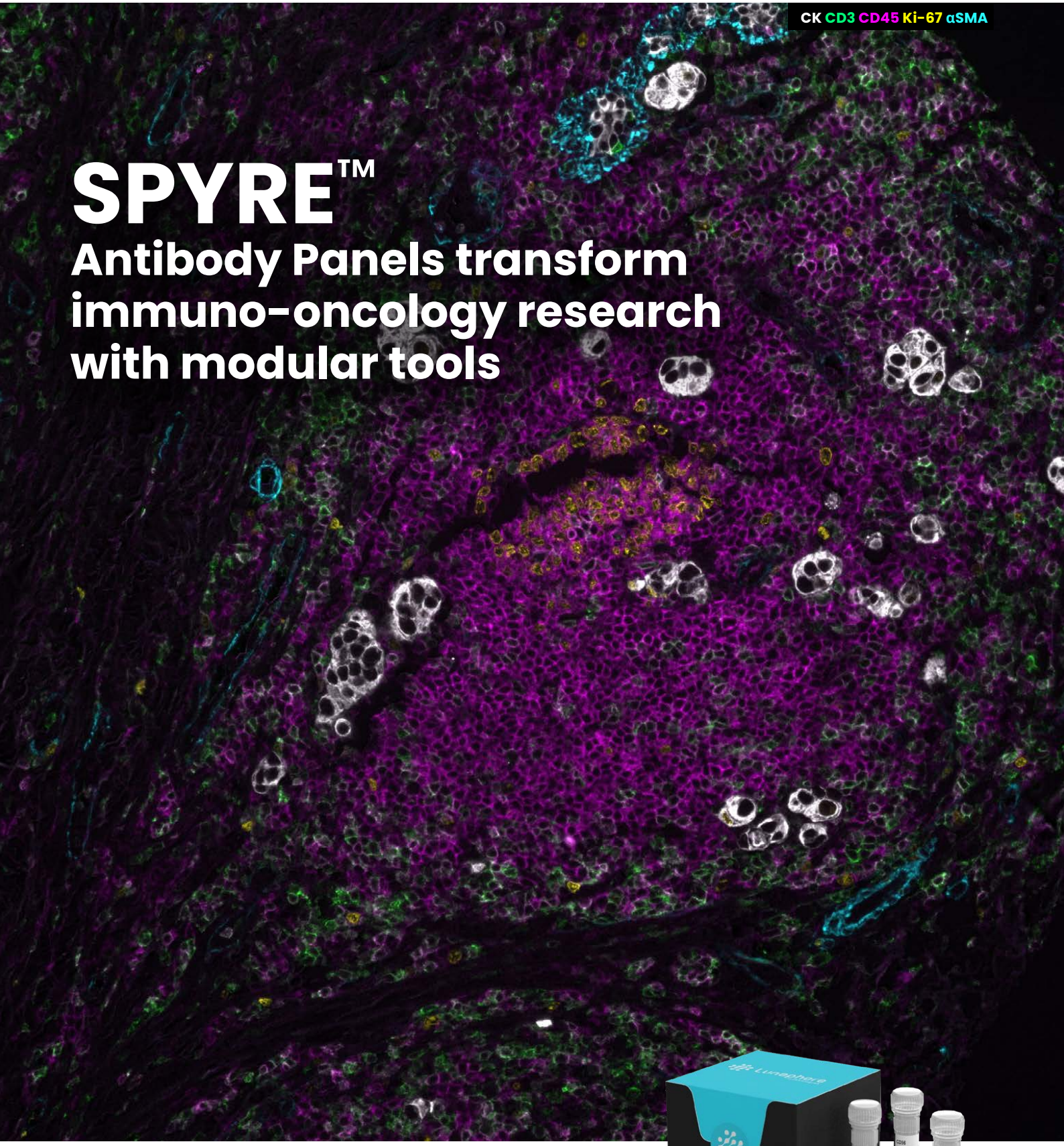
Lunaphore technologies

Human breast cancer stained with SPYRE™ Antibody Panels



SPYRE™

Antibody Panels transform immuno-oncology research with modular tools

SPYRE™ Technical Note

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Introduction

The accurate selection of cancer immunotherapies and the prediction of cancer prognosis are based on a deep understanding of the tumor microenvironment (TME). Multiplex immunofluorescence (mIF) has become an important tool in the immune profiling of the TME. Multiplex IF assays allow the study of multiple markers in the same tissue sample preserving the spatial information of tissue morphology. From basic to clinical research, mIF enables the discovery of complex cell interactions and the identification of predictive biomarkers to monitor the patient's response to immunotherapy.

Independent of the cancer type, a common approach in the characterization of the TME by mIF is to target a broad set of phenotypes aimed at identifying subclasses of immune and cancer cells and their functional statuses. Beyond the common immune markers, additional markers are commonly of interest to be investigated, such as tissue-specific markers, to characterize tumors that arise from different organs; previously unknown and new markers, to adapt to the evolving findings of the immunology research; validation markers from previous screens, to validate biomarkers identified from exploratory approaches.

Despite a fast-growing need for multiplex methods, mIF faces several barriers slowing its adoption in the field. Building multiplex panels requires fine-tuning of many interconnected conditions and can take up to several months when performed by manual staining methods. In addition, long and manual multiplex staining gives less reliable results due to user errors and tissue degradation. Upstream steps of antibody conjugation have a low-efficiency rate. Cross-validation is needed to combine barcoded antibodies every time a new panel is generated, thereby reducing the comparability and hence reproducibility of results across experiments, a downside particularly critical when analyzing large patient cohorts with different assays. In addition, once a panel is fully optimized on one tissue type, using the same panel on another tissue requires additional optimization steps to achieve high-quality results.

With SPYRE™ Antibody Panels Lunaphore has developed a simplified and flexible solution based on a modular approach to support the needs of every researcher and streamline the IO field with robustness and reproducibility.

SPYRE™ Antibody Panels as tools for IO research

COMET™ from Lunaphore is a fully automated system that performs sequential immunofluorescence (seqIF™) assays consisting of sequential cycles of staining, imaging, and elution of 2 markers per cycle. With 20 of these sequential cycles, runs of 40 markers can be performed fully automated in just a few hours on the same tissue sample and without user intervention. COMET™ is an open system and works with standard reagents and off-the-shelf antibodies.
Lunaphore has developed SPYRE™ Antibody Panels supporting COMET™ users to investigate scientific questions by mIF on COMET™. They are purpose-built tools to target commonly used immuno-oncology markers allowing scaling up immunofluorescence stainings to hyperplex assays with ease, because they can be easily expanded with customer-specific antibodies to build a panel matching the individual research question.

SPYRE™ Antibody Panels consist of label-free, primary antibodies to avoid complex and time-consuming upstream conjugation steps. SPYRE™ Antibody Panels are available as four different kits for broad immunophenotyping of the TME suitable for a large spectrum of IO and immunology research applications (see ***Table 1***):

- SPYRE™ T Cell Core Panel kit consists of 4 markers to target T cells, cytotoxic T cells, helper T cells, and regulatory T cells.
- SPYRE™ TIL Core Panel kit consists of 6 markers to identify tumor-infiltrating lymphocytes, such as T cells, B cells, and NK cells that have left the bloodstream and migrated towards the tumor site.
- SPYRE™ Immune Core Panel kit consists of 8 markers to identify main immune cell types from T and B cells to macrophages and dendritic cells.
- SPYRE™ Immuno-Oncology Core Panel kit consists of 13 inclusive markers to identify not-only immune cells (such as leukocytes, T cells, and B cells), but also stromal (fibroblasts), and functional cell statuses (such as active T cells, immunosuppressive and proliferative cells).

Panels can be easily combined with customer-specific antibodies to tailor panels to individual needs. This easily allows reaching the desired set of markers to complement spatial cell profiling of each tumor type for any biological question. In addition, COMET™ users will have exclusive access to the Panel Builder to generate customized panels by combining SPYRE™ Antibody Panels with Lunaphore-tested antibodies assembled into ready-to-use protocols that can be transferred and imported directly to COMET™.

Here, we show how SPYRE™ Antibody Panels have been optimized and validated on several human Formalin-Fixed Paraffin-Embedded (FFPE) tonsil tissues and screened across five tumor types, and how users can benefit from these tools to streamline their IO research without effort and difficulty.

SPYRE™ Antibody Panels				Marker	Cell lineage, type or function
SPYRE™ Immuno-Oncology Core Panel kit	SPYRE™ Immune Core Panel kit	SPYRE™ TIL Core Panel kit	SPYRE™ T Cell Core Panel kit	FOXP3	Regulatory T cell
				CD3	T cell
				CD8	Cytotoxic T cell
				CD4	Helper T cell
				CD56	Natural killer (NK) cell
				CD20	B cell
				CD68	Macrophage
				CD11c	Dendritic cell
				αSMA	Fibroblast, smooth muscle cell
				PD-L1	Immunosuppressive cell
				PD-1	Active / Exhausted T cell
				CD45	Leukocyte
				Ki-67	Proliferating cell

TABLE 1

Markers detected with SPYRE™ Antibody Panel kits.

SPYRE™ Antibody Panels are robust and validated on COMET™

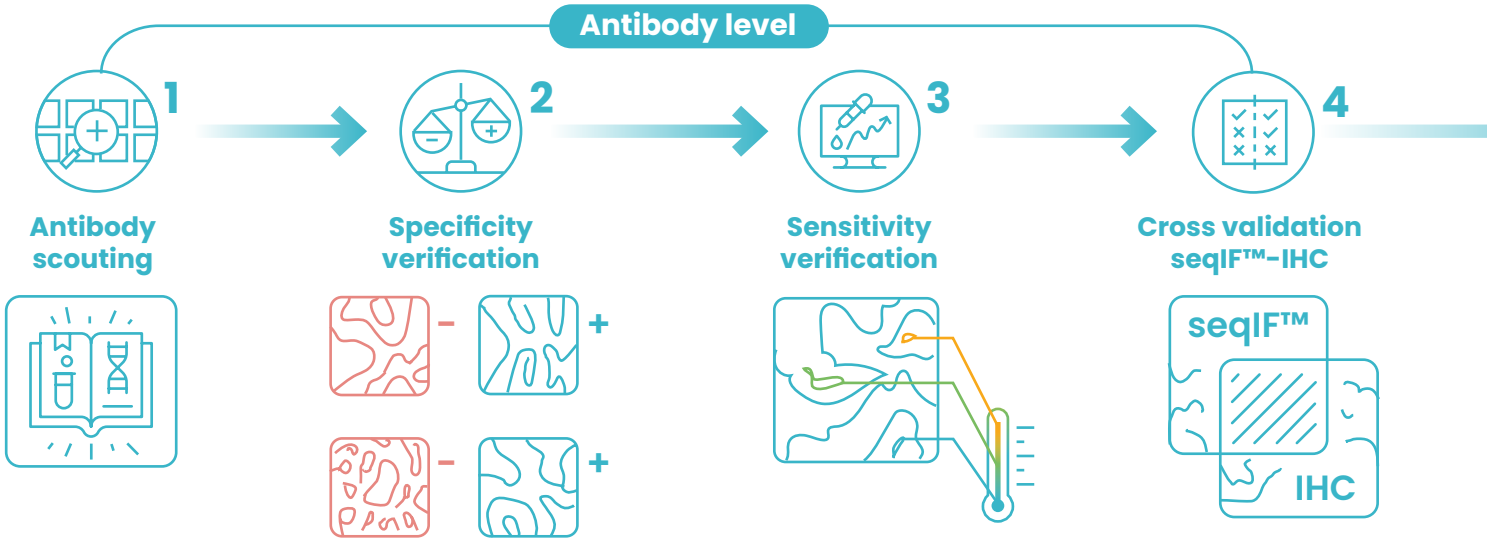


FIGURE 1

Steps of the antibody testing and validation process used to develop the SPYRE™ Antibody Panels.

SPYRE™ Antibody Panels and their COMET™ protocols are developed and optimized with the aim of studying subpopulations of immune and cancer cells as well as their functional statuses in tumors (***Table 1***). SPYRE™ Antibody Panels are assembled from primary antibodies with proven specificity, thoroughly tested at Lunaphore (***Figure 1***).

The SPYRE™ IO Core Panel, with the addition of cytokeratin (CK) marker (also available through Lunaphore as a stand-alone tumor marker antibody), was initially optimized on tonsil tissue and then transferred to different tumor types using a multiorgan tissue microarray (TMA). Using the same conditions optimized for tonsil tissue, with no further optimization steps, a high degree of transferability across multiple tissue types can be obtained for all markers with a satisfactory level of detection on five tumors (***Figure 2***): prostate, breast, melanoma, lung, colorectal cancers, as well as non-tumoral tonsil and appendix (used as positive control tissues).

The direct transferability from tonsil to tumors across different cancers shows the strength of the SPYRE™ Antibody Panels, confirming the robustness of these panels for applications in immuno-oncology research.

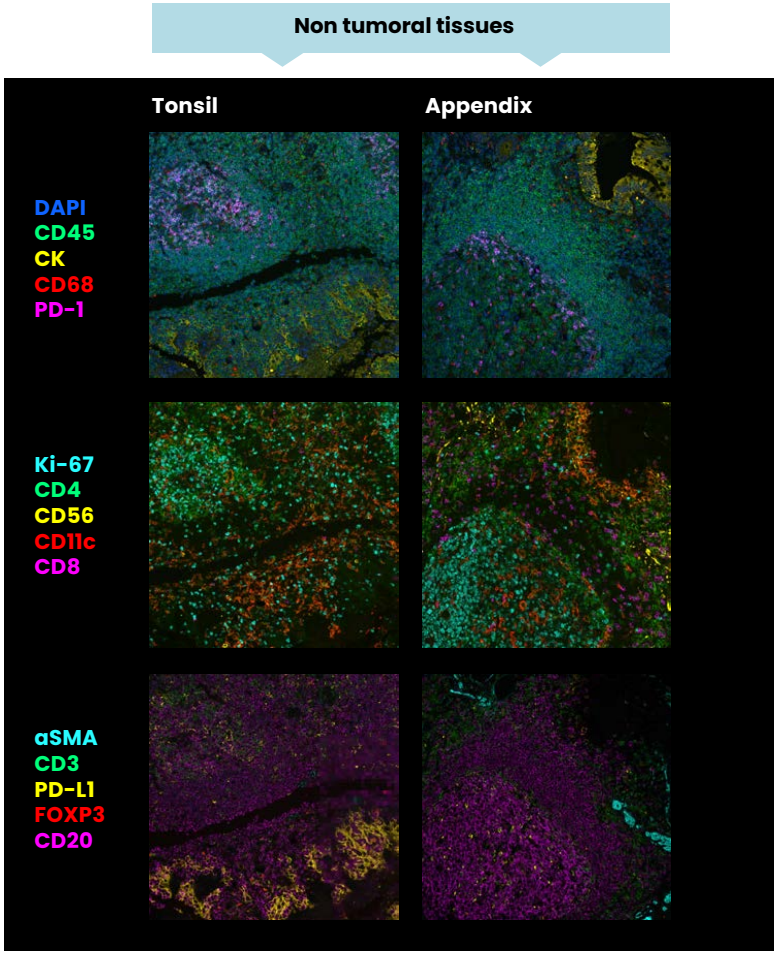
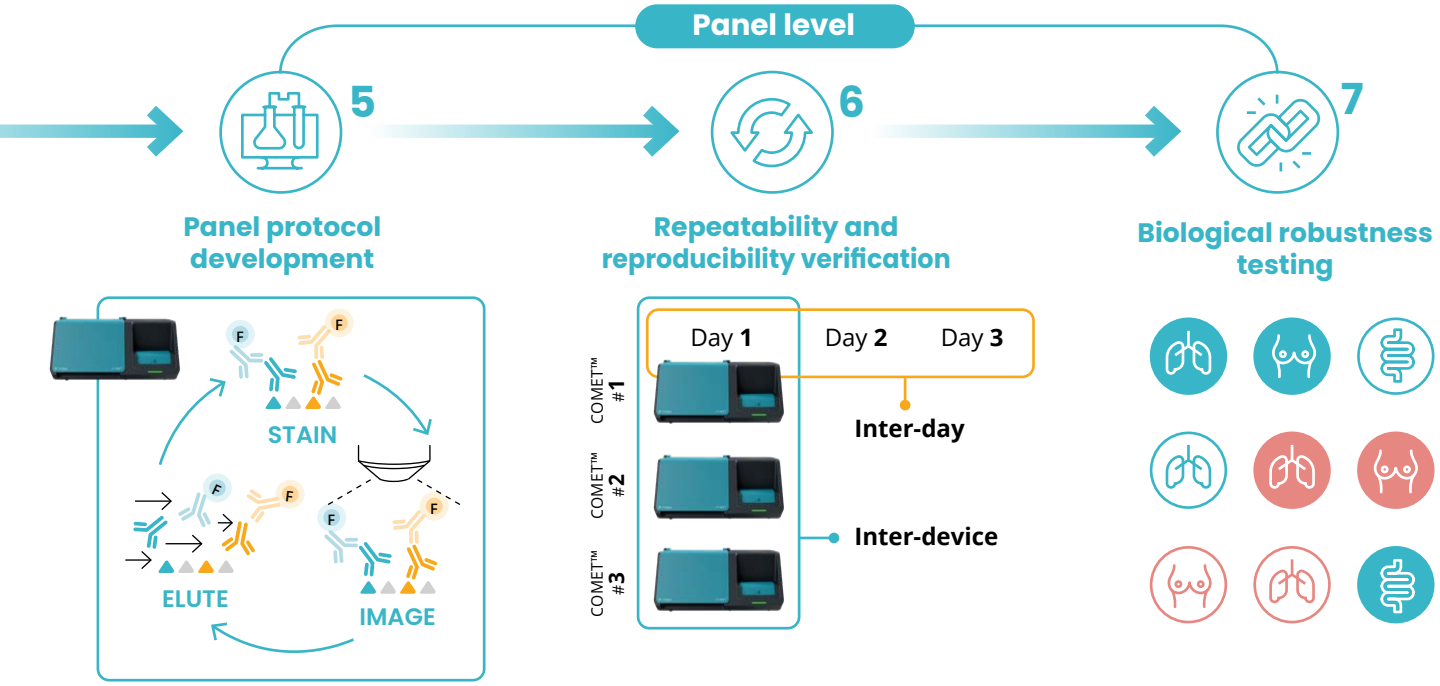
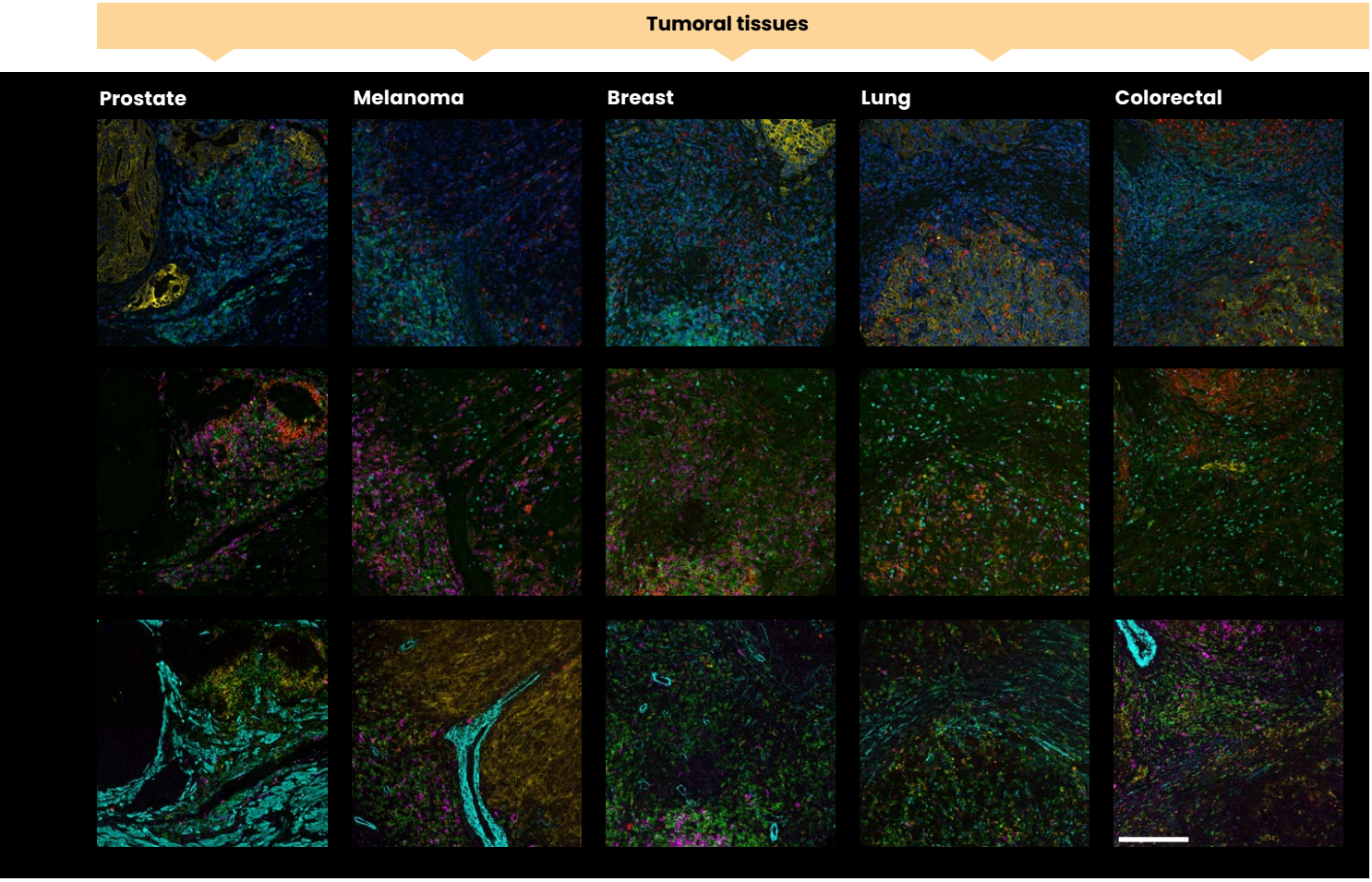


FIGURE 2

Validation of SPYRE™ Antibody Panels on multiple human tissues demonstrates biological robustness. For all images above, background was subtracted and brightness adjusted for visualization purposes. No contrast adjustment. Scale bar: 100 µm.


Panel level
5
6
7
Panel protocol development
Repeatability and reproducibility verification
Biological robustness testing
STAIN
ELUTE
IMAGE
Day 1
Day 2
Day 3
COMET™ #1
COMET™ #2
COMET™ #3
Inter-day
Inter-device


Tumoral tissues
Prostate
Melanoma
Breast
Lung
Colorectal

A powerful tool to streamline IO research

Using SPYRE™ Antibody Panels on COMET™, different cell phenotypes and cell statuses can be discriminated by comparing the co-expression of markers with high-quality staining data on a single sample, while preserving tissue integrity.

Measuring proliferation rate: differentiating low and high proliferative tumors to predict prognosis and aggressiveness

The TME consists of a complex network of tumoral and non-tumoral cells. Emerging evidence shows that the interaction between different cell populations in the TME can modulate the response to therapies. In addition, each TME has a unique composition, which can vary between tumor types and patients with the same tumor. Thanks to SPYRE™ Antibody Panels, the TME composition can be promptly analyzed as in the example shown in Figure 3, where subclasses of $CD45^+$ immune cells can be discriminated, such as B cells ($CD45^+CD20^+$) and NK cells ($CD45^+CD56^+$). The additional marker used to identify cells with epithelial origin, CK, allows the labeling of tumoral cells (***Figure 3***).

Proliferation is one of the most fundamental biological processes in tumors. Measuring the proliferation rate can provide useful information on the prognosis and aggressiveness of each cancer. Ki-67 is a nuclear protein that has been widely used as a marker to assess the proliferation rate. The co-expression of the proliferative marker Ki-67 with immune or epithelial markers helps identifying proliferating immune cells ($CD45^+Ki\text{-}67^+$) and proliferating tumoral cells from epithelial origin ($CK^+Ki\text{-}67^+$) (***Figure 3***).

Using SPYRE™ Antibody Panels on a TMA allows for analyzing multiple samples as well as tumor types simultaneously and under the same conditions. In this example, colorectal cancer (CRC) core shows high proliferative activity, whereas a prostate carcinoma shows lower tumor cell proliferation (***Figure 4***).

Mapping immune cell subpopulations: the dual role in promoting or inhibiting tumor growth

In tumor development different immune cells play crucial roles by triggering a response against cancer cells or inhibiting tumor growth. SPYRE™ Antibody Panels allow to phenotype immune cell populations at single-cell level.

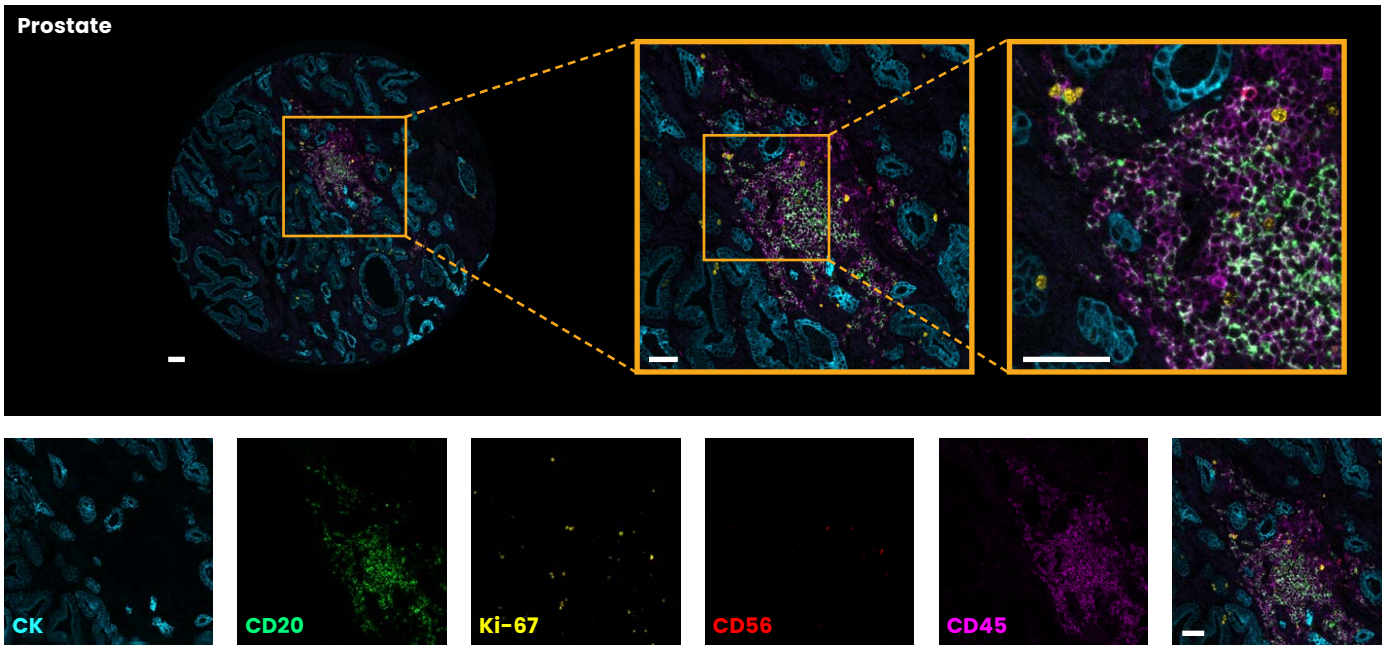


FIGURE 3

Proliferative and non-proliferative immune and cancer cells can be identified with SPYRE™ Immuno-Oncology Core Panel. For all images above, background was subtracted, and brightness adjusted for visualization purposes. No contrast adjustment. Scale bars: 50 µm.

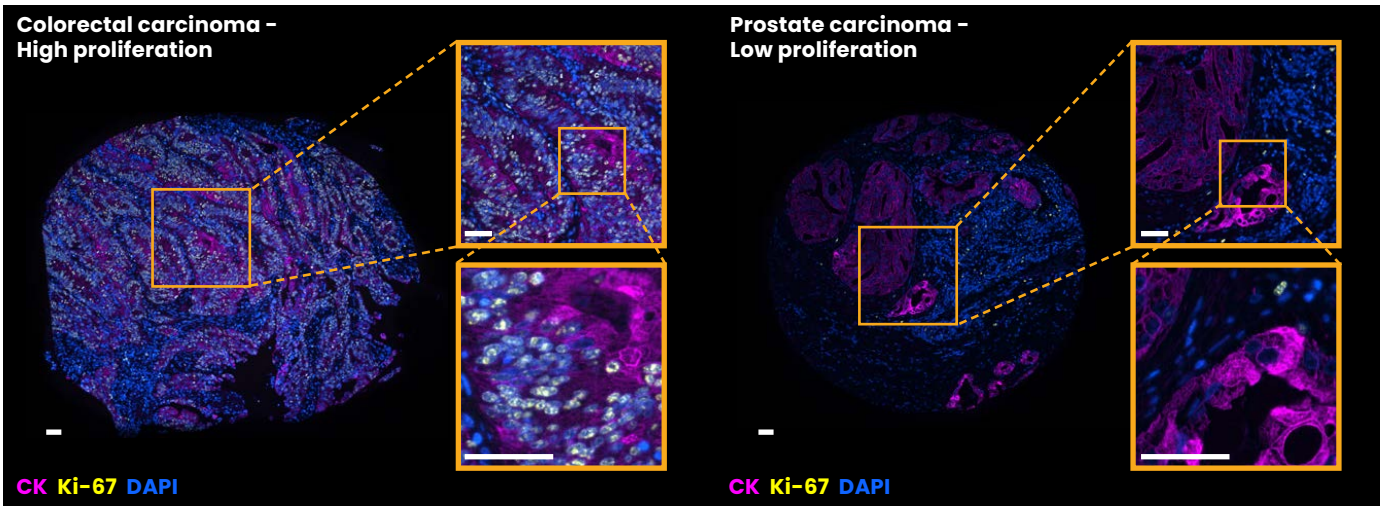


FIGURE 4

An example of two TMA cores that show high (left) and low (right) proliferation rate. Scale bars: 50 µm.

The expression of the master transcription factor FOXP3 characterizes regulatory T (Treg) cells. They play an essential role in immune homeostasis by preventing autoimmunity and suppressing exaggerated immune responses. However, Tregs are involved in tumor development and progression by inhibiting anti-tumor immunity because of their immune suppressive effect. High levels of Treg cells in the tumor microenvironment are associated with poor prognosis in many cancer types. In ***Figure 5***, the SPYRE™ T Cell Core Panel and the CK markers are used to stain a breast carcinoma core where Treg ($CD4^+FOXP3^+$) are identified near cancer cells (CK^+) and other T lymphocytes ($CD3^+$).

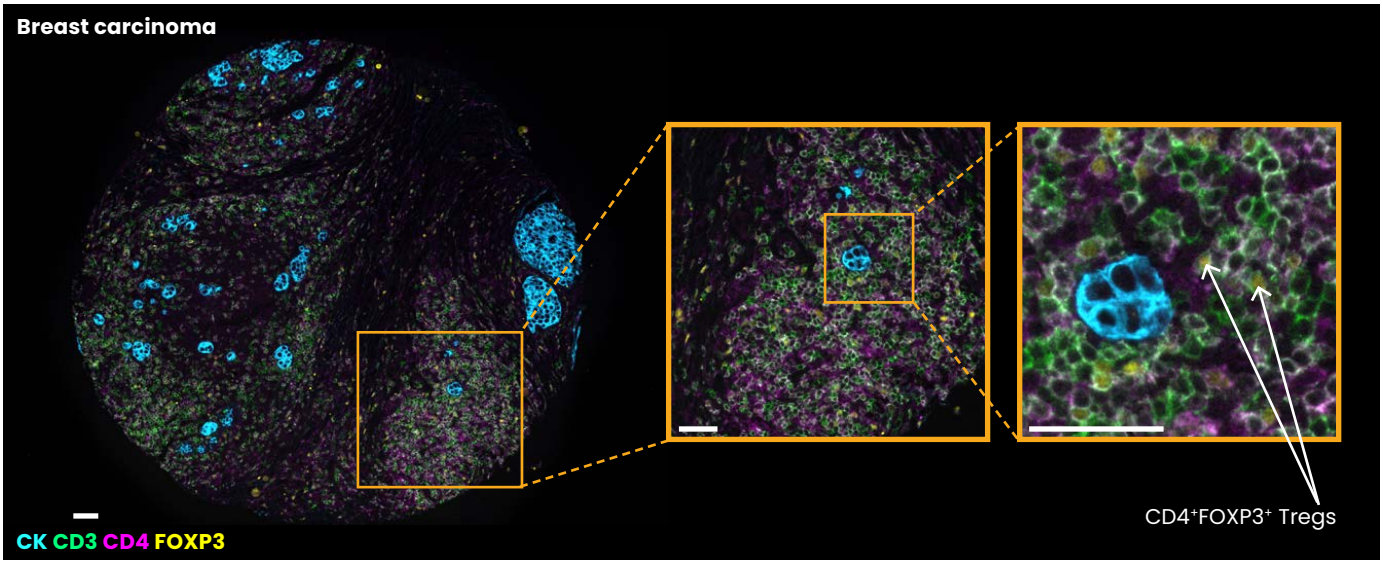


FIGURE 5

Tregs detected at single-cell resolution with SPYRE™ Antibody Panel on COMET™. Scale bars: 50 µm.

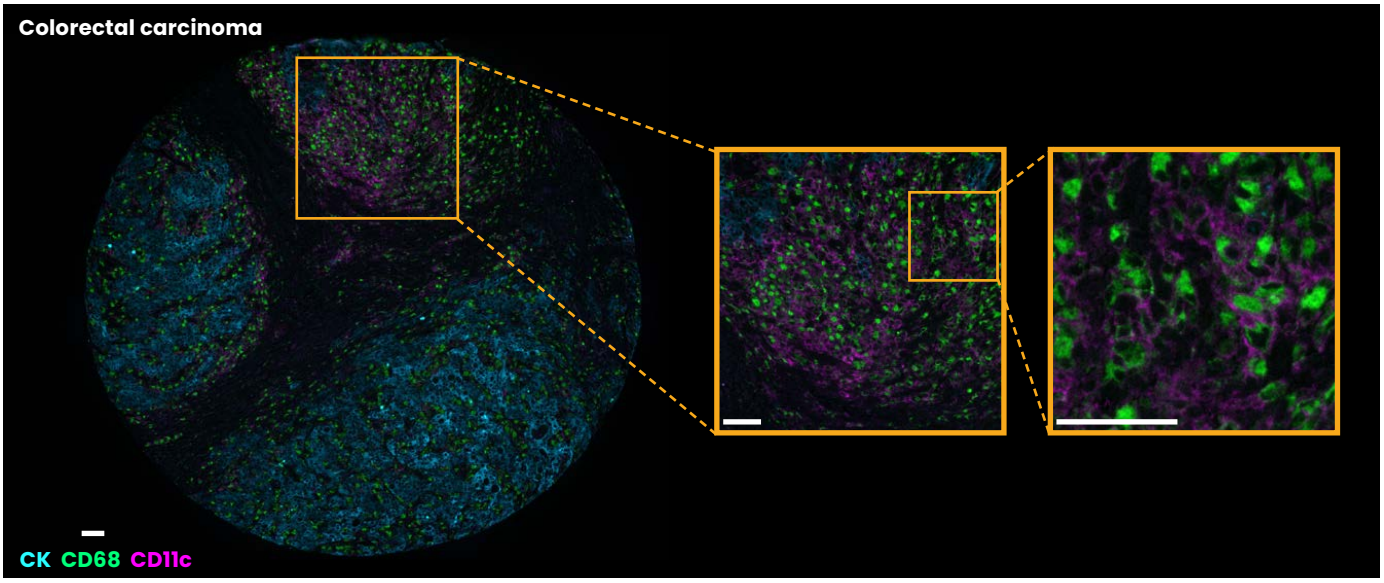


FIGURE 6

Macrophages (CD68⁺) and dendritic cells (CD11c⁺) can be identified with high quality in the TME using SPYRE™ Antibody Panels. Scale bars: 50 µm.

Dendritic cells and macrophages are additional important components of the immune system's response to cancer but play different roles. Dendritic cells are specialized, antigen-presenting cells that play a crucial role in initiating and regulating immune response against cancer. While macrophages can have a dual role in promoting or inhibiting tumor growth, depending on the context. Dendritic cells are usually identified by CD11c, an integrin expressed on the cell membrane. Macrophages are commonly identified by the expression of the CD68 marker, which is a glycoprotein found predominantly in the lysosomes. Using SPYRE™ Antibody Panels, both, dendritic cells and macrophages, can be discriminated with high quality on different tumor types, such as CRC as shown in ***Figure 6***.

Studying immune surveillance: how tumors can evade the immune system

PD-1 is a common immunosuppressive marker on the surface of T cells, indicating an activation and potential exhaustion of the lymphocyte. It plays a key role in downregulating the immune system and advancing self-tolerance. ***Figure 7*** shows how the SPYRE™ IO Core Panel allows for the straightforward identification of PD-1⁺ T cells (PD-1⁺CD3⁺) in close proximity to tumor cells (CK⁺).

PD-1's primary ligand is PD-L1 and is constitutively expressed, for example, on antigen-presenting cells. Some tumors make use of the immunosuppressive function and overexpress PD-L1 to evade immune surveillance. In such tumors, PD-L1 expression is normally associated with poor prognosis. In this melanoma sample, SPYRE™ IO Core Panel supports the identification of immunosuppressive environment where PD-L1 is broadly expressed. (***Figure 7***). At the same time, cytotoxic T cells (CD8⁺) and tumor vascularization (αSMA⁺ vessels) can be analyzed on the same sample.

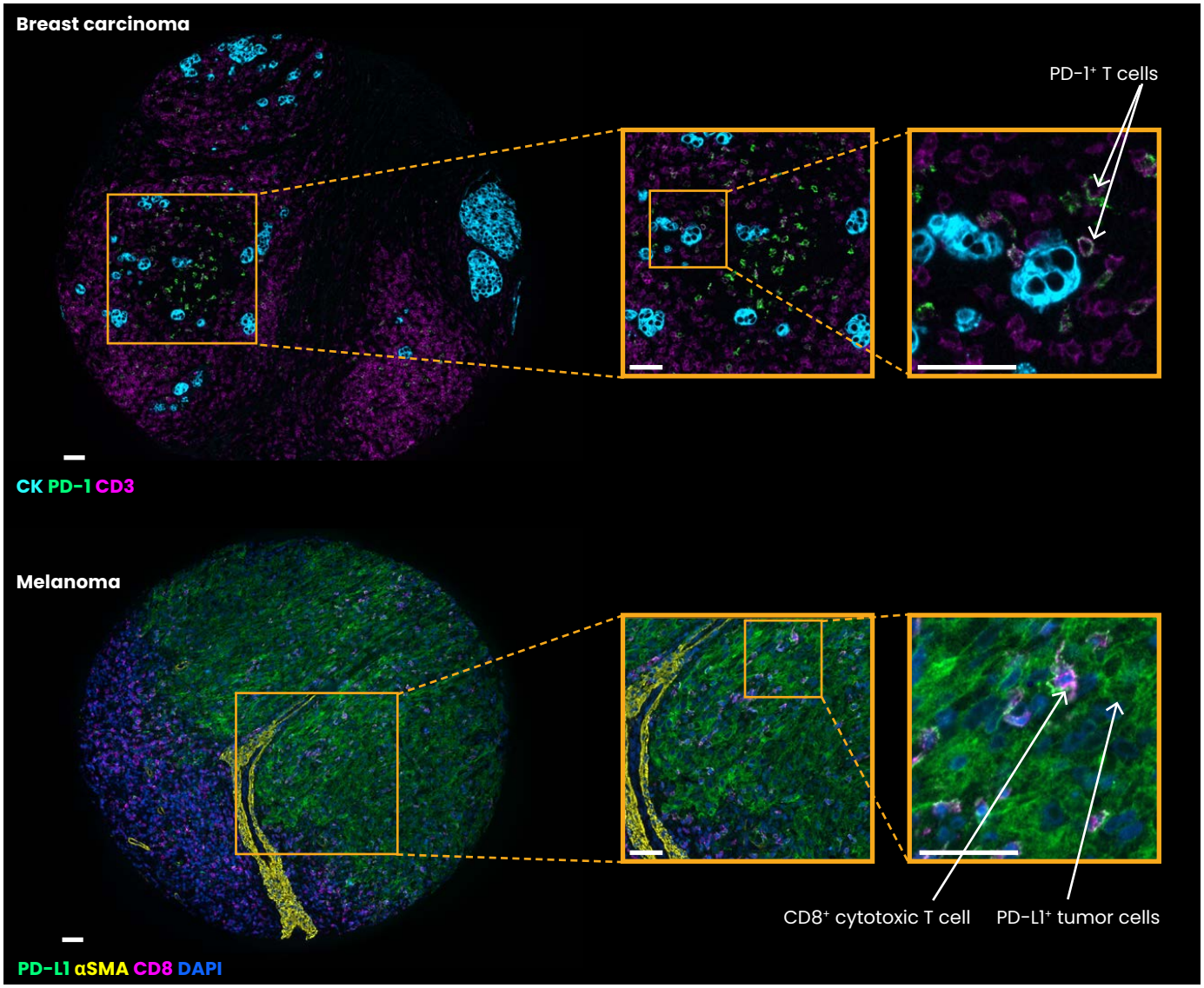
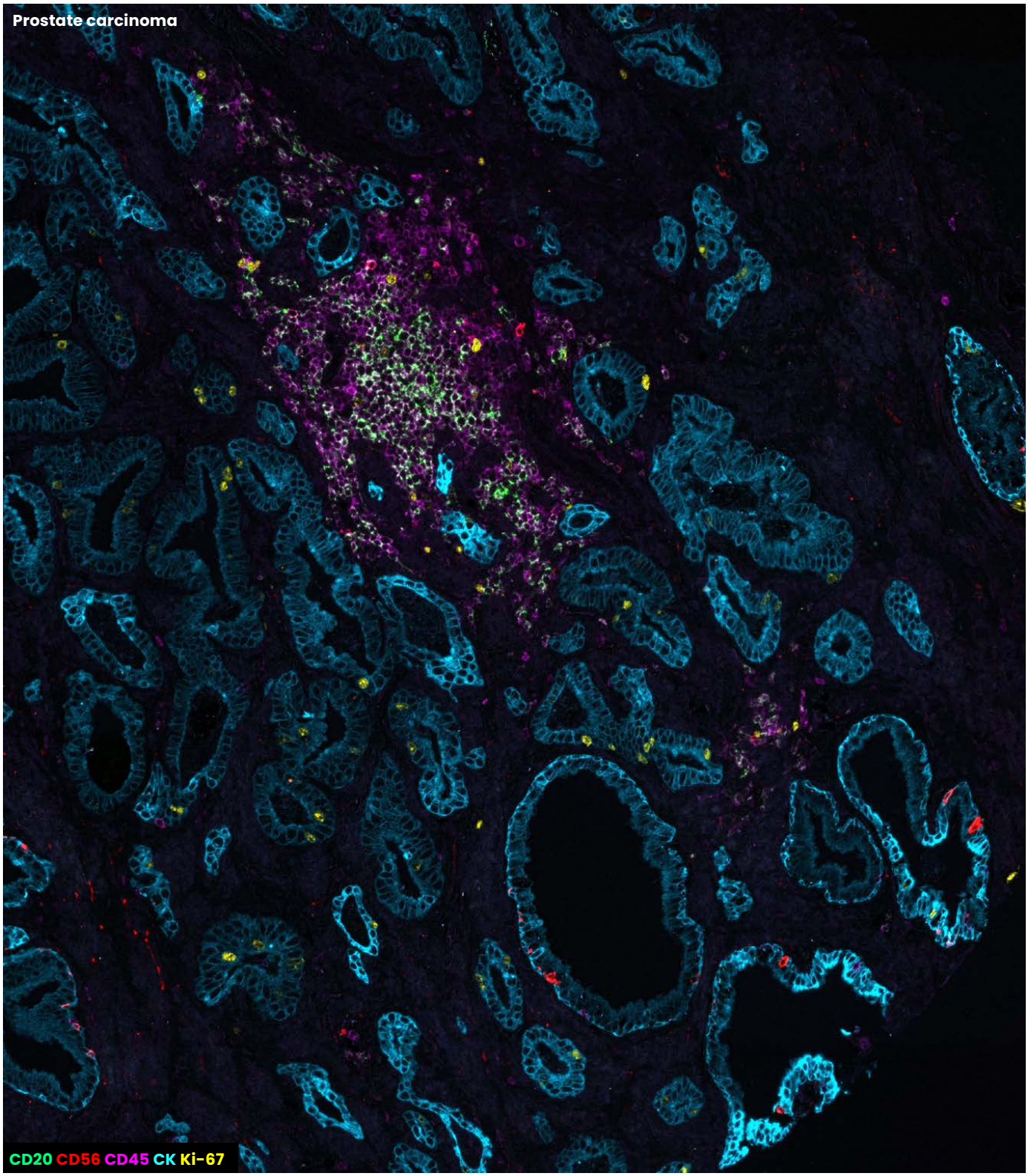


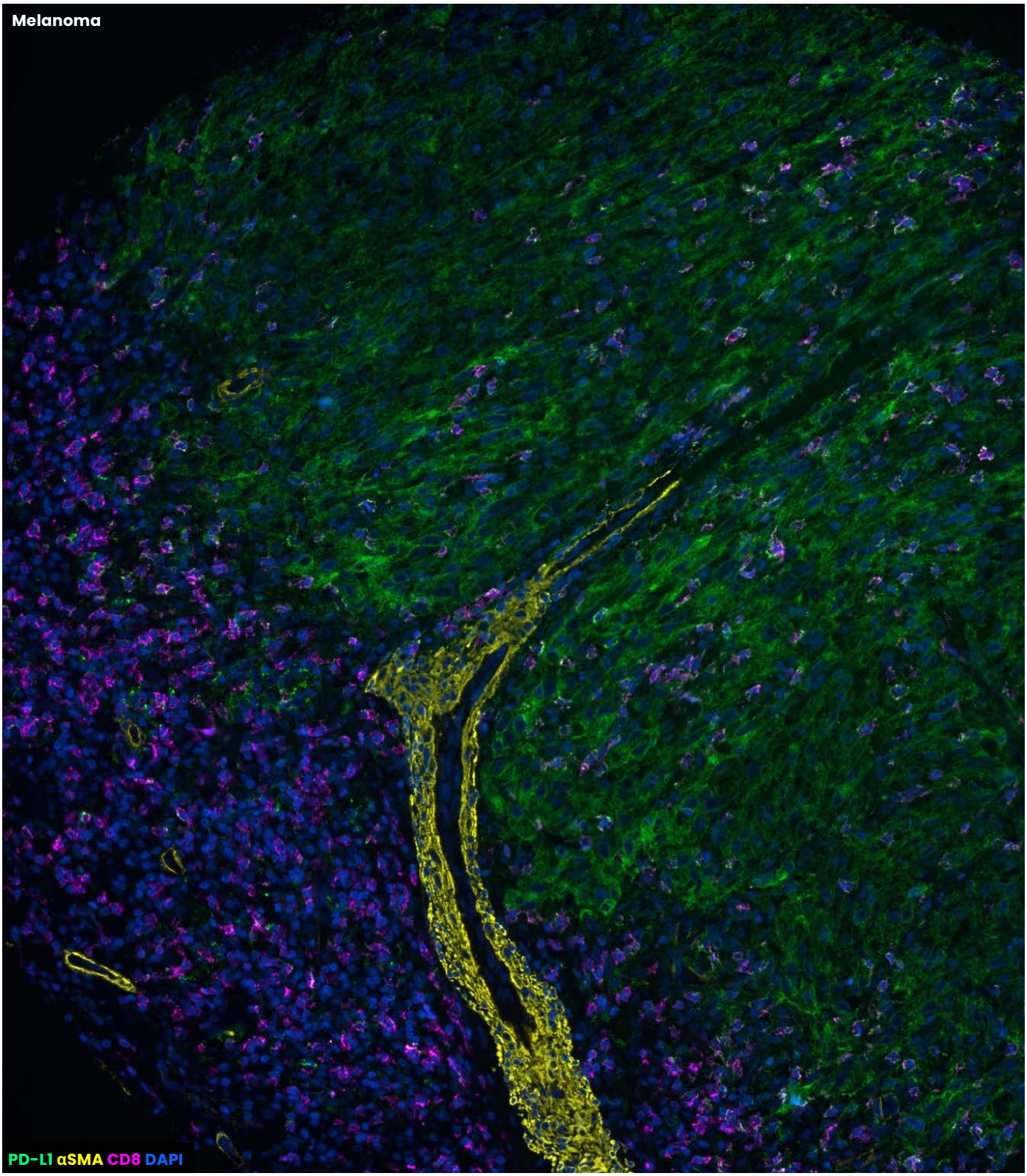
FIGURE 7

Immunosuppressive phenotypes such as PD-1⁺ T cells and PD-L1⁺ tumor cells can be identified with SPYRE ™ Antibody Panels. Scale bars: 50 µm.

Conclusions

Detailed immune profiling is crucial to improve predictions for patient response to checkpoint blockade immunotherapy. Multiplex immunofluorescence enables the study of multiple biomarkers and provides critical information on cell-cell interaction in the TME. Assays require robust validation but also a large degree of flexibility in order to be used across multiple tissue types. SPYRE™ Antibody Panels empower researchers to study the TME with ease and flexibility. These validated panels on COMET™ are ready-to-use tools to profile cancer and immune cells. SPYRE™ Antibody Panels are modular and can be quickly customized with 'add-on' markers either via the Panel Builder with Lunaphore-tested antibodies or any other commercially available and label-free, primary antibodies. SPYRE™ Antibody Panels can be easily transferred from one tissue type to another on COMET™, enabling researchers to identify core biomarkers across different tumors to develop novel targeted immunotherapies.


Prostate carcinoma
CD20 CD56 CD45 CK Ki-67


Melanoma
PD-L1 αSMA CD8 DAPI

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