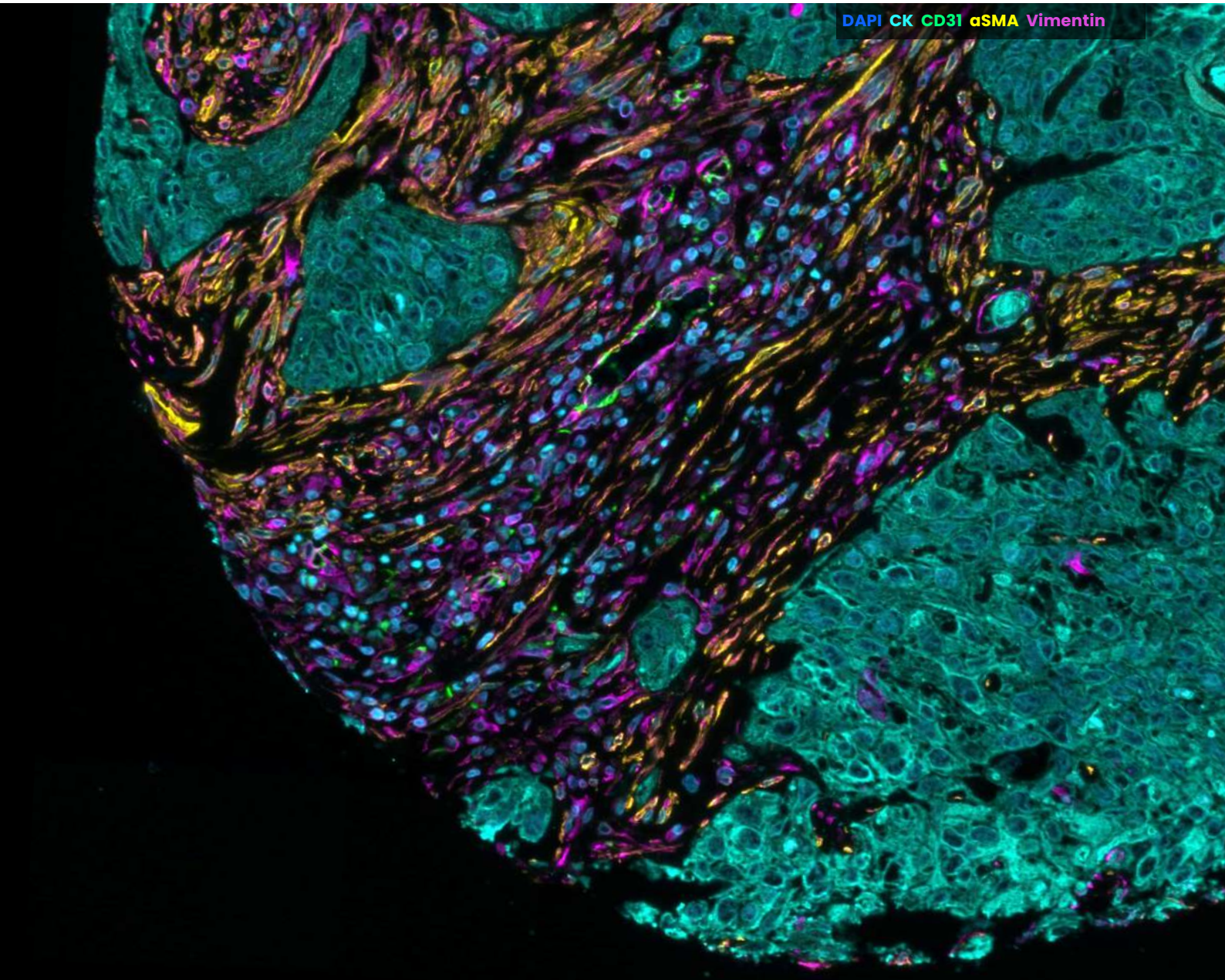
Lunaphore technologies

40-plex on TMA: human lung squamous cell carcinoma
Total protocol time: 16 hours



COMET™

streamlines hyperplex immunofluorescence at single-cell resolution

COMET™ Technical Note

Contents

Introduction

Spatial biology enables the study of the contextual relationship between different cell types. In the immuno-oncology (IO) field, spatial biology is used to study immune cells and tumoral cells in their spatial context. Understanding immune cell phenotypes, functional activities, and spatial relationships inside or around the tumoral mass is emerging as a crucial factor of immune responsiveness and resistance in the IO field.

The tumor microenvironment (TME) consists of cellular and non-cellular components. Cellular components include cancer cells, blood and lymphatic vessels, and immune cells. Non-cellular components include hypoxia, acidity, cytokines, and the extracellular matrix. Characterizing the TME can help classify tumors, aid in the understanding of tumorigenic pathways, and identify biomarkers that may ultimately lead to the development of reliable prognostic tools and targeted therapies.

One of the emerging approaches of studying multiple proteins in the TME is multiplex immunofluorescence (mIF), in which multiple proteins can be detected by several, specific antibodies applied to the same tissue slide. Despite the great need to develop mIF assays, basic and translational laboratories still struggle to integrate mIF approaches into their routine workflows. To make this possible mIF assays need to provide high-quality results within a short time and with minimal use of precious samples. However, long turn-around time, as well as tissue degradation due to reiteration of staining and signal removal steps, impact the ability to accurately detect more than 10-15 biomarkers in the same mIF assay. In addition, the great heterogeneity between tissue types and expression patterns, as well as error-prone approaches of manual or semi-manual techniques, have a significant impact on the reproducibility of mIF results.

Lunaphore has developed a revolutionary instrument, the COMET™. Based on patented microfluidic technology, COMET™ performs hyperplex assays using a sequential immunofluorescence (seqIF™) approach, where 40 protein biomarkers per run can be automatically detected without human intervention, in less than a day. It is an open system that allows users to directly create and customize panels with their off-the-shelf antibodies. A guided workflow for marker optimization helps users to find the best staining and imaging conditions. COMET™ is a robust system and supports researchers to address their biological questions on the TME with great flexibility and reproducibility.

COMET™ is a flexible solution for reproducible hyperplex stainings

COMET™ performs seqIF™ assays, that consist of sequential cycles of staining, imaging, and elution. During the staining step, two primary antibodies followed by fluorophore-labeled secondary antibodies detect two antigens simultaneously. An integrated microscope images without the need for sample manipulation and user intervention. After the imaging, a gentle and efficient elution step completely removes primary and secondary antibodies, preserving epitope stability without affecting tissue morphology. After elution the next cycle starts.

Thanks to the patented microfluidic technology, all conditions are finely controlled, enabling seqIF™ assays on COMET™ with outstanding reproducibility[1,2] and staining uniformity. Finely controlled conditions enable gentle staining and elution while preserving tissue and epitope integrity. Additionally, COMET™ is a reagent agnostic system, meaning users can use their already-validated off-the-shelf antibodies, without the need for upstream conjugation and the associated disadvantages. This enables first-time users to streamline the development of hyperplex assays thanks to the easy, marker optimization workflow.

COMET™ offers great flexibility on how to develop hyperplex assays (Figure 1). When building a new multiplex panel, guided optimization protocols[1] are available on COMET™ enabling straightforward marker optimization following these two approaches:

1) **single marker optimization:** one or two markers in the same staining cycle can be screened and optimized by testing different conditions per consecutive cycle on a single slide. This approach is particularly useful when there is no identified working condition for a marker;
2) **batch marker optimization:** if preliminary information of potential working conditions for several markers are known (e.g., working antibody/clone/concentration), they can be tested

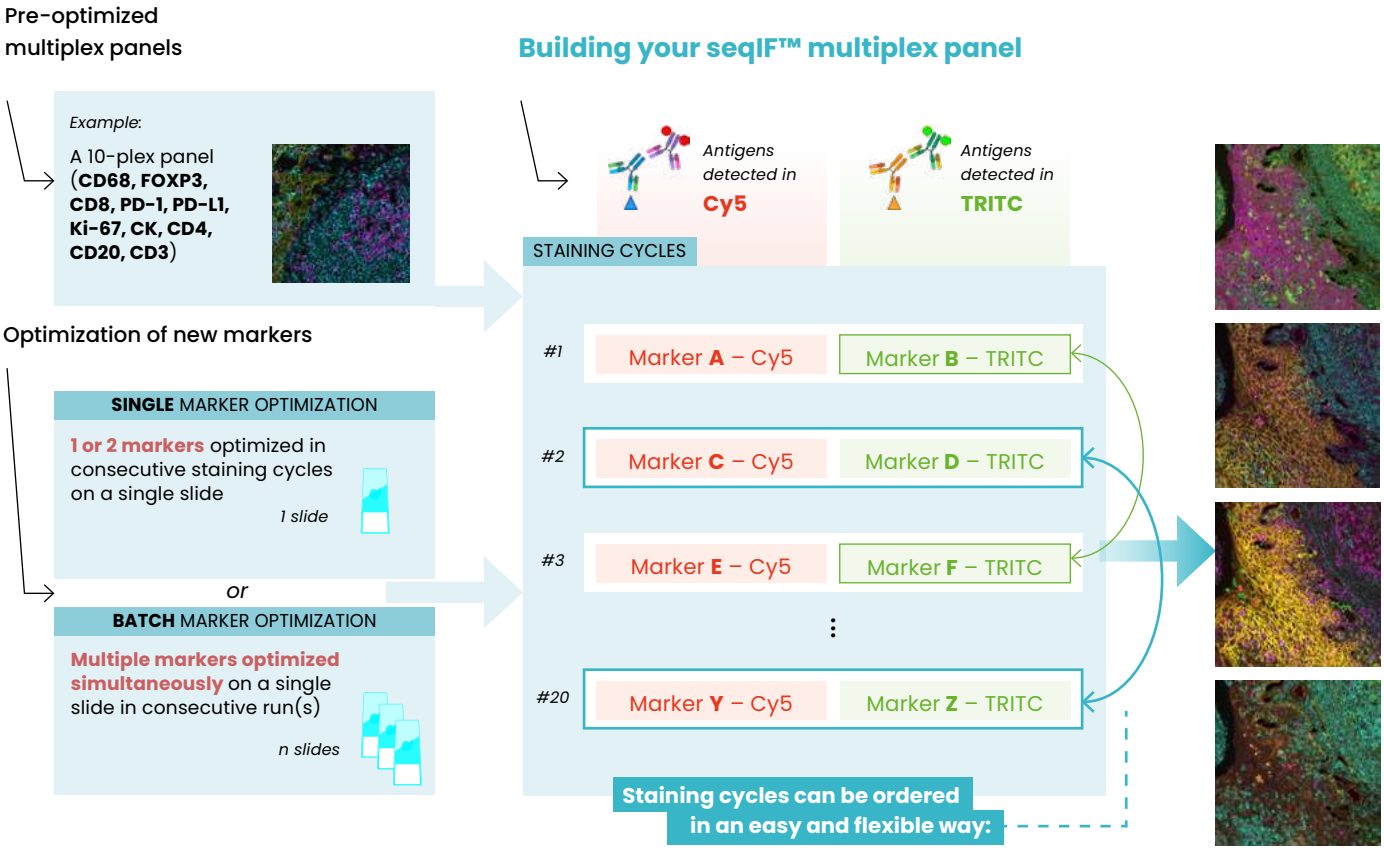


FIGURE 1

Hyperplex panels can be easily developed on COMET™ with guided protocols for optimization and flexible choice of marker positioning.

[1] Technical Note: Assay development of immuno-oncology panels with COMET™

[2] Technical Note: COMET™ Assay Validation

simultaneously on a single slide. Conditions for each cycle can then be adjusted in the following run(s) to optimize all markers together.

Previously optimized multiplex panels can simply be expanded by optimizing the desired new markers using one of the workflows described above. For example, our previously validated 10-plex panel can be expanded by adding one (or two) marker(s) at a time (single marker optimization) or by adding multiple markers simultaneously (batch marker optimization)[1] to speed up panel development in a few steps.

Once optimal staining conditions have been identified, users can build their seqIF™ hyperplex protocol with ease (Figure 1). The marker positioning within the hyperplex panel in the initial or final cycles is based on epitope stability and elution efficiency. However, thanks to superior elution efficiency along with preserved tissue morphology and excellent epitope stability[3], seqIF™ on COMET™ offers great flexibility for marker positioning. Amongst the 60 markers tested in-house so far, 93% could be freely positioned between cycles 1 to 20 supporting easy customization when building hyperplex panels.

How to develop a 40-plex panel on COMET™

To study the tumor and its microenvironment, we have selected 40 markers, indicated in Table 1, to build an inclusive hyperplex panel to streamline IO research. In this panel, markers to identify both the lymphoid (T cells, B cells, and natural killer cells) and myeloid lineage (such as neutrophils, dendritic cells, mass cells, basophils, and macrophages) of the immune system are selected. Complementary to lineage-specific markers, we have selected additional ones to characterize specific functional conditions, such as immunosuppressive status (IDO1, PD-L1, and VISTA), activation of immune cells (PD-1, ICOS, and LAG-3), or proliferative status (Ki-67). In addition to the immune profile of the TME, cancer and stromal cells, as well as blood and lymphatic vasculatures can be detected by specific markers in the panel. The panel also includes markers to study tumors with an epithelial origin (namely carcinomas), with which either tumoral or healthy epithelial cells can be detected by the expression of cytokeratins (CK) or E-cadherin.

TABLE 1

List of makers selected for the 40-plex panel and their corresponding cell phenotypes. The 10-plex panel used as a starting point to build the hyperplex panel on COMET™ is shown in orange.

Marker	Cell lineage, type or function
CD3	T cell
CD4	Helper T cell
CD8	Cytotoxic T cell
CD20	B cell
CD68	Macrophage
CK	Epithelial cell
Ki-67	Proliferative cell
PD-1	Activated T cell
PD-L1	Immunosuppressive cell
FOXP3	Regulatory T cell
αSMA	Vascular smooth muscle cell, fibroblast
BCL-6	B cell
CD11b	Myeoloid cell
CD11c	Dendritic cell
CD14	Monocyte, macrophage
CD15	Neutrophil
CD16	Neutrophil, monocyte
CD21	B cell, follicular dendritic cell
CD31	Endothelial cell
CD34	Immune cell progenitor, vascular endothelial cell
CD38	B cell, T cell, plasma cell
CD45	Leukocyte
CD45RA	Naïve/exhausted T cell
CD45RO	Memory T cell
CD56	NK cell, neuron, glia cell
CD107a	Lysosomes
CD138	Plasma B cell, epithelial cell
CD163	Macrophage
E-Cadherin (E-Cad)	Epithelial cell
Granzyme B	CD8+ T cell
HLA-DR	Antigen-presenting cell
ICOS	Activated T cell
IDO1	immuno-suppressive cell
LAG-3	Activated T cell
NaKATPase	All cell types
Podoplanin	Follicular dendritic cell, lymphatic endothelial cell, fibroblast
S100	Immune homeostasis
Tryptase	Mast cell
VISTA	Immuno-suppressive cell
Vimentin	Mesenchymal cell

[3] Technical Note: Hyperplex immunofluorescent stainings with COMET™ show high antibody elution efficiency, intact tissue morphology, and epitope stability

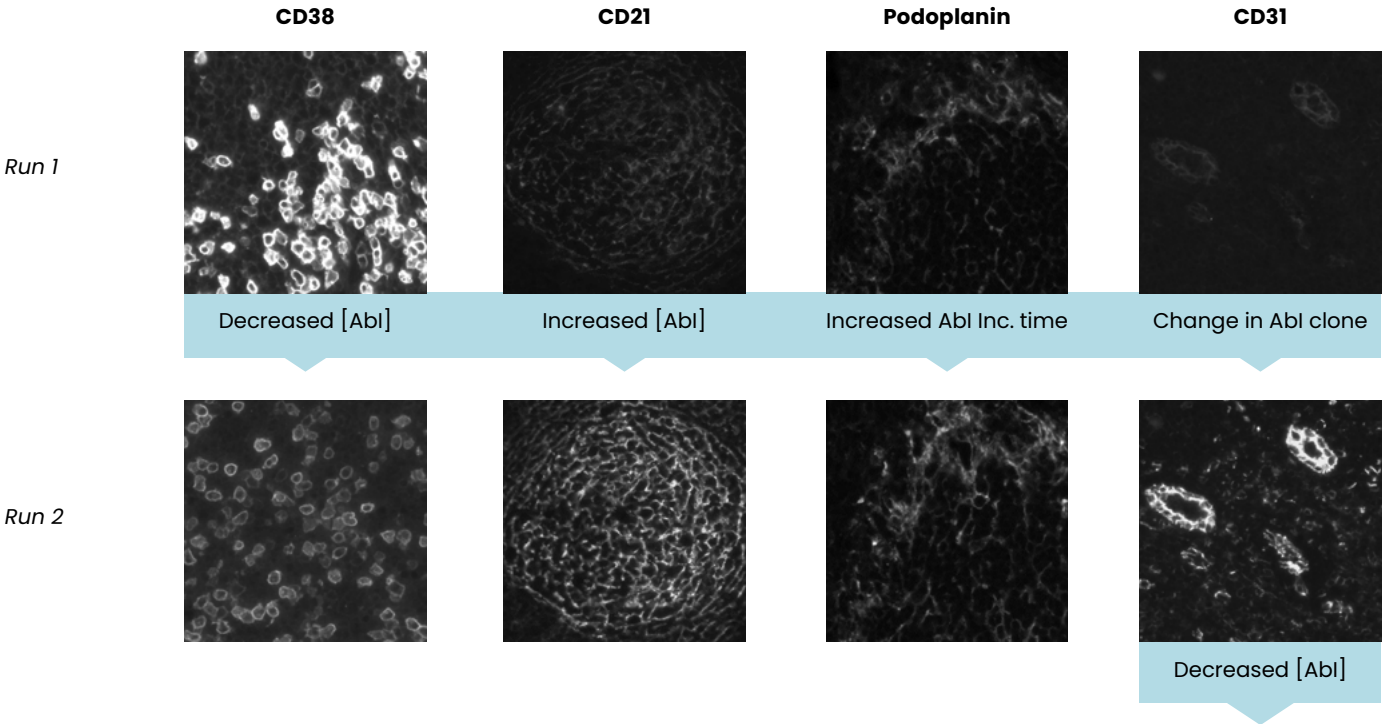


FIGURE 2

Example of batch marker optimization on COMET™. When building hyperplex panels, multiple markers can be tested, optimized, and re-assessed simultaneously on COMET™. Here, four markers are shown out of the 40-plex panel tested in the first run. In the second run, staining conditions were adjusted for each marker shown, such as decreasing or increasing primary antibody ([Abl]) concentration, increasing Abl incubation time, or changes of the Abl clone. An additional optimization step was performed in the third run by decreasing the Abl concentration to improve the staining of one marker (CD31). Podoplanin brightness was increased for visualization; no contrast adjustment.

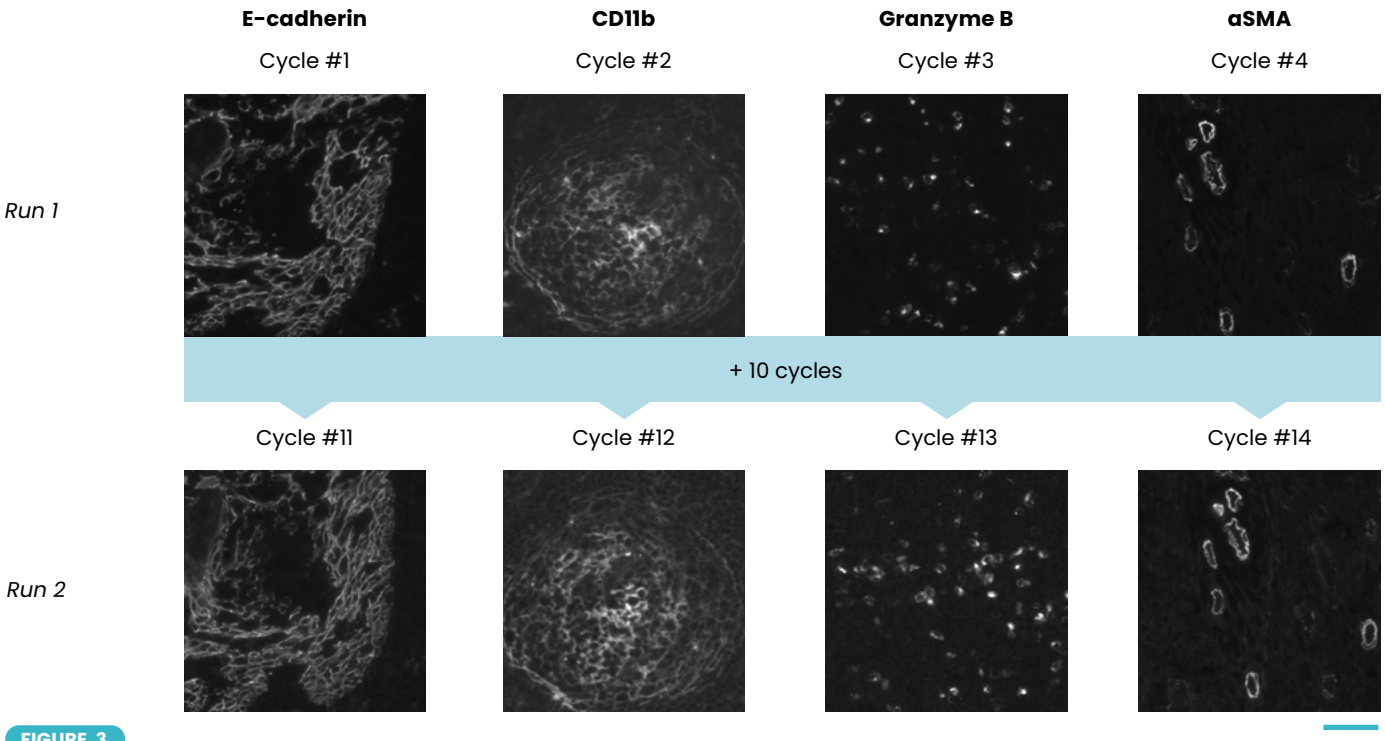


FIGURE 3

Example of flexibility in the positioning of markers in a seqIF™ assay on COMET™. CD11b brightness was increased for visualization; no contrast adjustment.

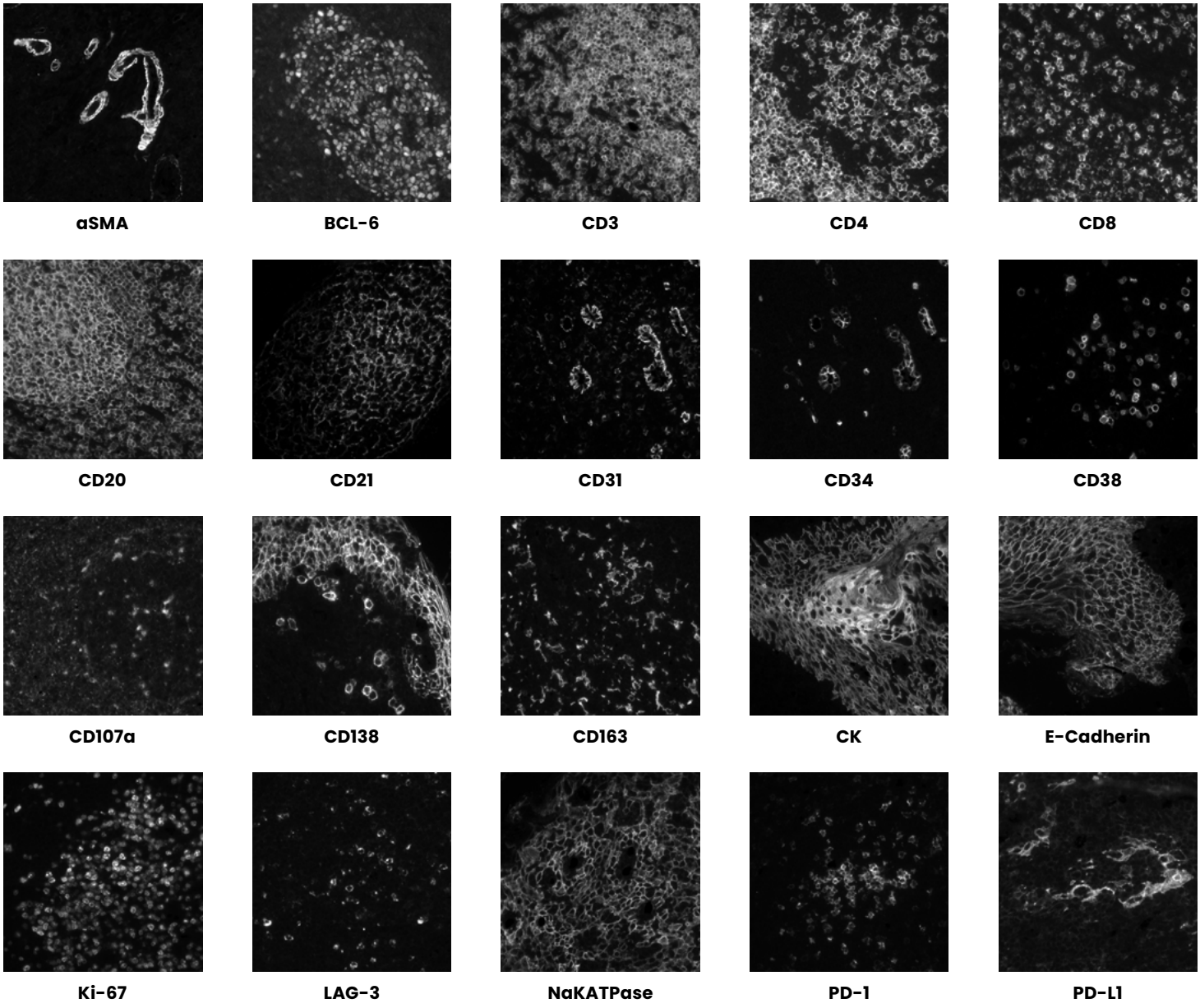
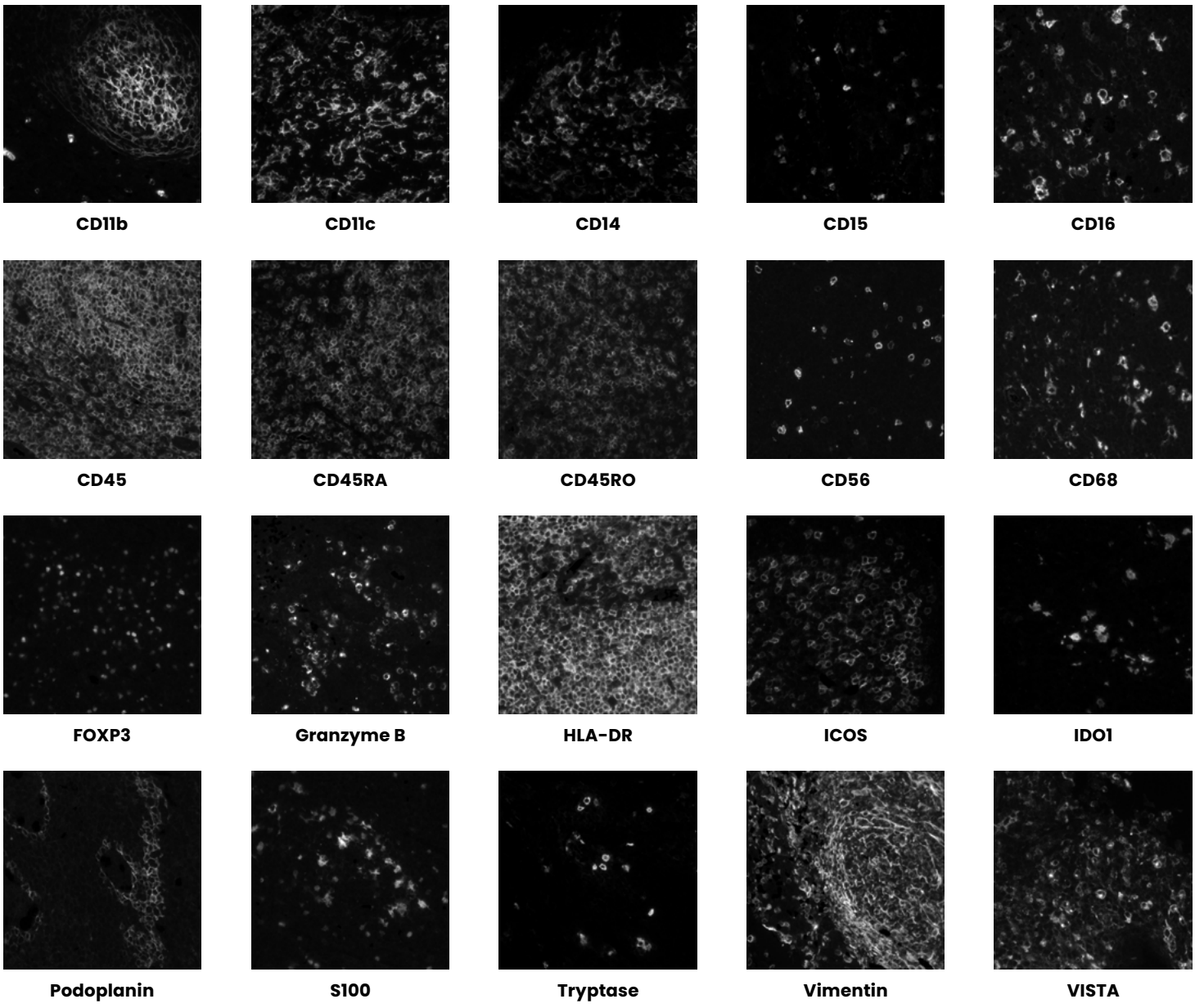


FIGURE 4

High-quality staining for each marker in the 40-plex panel on human FFPE tonsil. Staining and imaging were performed automatically in only 16 hours. All images: Autofluorescence subtracted and brightness adjusted for visualization. No contrast adjustment.

Our 40-plex panel was developed on formalin-fixed paraffin-embedded (FFPE) sections of human tonsil, a commonly available tissue that is rich in immune cells and therefore very useful for the development of immuno-oncology assays.

We used a previously established 10-plex panel[1] as a starting point. The optimization of new markers was done using the 'batch marker optimization' workflow on COMET™. Staining conditions of multiple markers were assessed and re-optimized simultaneously on the same tissue slide. As shown in Figure 2, on a few representative examples, new markers were optimized easily in only a few steps. During the optimization phase, COMET™ offers great flexibility in



the choice of marker positioning when building a hyperplex panel. Markers that were initially tested at a low-plex level were moved to higher cycles (Figure 3), confirming that seqIF™ assays preserve excellent epitope stability.

Once staining conditions were identified for all markers and the seqIF™ protocol was defined, the 40-plex panel was used to stain human tonsil tissue. All 40 markers were detected with high quality and uniformity, independently of their subcellular localization and expression levels (Figures 4 and 5). Good results were obtained in terms of specificity, signal-to-background ratio, sensitivity, and elution efficiency, while tissue morphology and epitope stability were well preserved and optimal hyperplex staining was produced.

The 40-plex assay was run on COMET™ in only 16 hours with automated staining and imaging.

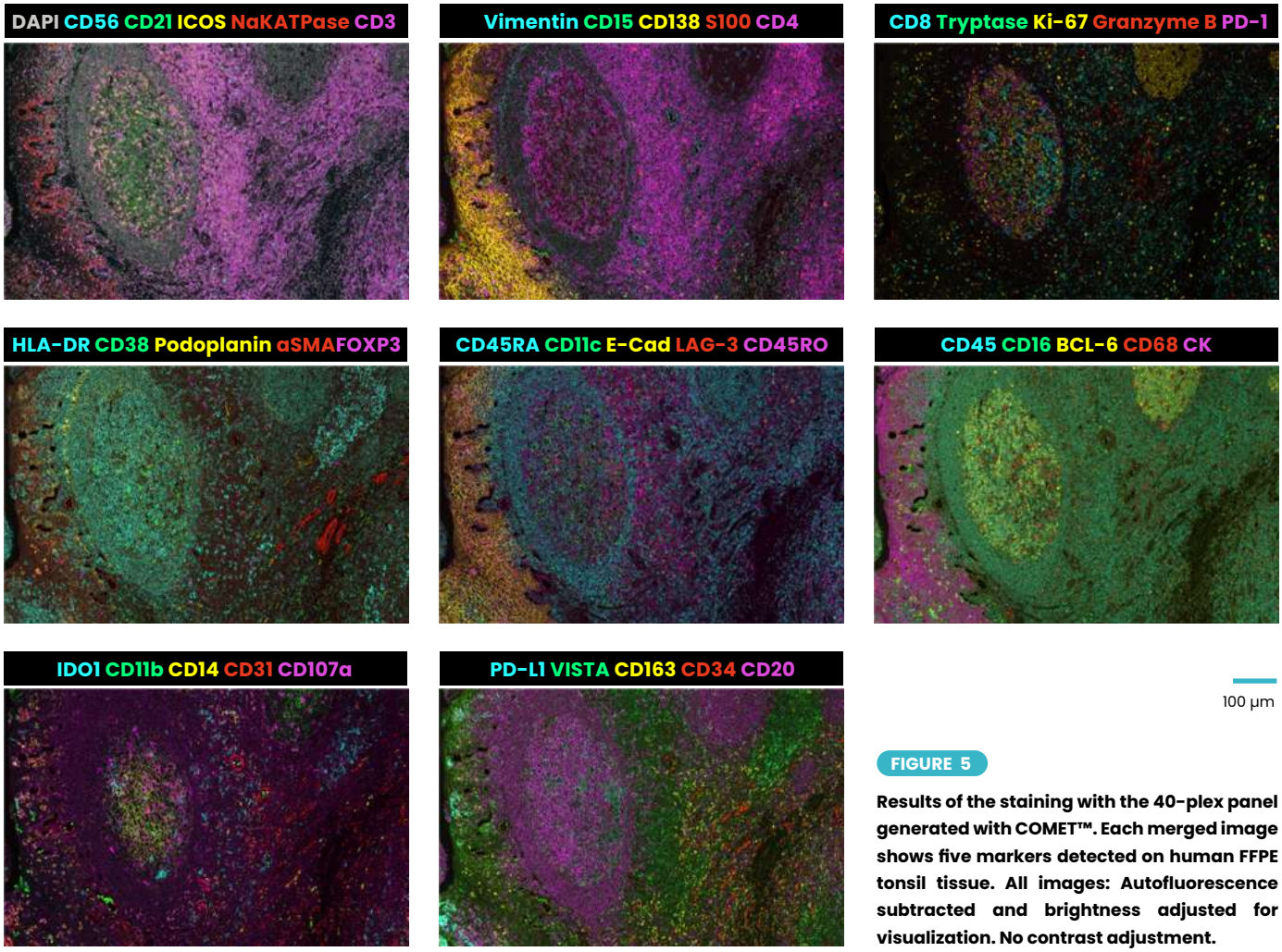


FIGURE 5

Results of the staining with the 40-plex panel generated with COMET™. Each merged image shows five markers detected on human FFPE tonsil tissue. All images: Autofluorescence subtracted and brightness adjusted for visualization. No contrast adjustment.

In order to study different tumors and profile their immune populations, we used the 40-plex panel to stain tissue microarrays (TMA), including human primary tumors and their metastasis, as well as healthy tissues (Table 2).

TABLE 2 **List of tumoral and non-tumoral tissues in the analyzed TMA, containing 48 cores.**

Patient	Primary tumor	Metastasis site
1	Lung squamous cell carcinoma (SCC)	lymph node
2-5	Lung adenocarcinoma (AC)	lymph node
6	Nodular Sclerosis Classical Hodgkin lymphoma (NSCHL)	N/A
7	Follicular lymphoma (FL)	N/A
8	Chronic / Small Lymphocytic (CLL/SLL) lymphoma	N/A
9	Diffuse large B cell lymphoma	N/A
Other patients	**Non-tumoral tissues**	
	Spleen, tonsil, skin, lymph node, brain, appendix, liver	

Hyperplex panels to streamline IO research

The staining protocol showed a high degree of transferability across multiple tissue types on the TMA with a satisfactory level of detection for all markers as shown in Figure 6. This proves the robustness of our 40-plex to address relevant questions in the IO field.

The ease of transferability of this assay allows researchers to directly compare the immune profile of control versus targeted tissues, healthy versus tumoral samples, or among a small patient cohort in the same slide in a single staining run.

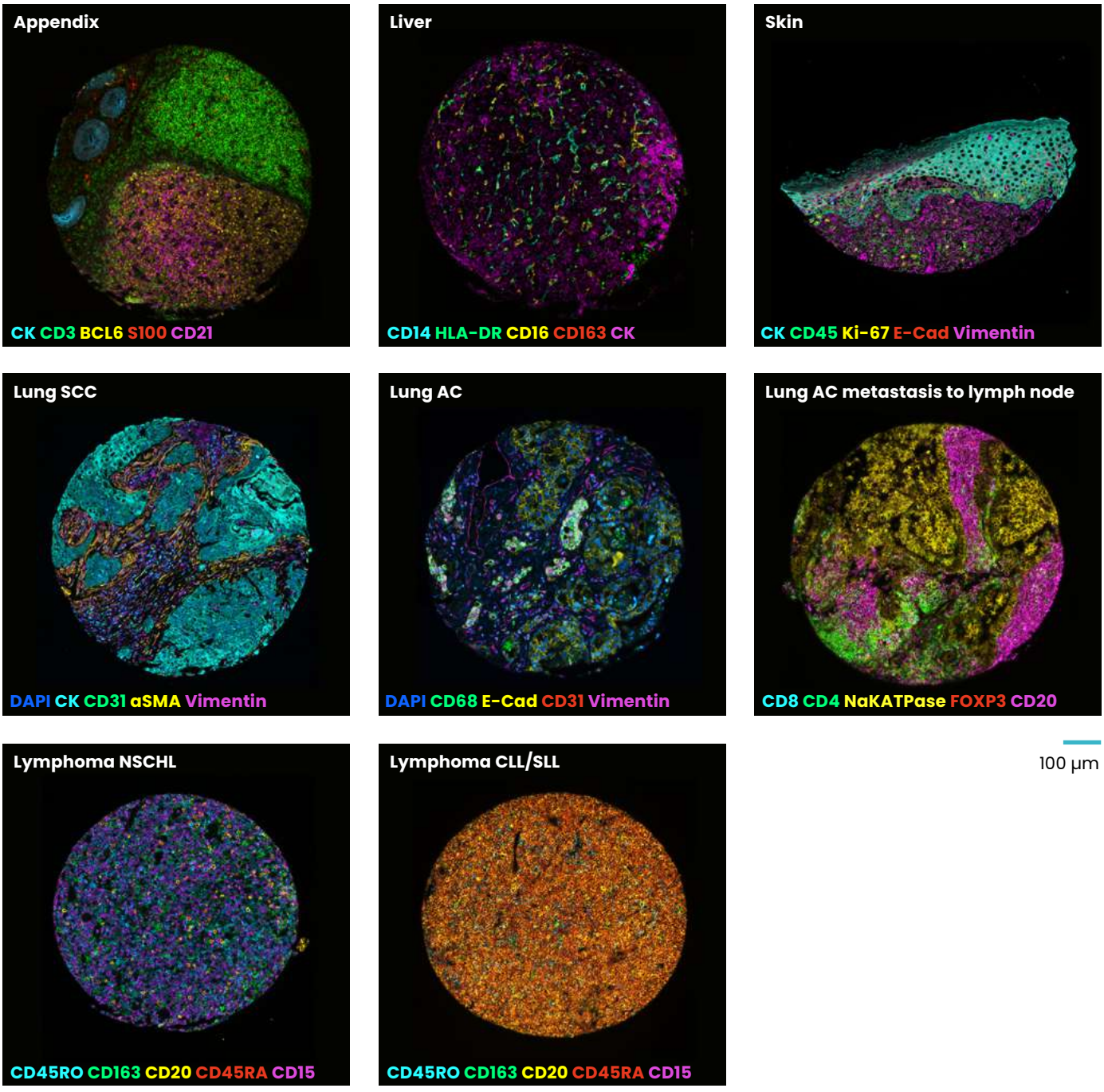


FIGURE 6 **Examples of 40-plex staining on COMET™ using human TMA cores. A selection of markers characterize different TME components or cell phenotypes. All images: Autofluorescence subtracted and brightness adjusted for visualization. No contrast adjustment.**

In IO research, critical information can be obtained by understanding which cell populations contribute to the different tumoral stages. For instance, during the formation of tumor metastasis, studying which cell types are invading surrounding tissues is highly relevant. In Figure 7, we applied our 40-plex panel to stain TMAs containing primary tumor cores and their respective lymph node metastasis. Using a different set of markers, morphological features (detected with E-cadherin, Vimentin, NaKATPase, CK, and CD31), immune cell classification (detected with CD3, CD4, CD16, CD20, CD68, CD11c, and HLA-DR), and immune cell state (detected with FOXP3 and PD-L1) were compared between the primary tumor front and metastatic tissue of the same patient (Figure 7).

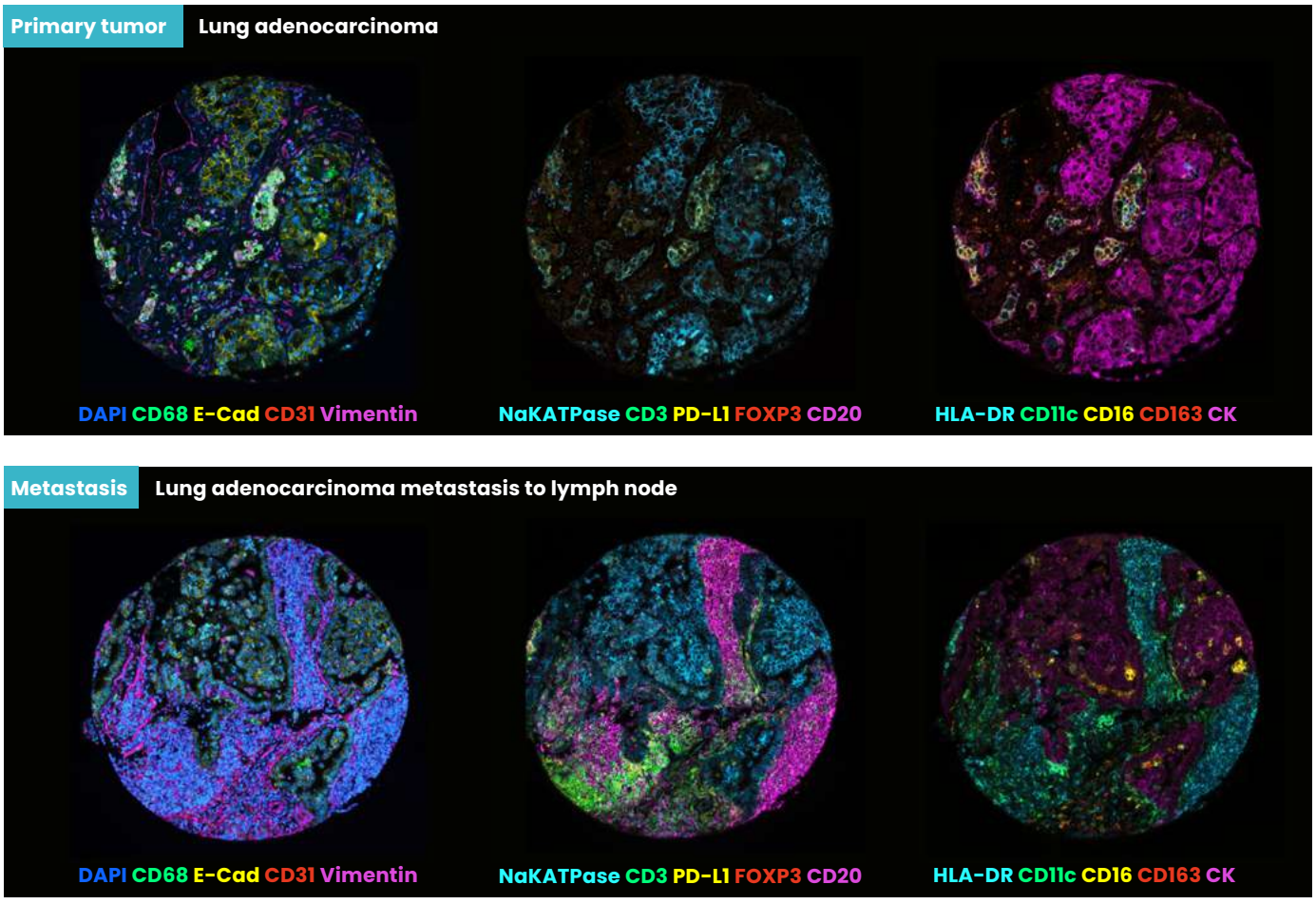


FIGURE 7

Staining of primary tumors and respective lymph node metastasis. TMA cores were stained with a 40-plex panel on COMET™ and a selection of markers is shown to characterize different TME components and cell phenotypes. All images: Autofluorescence subtracted and brightness adjusted for visualization. No contrast adjustment.

Hyperplex staining on COMET™ enables analysis of marker colocalization at single-cell level. As shown in Figure 8, B cells (CD20+) and T cells (CD4+) are shown in the proximity of epithelial tumor cells (E-Cad+). Among the immune cell populations, functional cells can be detected by marker co-expression, such as immunosuppressive cells (CD4+IDO1+) and regulatory T cells (CD4+FOXP3+). Thus, hyperplex staining on COMET™ enables immune profiling of tumoral and peritumoral regions without compromising on single-cell resolution.

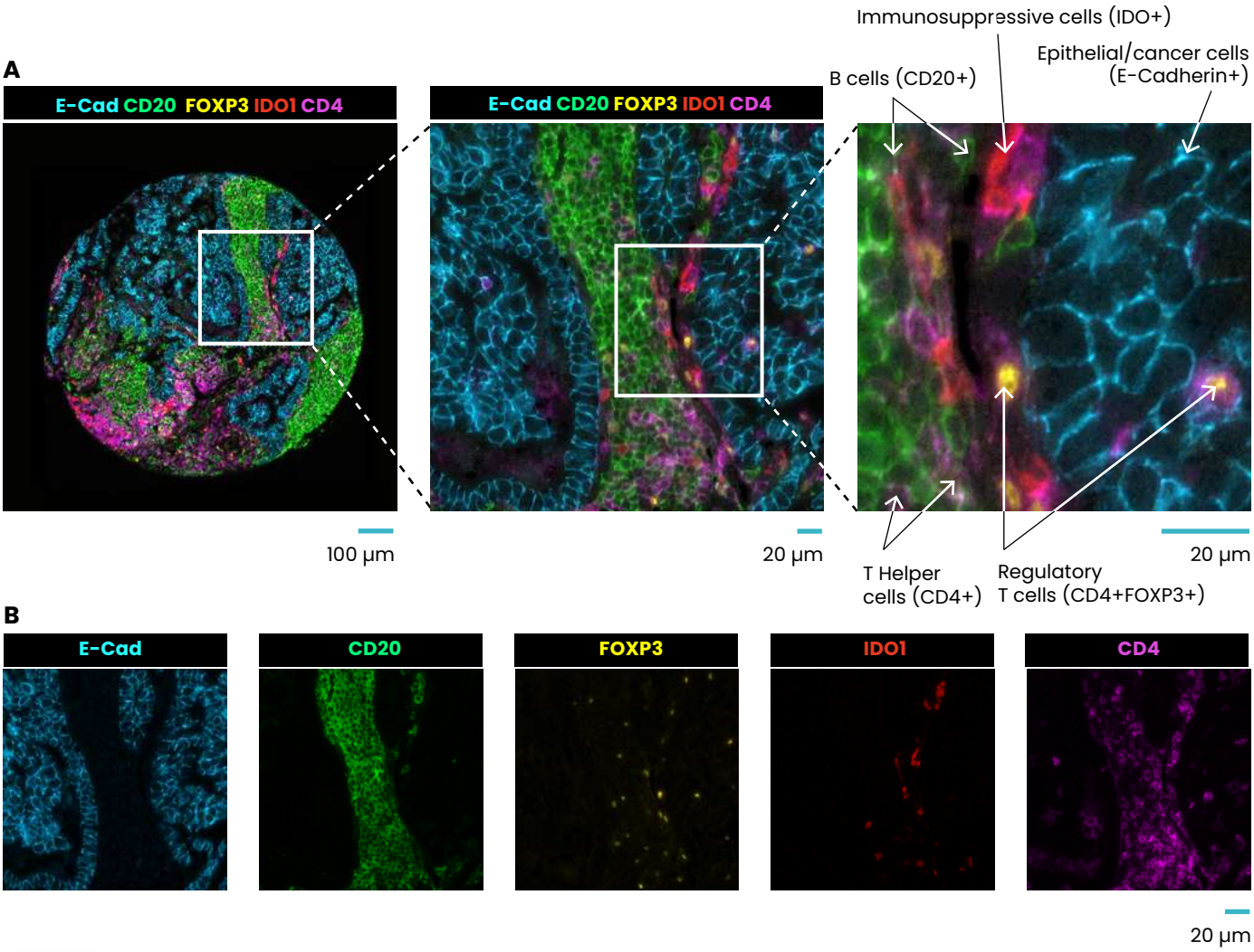


FIGURE 8

Single-cell resolution achieved with 40-plex staining on COMET™. A. Left panel shows five markers detected on a TMA of lung adenocarcinoma with lymph node metastasis, middle and right panels show a selection of regions of interest (ROIs) to identify different cell types. B. Each panel shows magnified staining for each of the five markers in panel A. All images: Autofluorescence subtracted and brightness adjusted for visualization. No contrast adjustment.

Conclusion

The TME is a key driver of response and resistance to treatment of cancer patients. Understanding the molecular composition of it will help in developing targeted therapies for each tumor type. Multiplex immunofluorescence enables the study of multiple biomarkers and provides critical information on cell-cell interaction within the TME. The development of mIF assays is challenging as it requires long optimization times and reproducible results are difficult to achieve. Researchers are also limited in the degree of flexibility to customize panels to address their specific, biological questions. COMET™ enables users to quickly develop multiplex panels by allowing researchers to use any commercially available antibody on the instrument. The simultaneous optimization of multiple markers increases the speed to establish a functional, reproducible assay and reduces the use of precious samples and reagents. Hyperplex IO panels developed on COMET™ can be applied to study core biomarkers across different tumors. Each hyperplex panel can be customized with a large degree of flexibility. Lunaphore's COMET™ supports users across basic and translational research to understand the TME and classify different tumor types, with the ultimate goal to develop targeted and efficient therapies.

Lunaphore technologies

Interested in COMET™? Ask our scientists.

info@lunaphore.com
www.lunaphore.com

LMK_TN11_032023