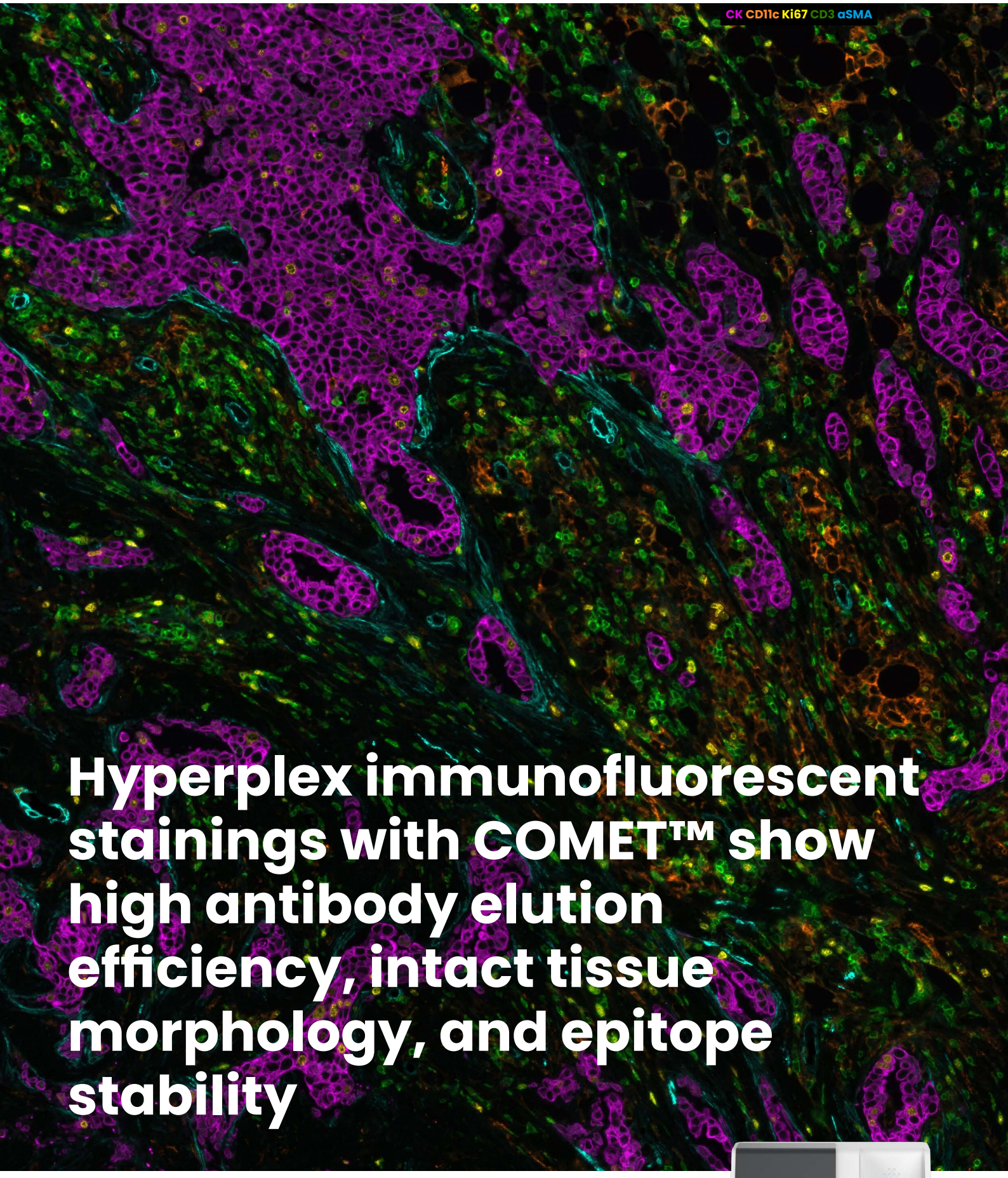
Lunaphore technologies

15-plex on human breast cancer. Total protocol time 9 hours



Hyperplex immunofluorescent stainings with COMET™ show high antibody elution efficiency, intact tissue morphology, and epitope stability

COMET™ Technical Note

Summary

Sequential indirect immunofluorescence (seqIF) is a method that enables multiplex spatial proteomics, without the need for highly manual and complex upstream barcoding and conjugation of primary antibodies.
Since seqIF relies on repeated cycles of staining, imaging and antibody elution (i.e. step for removal of antibodies with a dedicated buffer and an optimized protocol) over the same tissue section, high quality results are obtained by (i) optimal elution efficiency on each cycle, (ii) tissue morphology preservation throughout the experiment, and (iii) target tissue epitopes that would remain intact and stable over multiple cycles.
In this technical note, we present data and results showing:

- An optimal elution efficiency (>95%) across more than 15 tissue types and at least 40 antibodies tested on COMET™. This includes both formalin-fixed paraffin-embedded (FFPE) and frozen sections (FS).
- The epitope stability on COMET™, tested on 60 markers across different tissue types, species, and preparation, demonstrating excellent stability in all 20 cycles for >93% of markers.
- An automated workflow for measuring elution efficiency, epitope stability and tissue morphology, as well as guiding the user in efficiently developing multiplex panels.

1. Introduction

Multiplex staining methods enable the study of intercellular interactions by simultaneously detecting proteins expressed on different cell types in their pathophysiological environment. The detection and quantification of multiple proteins allows to study not only morphological changes due to pathological or therapeutic conditions, but also to characterize phenotypes at single cell level, to examine cell distance and to measure cell density in a specific niche. Thus, multiplex staining enables the creation of a cell-by-cell map to decipher complex interactions.

Cyclic immunofluorescence is one of the most used multiplex methods and consists of repeated staining and imaging on the same tissue slide. In order to accurately detect every marker of a multiplex panel, several parameters have to be measured and optimized for, i.e. different antibody clones and dilutions are tested, the tissue morphology has to be preserved throughout the cycles and the epitope stability for every target marker has to be assessed to decide on the optimal sequence in the panel. When increasing the number of markers in a panel, the number of steps that affect tissue integrity and epitope stability increases as well. In addition, the specificity of each staining step is dependent on a proper removal of both primary and secondary antibodies after every cycle. Various cyclic immunofluorescence techniques rely on organic denaturants or photobleaching steps for antibody removal that irreversibly damage tissue morphology and affect the accuracy and the reproducibility of each staining.

COMET™ is a fully automated staining and imaging platform based on a patented microfluidic technology that combines rapid hyperplex staining with integrated microscopy. COMET™ performs sequential immunofluorescence (seqIF) assays, which consists of sequential cycles of staining, imaging, and elution of 2 markers per cycle, where elution is a gentle step to remove antibodies with a dedicated buffer and an optimized protocol. Standard, off-the-shelf primary and secondary antibodies can be used, removing the need for highly manual and complex upstream barcoding and use of conjugated primary antibodies. With COMET™, the identification of the best staining conditions can be easily determined using an automated workflow. A gentle but efficient elution step is performed after each staining cycle without affecting tissue morphology. Epitope stability can be assessed during panel optimization, enabling users to easily develop up to 40-plex panels in a few automated steps.

2. COMET™ enables an easy analysis of the elution efficiency

A high quality seqIF assay primarily relies on the efficiency of antibody elution to warrant both staining specificity and repeatability.

COMET™ is a flexible system and supports the use of off-the-shelf reagents and buffers. Users can optimize the elution step using their preferred buffer across many conditions and parameters. However, it is key to find the conditions that bring the best overall benefit. While assessing the elution conditions, two parameters are important: i) efficiency of signal removal compared to staining, where a value below <95% is considered suboptimal; ii) tissue integrity preservation, here shown as stability of DAPI staining. Figure 1 shows that among the different buffers and protocols, one alternative elution buffer showed very low elution efficiency, while another alternative buffer affected DAPI stability thus not preserving tissue integrity. Lunaphore has developed and validated its own elution buffer, which ensures superior elution efficiency (>95%) without affecting tissue integrity.

When performing seqIF assays using COMET™, Lunaphore's elution buffer has shown highly efficient removal of antibodies. Independent of tissue preservation (formalin-fixed paraffin embedded (FFPE) or frozen section (FS)) Lunaphore's elution buffer resulted in outstanding elution efficiency of 95% and above on tissue from various organs, and with both human and mice origin. Figure 2 shows example stainings, post-elution images of the same tissue and their corresponding elution

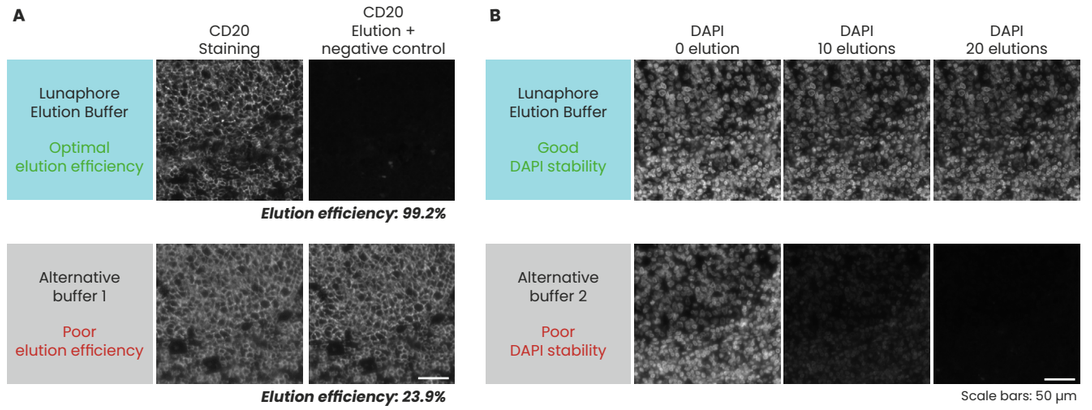


FIGURE 1 **Comparison of elution conditions between Lunaphore elution buffer and alternative buffers on formalin-fixed paraffin-embedded (FFPE) human tonsil. Panel A shows (i) CD20 staining and (ii) tissue image following elution plus additional incubation with the same secondary antibody (negative control). Such additional secondary antibody incubation ensures that both primary and secondary antibodies are removed. Quantification is shown as efficiency of signal removal compared to staining. A value of 95% or above is considered as optimal elution. Panel B shows comparison of DAPI signal between Lunaphore elution buffer and an alternative buffer 2. Lunaphore elution buffer ensures optimal DAPI stability even after 20 elutions, while DAPI staining is strongly decreased after 10 elutions with alternative buffer 2.**

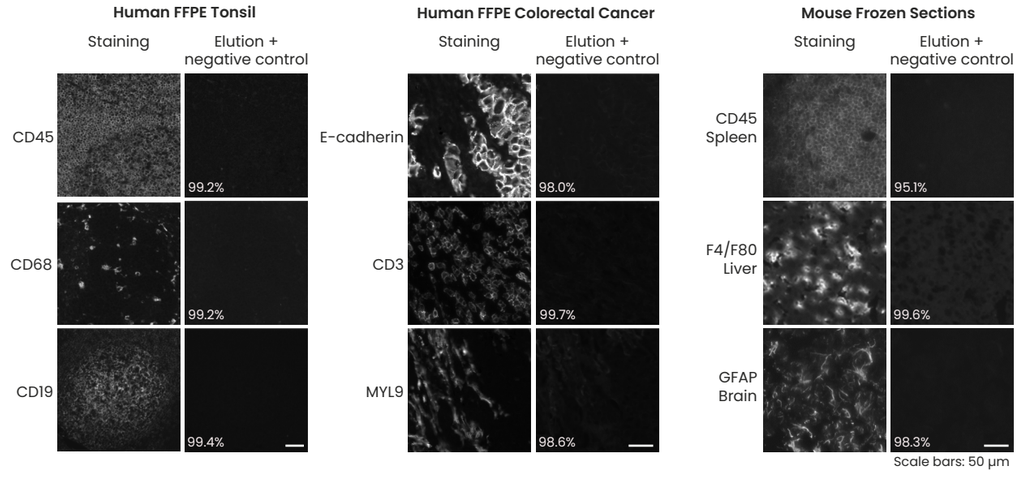


FIGURE 2 **Elution efficiency was tested across more than 15 tissue types. All markers tested have optimal elution efficiency (>95%) on COMET™.**

performance following our COMET™ elution protocol. Table 1 lists tissue types on which elution efficiency was successfully tested for more than 50 markers.

The elution step performed on COMET™ is efficient as well as gentle which ensures a well-preserved tissue integrity.

TABLE 1 **Elution efficiency was tested across more than 15 tissue types including Tissue Micro Arrays (TMA) with cores from different organs. All markers tested have optimal elution efficiency (>95%) on COMET™.**

Species	Preparation	Tissue
Human	FFPE	Tonsil
		Lung cancer
		Head and neck cancer
		Breast cancer
		Colorectal cancer
		Skin melanoma
		Pancreatic cancer
		TMA with multiple cores
	FS	Tonsil
Mouse	FS	Liver
		Lung
		Spleen
		Brain
> 50 markers tested		

3. Assessment of epitope stability and morphology preservation with COMET™

The development of robust multiplex assays requires the identification of the right sequence of markers, based on the stability of each epitope to be detected in the panel.

The assessment of epitope stability is performed by comparing the signal intensity of the initial staining (cycle 1) with the intensities obtained at subsequent staining cycles as illustrated in Figure 3. The signal intensity is calculated as signal-to-background ration (SBR) of positive cells. The SBR of each cycle is normalized to the SBR of the initial staining (cycle 1). After 20 elutions, the epitope stability is defined as excellent if the SBR is >80%, intermediate if between 60-80%, and poor if <60% compared to the SBR of the initial staining (Table 2).

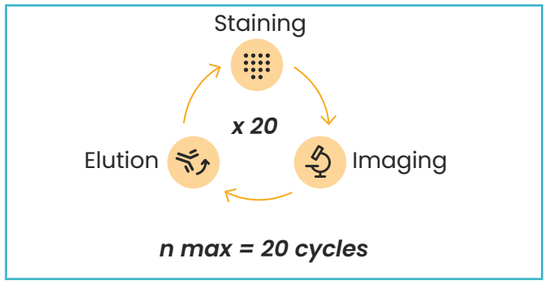


FIGURE 3 **The assessment of epitope stability consists of sequential cycles of staining, imaging, and elution.**

We assessed the epitope stability with COMET™ on several markers and across various tissue types. Figure 4 shows the study of epitope stability for four markers on FFPE sections of human tonsil: (i) CD45, a membrane marker ubiquitously expressed by immune cells; (ii) FoxP3, a transcription factor expressed in the nuclei of regulatory T cells; (iii) panCK, a membrane marker for all epithelial cells; (iv) Vimentin, a cytosolic marker expressed by mesenchymal cells. The intensity of the four markers remained almost identical from cycle 1 to 20, with <4% standard deviation of SBR-among cycles. The gentle and fast incubation protocol performed with COMET™ preserves membrane, cytoplasmatic and nuclear epitopes up to 20 cycles.

TABLE 2 **Definition of epitope stability and recommendations for seqIF assay development.**

Epitope stability	% SBR after 20 elutions	Recommendations for seqIF assay development
Excellent	>80%	This marker can be efficiently detected in hyperplex seqIF assays (from cycle 1 to 20)
Intermediate	60%-80%	It is recommended to check stability at intermediate cycles (between cycle 5 and 15)
Poor	<60%	It is recommended to check stability at medium-low cycles for accurate detection (cycles 1-5).

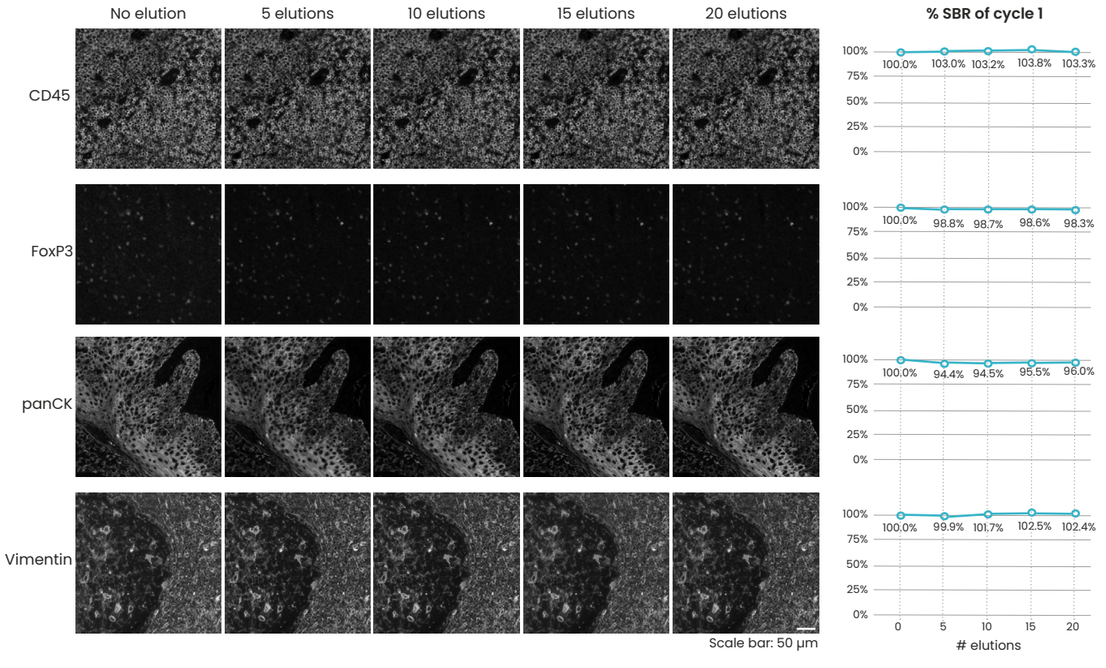


FIGURE 4 **Quantification of marker epitope stability on FFPE human tonsil. Graphs show the signal intensity change as percentage of SBR when compared to the pre-elution staining at cycle 1. For each marker, standard deviation between four different measures of different areas of the tissue is less than 4%.**

Figure 5 and Figure 6 show visual examples of the epitope stability deemed as excellent for six markers on two different human FFPE tissues. In tonsil, the study focused on the following markers: CD4, HLA-DR, CD31, CD38, CD56, and CD68. While on colorectal cancer, the following markers were assessed: CD8, CK, MYL9, CD3, CD45RO, and Vimentin. Tissue antigenicity was well preserved up to cycle 20 in both non-tumoral and tumoral tissues, and independently of their subcellular localization. This demonstrates that COMET™ enables the development of multiplex panels that can support users in their study of immune and cancer cells.

The stability of COMET™ has been proven across several tissue types, from both human and mouse (Table 3). The epitope stability for 60 markers has already been tested on COMET™ and 93% of markers (56/60 markers) have excellent stability (>80% SBR after 20 elutions) on COMET™.

COMET™ enables an easy assessment of epitope stability and simplifies panel development as the majority of markers can be detected in any of the different 20 cycles on different species, fixation and tissue types.

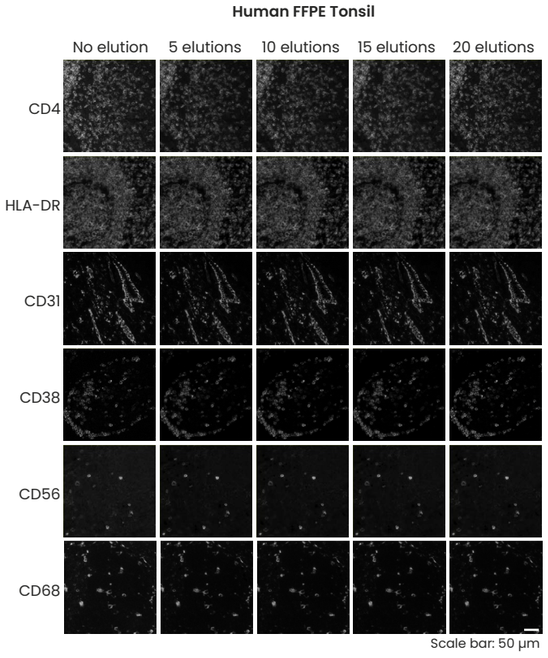


FIGURE 5 **Marker epitope stability over 20 elutions on human FFPE tonsil sections.**

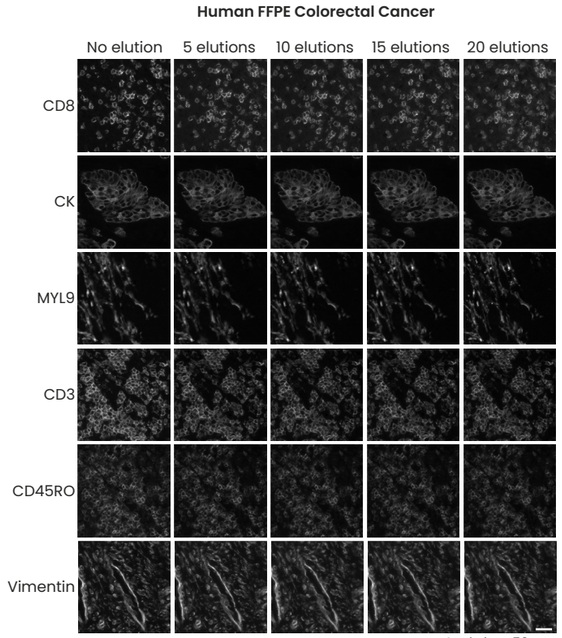


FIGURE 6 **Marker epitope stability over 20 elutions on human FFPE colorectal cancer sections.**

TABLE 3 **The epitope stability on COMET™ was tested on 60 markers across different tissue types, species, and preparation.**

Species	Preparation	Tissue	# tested markers
Human	FFPE	Tonsil	34
		CRC	11
Mouse	FS	Liver	2
		Lung	2
		Spleen	6
		Brain	5

56/60 markers with excellent stability (>80% SBR with respect to cycle 1 after 20 elutions)

4. COMET™ automated workflow streamlines panel development

New panels of up to 40 markers can be easily optimized on COMET™ using automated workflow templates found in the COMET™ Control Software that consists of three parts:

1. Characterization Part 1 is intended to assess baseline signal using default parameters suggested by the instrument and primary antibody concentration recommended by the manufacturer.
2. Characterization Part 2 allows for accelerated staining optimization: multiple conditions can be tested, evaluated and readjusted to optimize the detection of each marker.
3. Characterization Part 3 assists users in the evaluation of elution efficiency of selected staining conditions and epitope stability.

Using Characterization Part 3, COMET™ offers the possibility to evaluate the elution efficiency for the selected markers, thanks to dedicated steps in the first cycle of the protocol (Figure 7), as follows:

Step 1. Imaging to detect tissue autofluorescence.
Step 2. An optional, negative control staining (incubation with only secondary antibodies) and imaging.
Step 3. Staining for the marker of interest and imaging.
Step 4. Elution step and imaging.
Step 5. An optional, negative control staining and imaging.

Based on the results of steps 1-5 of the Characterization Part 3, users can determine if the elution of their antibodies is satisfactory. If the elution is not optimal, the user can either optimize it further using the Characterization Part 1 or Part 2 protocols, change different parameters, or place the marker in the last cycle of the multiplex panel.

To achieve a fast evaluation of epitope stability over multiple cycles, groups of 5 elution steps can be added in the Characterization Part 3 template. This enables the user to assess if the staining quality is preserved from cycle 1 upon either 5, 10, 15, or 20 elution cycles (Figure 7). The protocol can be run with either a single marker, or a mix of two markers simultaneously.

COMET™ simplifies panel development using a guided workflow to assess elution efficiency and epitope stability. COMET™ allows the development of hyperplex panels up to 40 markers (2 markers per cycle) in only a few weeks.

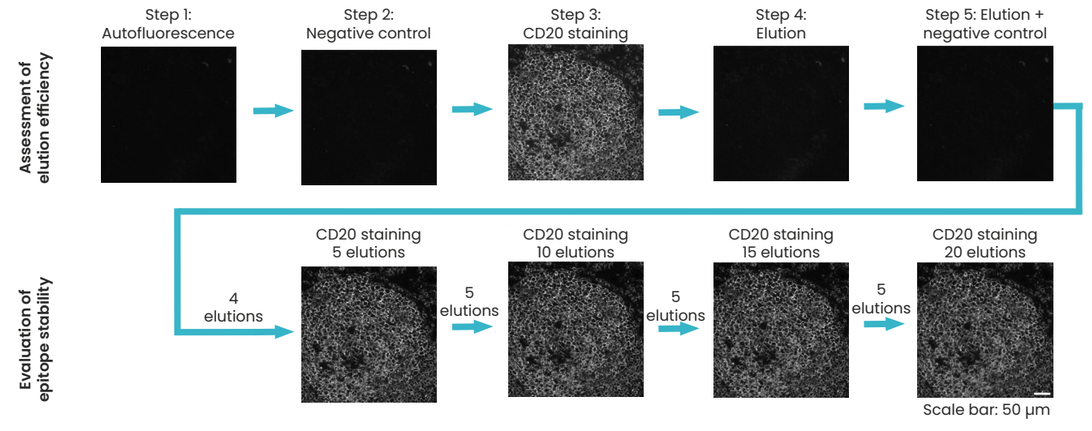


FIGURE 7 **Example of the COMET™ workflow to evaluate elution efficiency of both primary and secondary antibodies as well as the marker's epitope stability over elution cycles.**

5. Conclusion

The immunolabeling assessment of multiple biomarkers is essential to characterize different cell populations and their spatial organization within the tissue microenvironment. Bringing excellent elution efficiency, epitope stability and tissue morphology preservation, COMET™ enables high quality hyperplex seqIF assays. In addition, COMET™ provides an automated workflow to optimize multiplex panels in a few days only. In contrast, traditional techniques would require highly manual optimization repeats that can take months to develop, before starting to run experiments.

A strong advantage of the seqIF approach on COMET™ is the possibility to use any standard, non-conjugated primary antibodies without the need for any modification or conjugation, thus providing the opportunity to leverage previously validated antibody libraries. This flexibility greatly widens the scope of applications that can benefit from hyperplex, spatial proteomics, allowing an easy integration of new markers outside the known panels. COMET™ is a tool that will bring seqIF to mainstream use, enabling access to spatial biology in every laboratory.

In this technical note, we showed data demonstrating that, thanks to its patented technology, COMET™:

(1) turns seqIF into a powerful spatial biology workhorse with excellent quality in results to accomplish up to a 40-plex panel;

(2) brings a unique, automated workflow to streamline panel development, thereby eliminating the dependency of researchers on (i) antibody conjugation processes, which pose significant upstream complexity; and (ii) tissue-damaging antibody removal steps, such as photobleaching, typical to other spatial proteomics techniques.

Interested in COMET™? Ask our scientists.

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