instruments and over multiple days (Figure 3). To illustrate the potential of the multiomics approach in uncovering the complexity of the TME, we designed a panel of **12-RNA targeting probes** for key biomarkers of **tumor-infiltrating lymphocytes (TILs) and their activation status**, including multiple secreted molecules (*GZMB*, *GZMK*, *HAVCR2*, *IFNG*, *IL2*, *IL4*, *IL6*, *IL10*, *LAG3*, *TCF7*, *TGFB1*, *TNFA*). This RNA panel was combined with a **24-protein panel** for the detection of protein biomarkers selected to enable the single-cell profiling of different players within the TME, such as macrophages, dendritic cells, fibroblasts, and different types of lymphocytes. We applied this multiomics panel to characterize human FFPE breast cancer tissue and showed that the **co-detection of RNA and protein biomarkers on the same section** allows a better characterization of key cellular components involved in tumor progression and immune response (Figure 4). With **COMET™ capability of acquiring z-stacks**, a maximum intensity projection (MIP) was generated automatically to demonstrate the increase in RNA signals acquisition from HeLa cell pellet (Figure 5). Finally, **well-preserved tissue morphology** was observed throughout multiple tissue types with hematoxylin and eosin (H&E) staining after a 12-plex RNA and 24-plex protein multiomics protocol (Figure 6).

FIGURE 1. COMET™, a walk-away automated platform for same-section multiomics assays



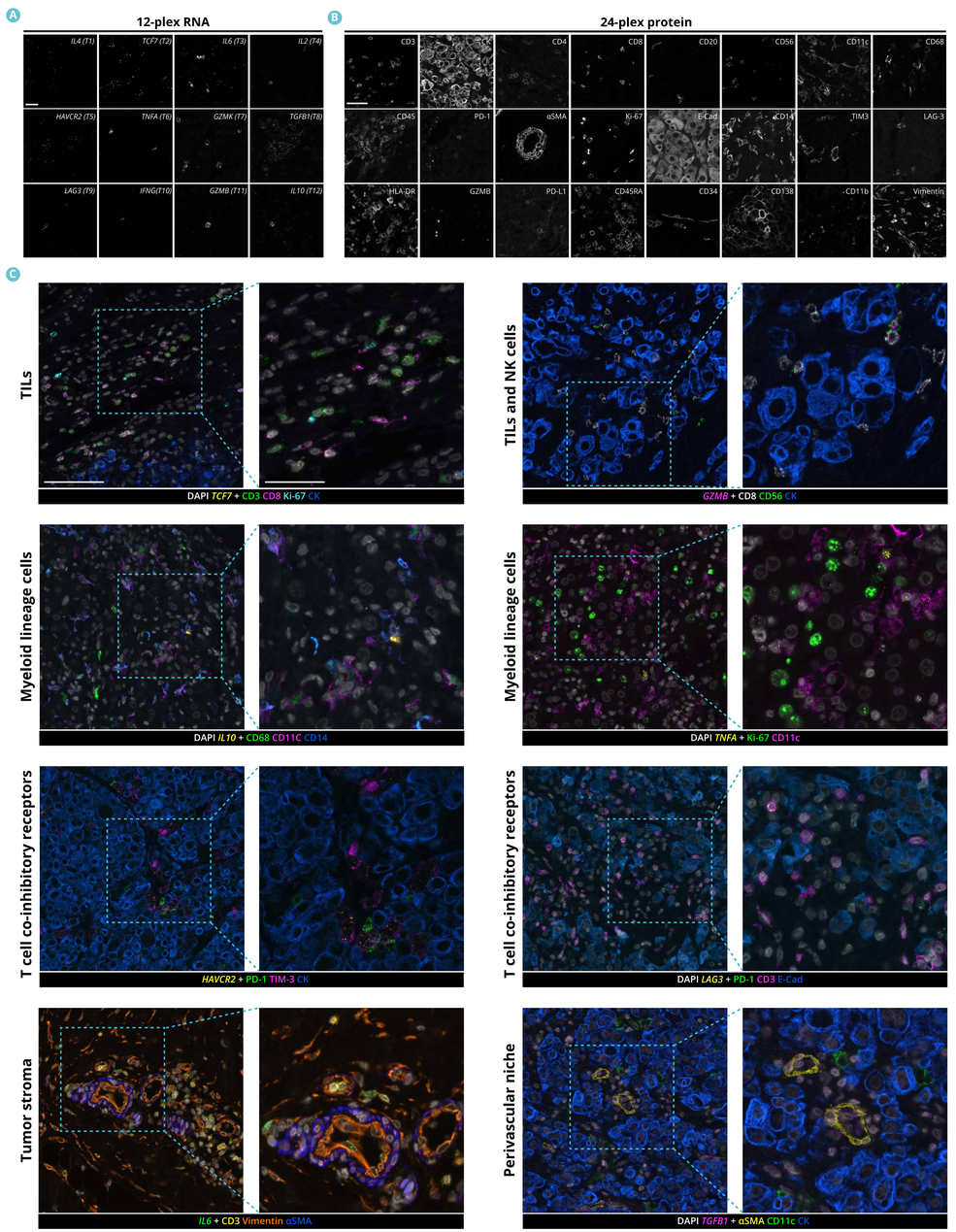
A COMET™ is an automated platform providing a slide-in, data-out workflow for spatial biology analysis on up to 4 tissues simultaneously. **B** Lunaphore FFeX™ technology allows fast and uniform delivery of reagents to a closed staining chamber, enabling high-quality results within minutes instead of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

FIGURE 2. RNAscope™ HiPlex Pro automation on COMET™ for RNA detection on HeLa cell pellet



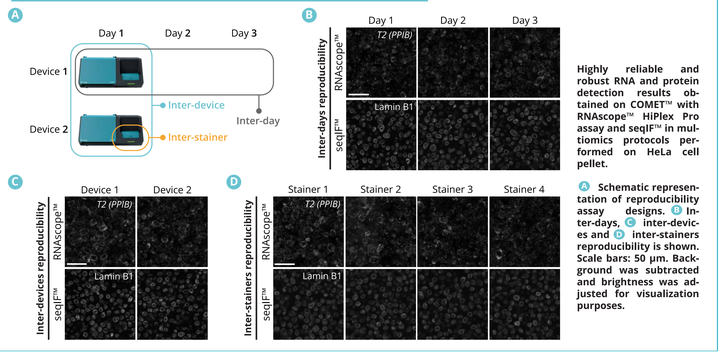
Automated detection on COMET™ of 12-plex RNAscope™ HiPlex positive and negative control targets utilizing T1 to T12 probe tails in three consecutive cycles on HeLa cell pellet. Each cycle uses four fluorescent channels (FITC, TRITC, Cy5 and Cy7). Positive control probes consist of housekeeping genes, whereas negative control probes target the bacterial gene *dapB* with T1-T12 tails. A Composite image of the overview of the HeLa cell pellet showing DAPI and two RNA targets detected with RNAscope™ assay. Scale bars: 1 mm and 200 µm for zoomed-in image. B Side-by-side comparison demonstrates the specificity of the 12 probes (T1-T12). Scale bar: 20 µm. Background was subtracted, and brightness was adjusted for visualization.

FIGURE 4. Co-detection of RNA and protein biomarkers on the same section of human FFPE breast cancer tissue



Automated detection on the COMET™ platform of 12 RNA targets with RNAscope™ HiPlex Pro and 24 protein biomarkers with seqIF™ on human FFPE breast cancer tissue through multiomics protocol. Example images of the 12-plex RNA panel detected by RNAscope™ HiPlex Pro panel A , and of the 24-plex protein panel detected by seqIF™ B on the same section on the COMET™. Scale bars: 20 µm and 50 µm respectively. C Composite images of breast cancer tissue illustrating examples of cells co-detected by RNAscope™ and seqIF™ technologies. TILs, NK cells, myeloid lineage cells, T cells expressing co-inhibitory receptors, and multiple stroma cells are identified with protein markers, and characterized by staining for their expression of specific transcripts, including secreted molecules. Scale bars: 100 µm and 50 µm for zoomed-in images. RNA probes and antibody background signals were subtracted, and brightness was adjusted for visualization purposes.

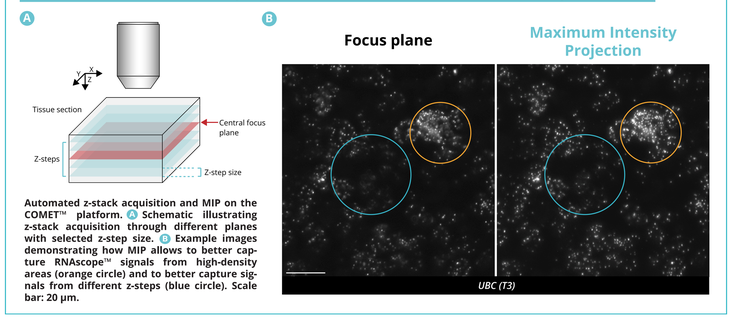
FIGURE 3. High reproducibility of multiomics protocols on COMET™



Highly reliable and robust RNA and protein detection results obtained on COMET™ with RNAscope™ HiPlex Pro assay and seqIF™ in multiomics protocols performed on HeLa cell pellet.

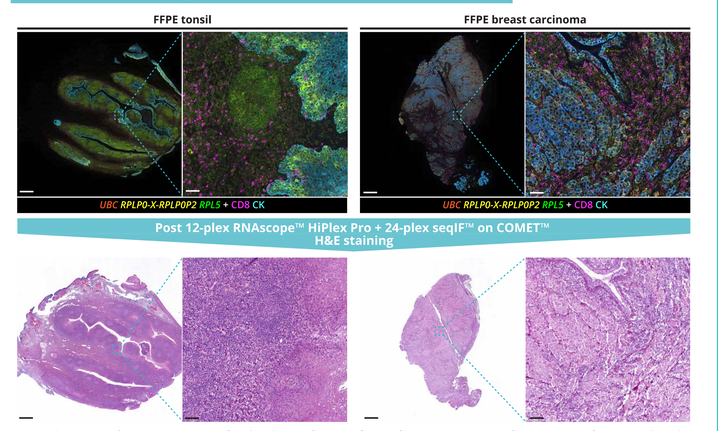
A Schematic representation of reproducibility assay designs. B Inter-days, C inter-devices and D inter-stainers reproducibility is shown. Scale bars: 50 µm. Background was subtracted and brightness was adjusted for visualization purposes.

FIGURE 5. Automated z-stacks acquisition and MIP visualization on COMET™ for RNAscope™ HiPlex Pro



Automated z-stack acquisition and MIP on the COMET™ platform. A Schematic illustrating z-stack acquisition through different planes with selected z-step size. B Example images demonstrating how MIP allows to better capture RNAscope™ signals from high-density areas (orange circle) and to better capture signals from different z-steps (blue circle). Scale bar: 20 µm.

FIGURE 6. Tissue morphology is preserved after multiomics protocol on COMET™



Composite images of two tissue types analyzed with a 12-plex RNA plus 24-plex protein targets multiomics protocol automated on the COMET™ platform. Both tissues underwent hematoxylin and eosin (H&E) staining after the multiomics run, confirming tissue integrity preservation and the potential for further downstream applications. Multiomics staining images are background subtracted, and brightness was adjusted for visualization. Scale bars: 1 mm and 50 µm for zoomed-in images.

Conclusions

Our results highlight the potential of spatial multiomics in enhancing research on immune cell biology and improving the understanding of the cellular interplay within the TME. Full automation of RNA and protein co-detection on a platform such as COMET™ will accelerate the analyses and increase the robustness of findings by minimizing user intervention. Ultimately, spatial multiomics assays will help in the development of prognostic and predictive biomarkers, in the refinement of cancer diagnoses, and in the selection of novel personalized therapies.

References

1. Wang, F., Flanagan, J., Su, N., et al. RNAscope: A Novel In situ RNA Analysis Platform for Formalin-Fixed Paraffin-Embedded Tissues. Journal of Molecular Diagnostics, 14:22-29 (2012).
2. Rivest, F., Eroglu, D., Pelz, B. et al. Fully automated sequential immunofluorescence (seqIF) for hyperplex spatial proteomics. Scientific Reports. 13, 16994 (2023).