From a tissue to another one:
easy adaptation of a core immuno-oncology panel

The development of novel immune-therapies and diagnostic tests requires a detailed understanding of the immune system and tumoral cells in the tumor microenvironment (TME). Multiplexed immunofluorescence (IF) is one of the emerging technologies to study the spatial distribution of TME biomarkers in their topographic context and in a quantitative manner.

Developing a multiplex IF panel of TME biomarkers usually requires weeks of work with traditional procedures. In addition, when studying several tissue types, a multiplex IF panel cannot be easily adapted to other tumors for reasons such as heterogeneity between tumor types, variability among tumor sections, different patterns of biomarker expression, diverse tissue architectures, as well as variation of tissue autofluorescence.

Thus, when transferring a multiplex staining protocol to a new tissue type, the optimization of many conditions needs to be taken into consideration. Important optimization parameters to consider include antibody concentration, incubation time, and antibody elution. With conventional manual methods, optimization can take several weeks of work and requires the use of large amounts of expensive reagents and precious sample.

LabSat® allows a significant reduction in time as well as lowering the barrier of optimization of tissue staining. With outstanding flexibility to control a broad spectrum of IF protocol parameters, the user-friendly interface allows easy modification of workflow conditions such as antigen retrieval temperature incubation time and many others. This enables a fast and simple protocol optimization to a new tissue type.

In this study, a 6-plex panel was transferred from tonsil to non-small-cell lung carcinoma (NSCLC) whole-tumor, in two simple steps and only two days.

A validated 6-plex immuno-oncology panel

Tonsil is commonly used as positive control tissue for immune panels since it contains several immune cell types and offers easier marker optimization. Tonsil has previously been used to establish a core 6-plex panel on LabSat® using FFPE sections. This core 6-plex panel consists of markers to identify several TME components, such as CK for tumoral cells, CD8 for cytotoxic T cells, and CD68 for macrophages (***Table 1***). In the same panel, functional information is given by staining FoxP3 as regulatory T cell marker and PD-L1 or PD-1 as checkpoint markers.

A 6-plex and DAPI staining was performed using Opal® 7-color IHC kit in a total staining time of 4 hours and 14 minutes on LabSat® (***Figure 1***). Images were acquired with Mantra™, a spectral microscope with deconvolution capability of overlapping emission/excitation spectra, thus allowing to analyze up to 6 markers.

Marker	TME Component	Cell lineage	Subcellular localization	Fluorophore
CD68	Membrane	Myeloid	Membrane	Opal® 620
FoxP3	TIL	Lymphoid	Nuclear	Opal® 570
CD8	TIL	Lymphoid	Membrane	Opal® 540
PD-1	Checkpoint	Lymphoid, Myeloid	Membrane	Opal® 690
PD-L1	Checkpoint	Lymphoid, Myeloid, Cancer, Epithelial	Membrane	Opal® 520
CK	Tumor	Epithelial	Membrane	Opal® 650

TABLE 1 **A 6-plex immuno-oncology panel for the study of tumor microenvironment (TME). TIL: Tumor-infiltrating lymphocyte cells**

Performance evaluation and identification of re-optimization needs

The 6-plex panel described above was used to stain FFPE sections of NSCLC in order to investigate whether preliminary staining quality was sufficient or needed to be optimized.

For this, the 6-plex protocol was run sequentially on one tonsil and one NSCLC section. As a control, immunohistochemistry (IHC) of the corresponding tissue was used as reference of marker expression.

All staining was performed on the same day, with IHC staining being performed on a separate instrument.

Figure 2 shows all markers to display lower intensity levels on NSCLC compared to tonsil tissue and compared to IHC control staining when a protocol optimized for tonsil is used. CD8 (Opal 540®) was poorly detected, compared to the IHC control on NSCLC, indicating need for optimization.

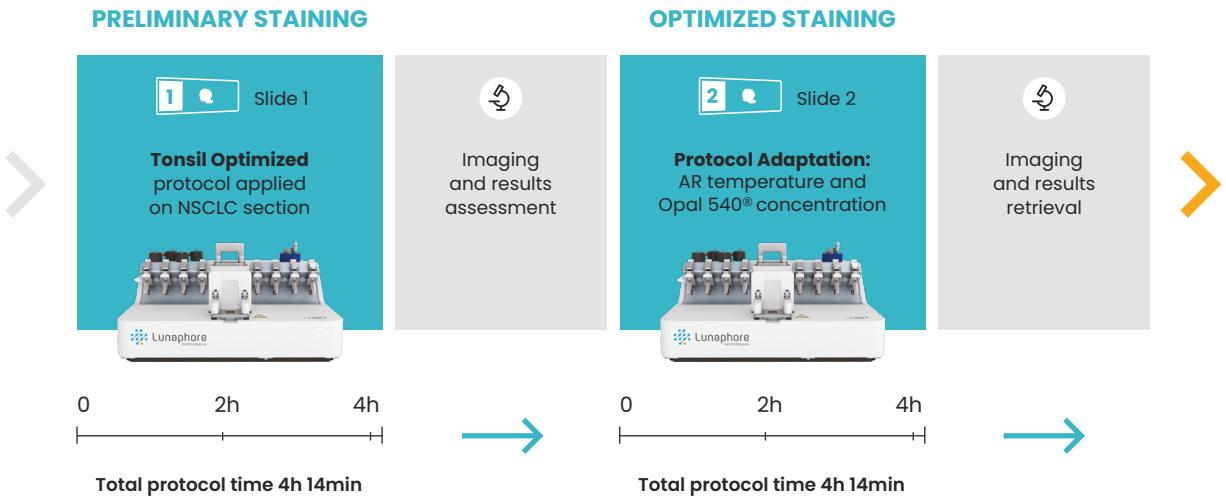

FIGURE 1 **Workflow: 6-plex with Opal® 7-color IHC Kit. Indicated the two steps to optimize the protocol on NSCLC. (AR: antigen retrieval)**

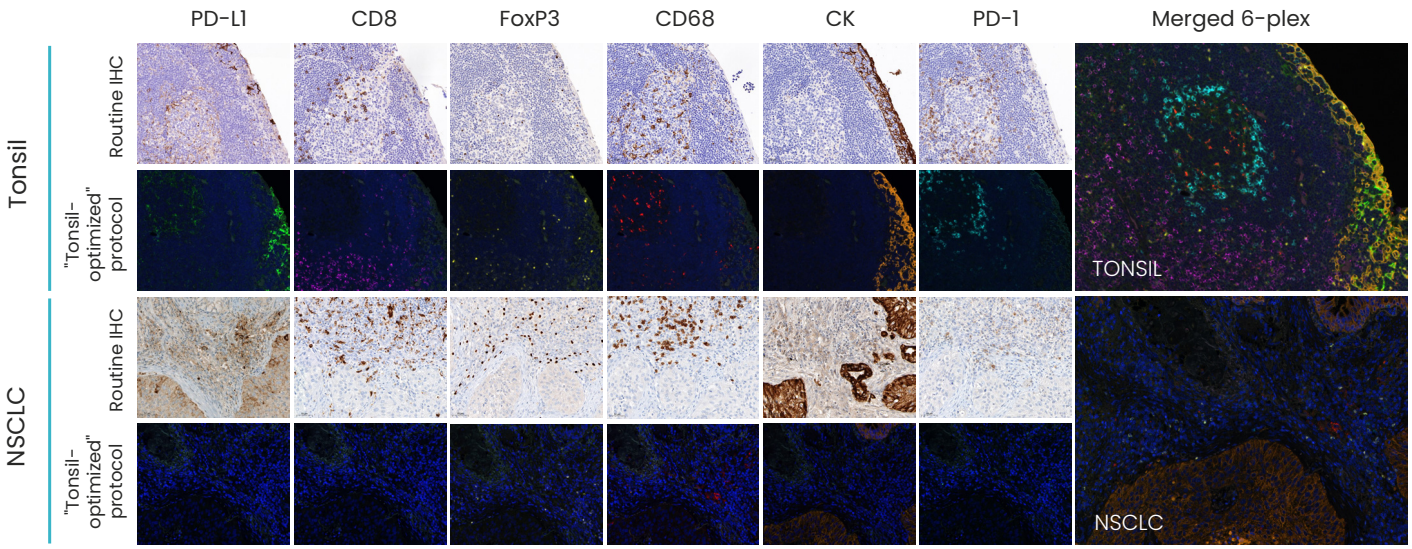

FIGURE 2 **Application of a previously established protocol for tonsil to NSCLC showed lower intensity levels than expected for all markers compared to tonsil.**

Protocol adjustment in 2 steps

The lower intensity for all markers suggests a lack in antigen retrieval efficiency for the lung compared to tonsil tissue than an issue with primary antibody conditions. In addition, only one marker (CD8) was particularly weak compared to the others, indicating the need for further optimization of the staining with this marker in particular. Therefore, two consecutive protocol adjustments were applied (***Figure 1***):

i) Increase of antigen retrieval temperature in the first 2 cycles, in order to improve the antigen retrieval efficiency for all markers.

ii) Increase of Opal 540® concentration, in order to improve the detection of CD8. The Opal 540® concentration remained low enough to avoid saturation of tyrosine residues and therefore, did not affect subsequent cycles.

Both adjusted conditions were then applied within the same protocol to a new tissue sample. The staining time of 4 hours and 14 minutes was remained unchanged as the incubation time for each cycle was unaffected by the new conditions.
These changes obtained a high-quality detection for all markers in NSCLC (***Figure 3***) already on the second day of protocol optimization and by using only two tissue samples.

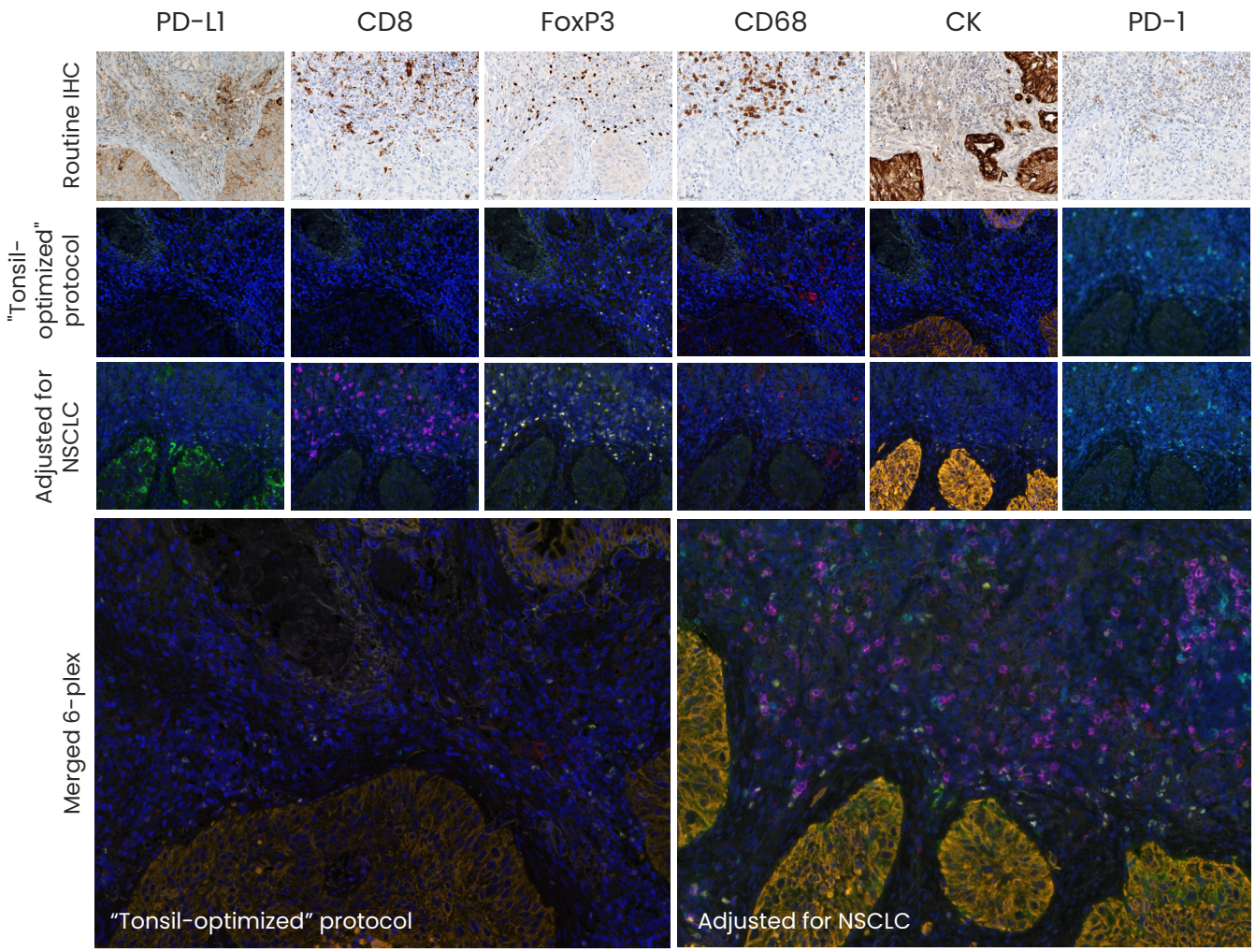

FIGURE 3 **Optimized TSA-based 6-plex protocol resulted in high-quality detection of all markers on NSCLC.**

Robust results on multiple tissue patterns

The same protocol was applied to a tissue microarray (TMA) of NSCLC to assess staining results on different tissue patterns. ***Figure 4*** and ***Figure 5*** show high-quality staining of all markers on different samples of the NSCLC TMA. Despite the tumor and sample heterogeneity, automated staining gave fast, robust, and reproducible results. All cell types, including macrophages (CD68), cytotoxic T-cells (CD8), and tumoral cells (CK) were reliably identified. Furthermore, T-reg (FoxP3), PD-L1+, and PD-1+ cells were clearly detectable as established markers of immunomodulation.

Conclusions

LabSat® allows easy protocol adaptation through a user-friendly interface and easy adjustment of parameters. With only a few and simple optimization steps, LabSat® significantly reduces time as well as the amount of sample and reagents needed for protocol transfer. Within two days and using only two tissue samples, a core 6-plex immuno-oncology panel was transferred from tonsil to NSCLC sections making LabSat® a flexible solution to advance immune-oncology research.

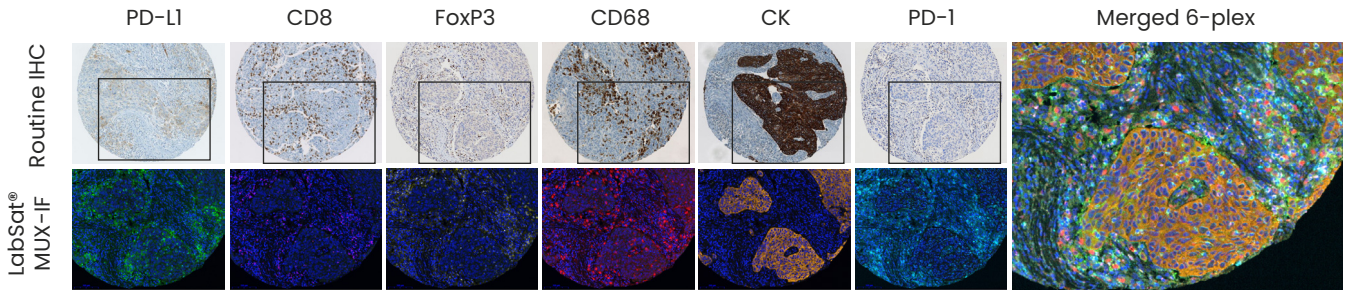

FIGURE 4 **Optimized and automated TSA-based 6-plex protocol gives robust results on NSCLC TMA.**

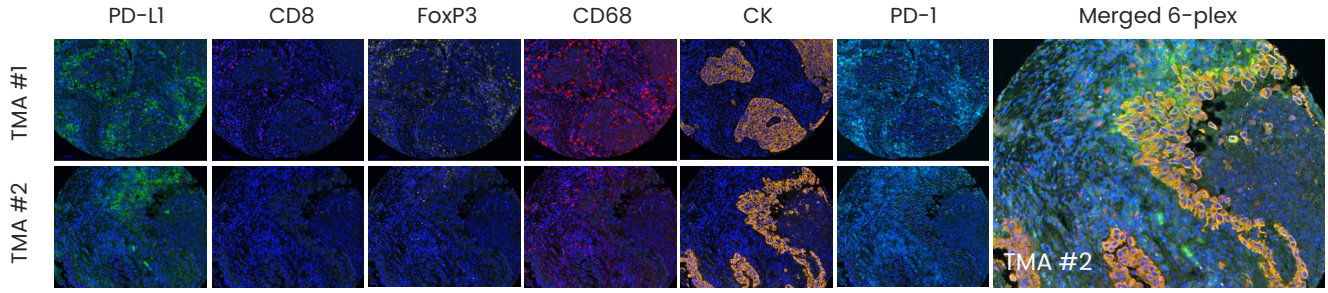

FIGURE 5 **Automated and fast staining of NSCLC TMA.**

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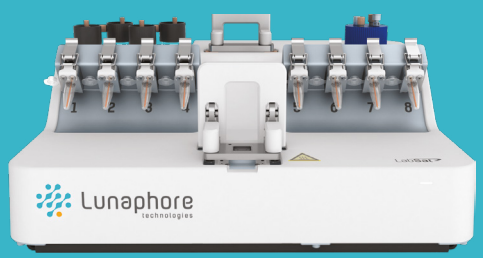

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