Enhanced analysis of tumor microenvironment and immune regulation via an automated adjustable signal amplification technique for multiplex immunofluorescence

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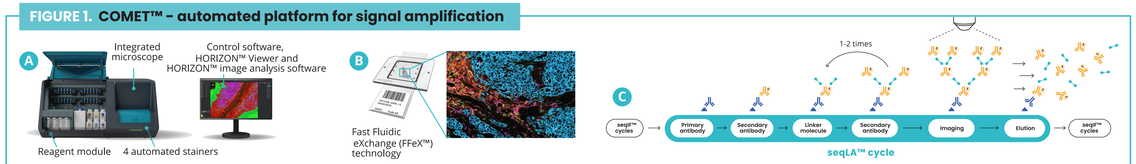
Introduction

Multiplex immunofluorescence (mIF) has become fundamental for tumor microenvironment (TME) and biomarker research [1]. Therein, different marker expression levels often have biological meaning, and the detection of markers expressed at low vs. high levels can provide crucial insights [2]. However, detecting antigens expressed at lower levels can be challenging and often benefits from signal amplification [3]. Here, we demonstrate a new automated method for COMET™ instrument called sequential layered amplification (seqLA™) enabling the amplification of individual markers within multiplex panels. This technique provides an adjustable level of amplification and an efficient elution for subsequent staining cycles.

Material and Methods

COMET™ is an automated platform that performs sequential immunofluorescence (seqIF™) assays [4]. Based on this, the novel amplification method increases the number of detection antibodies per primary antibody in a cyclic manner leading to a stronger signal. The amplification complex can be eluted, enabling subsequent staining cycles while preserving tissue integrity. Formalin-fixed paraffin-embedded (FFPE) tissue sections from three human colorectal carcinoma cases were stained using a 26-plex panel including 20 markers in standard seqIF™ and 6 amplified markers, together covering most basic immune cell types, functions, and stroma compartments. The performance was assessed by comparing marker expression with and without amplification. Image post processing and immune cell classification were done using Lunaphore HORIZON™ image analysis software. Amplified stainings were also compared to single-plex chromogenic immunohistochemistry (IHC) for each marker [5].

FIGURE 1. COMET™ – automated platform for signal amplification

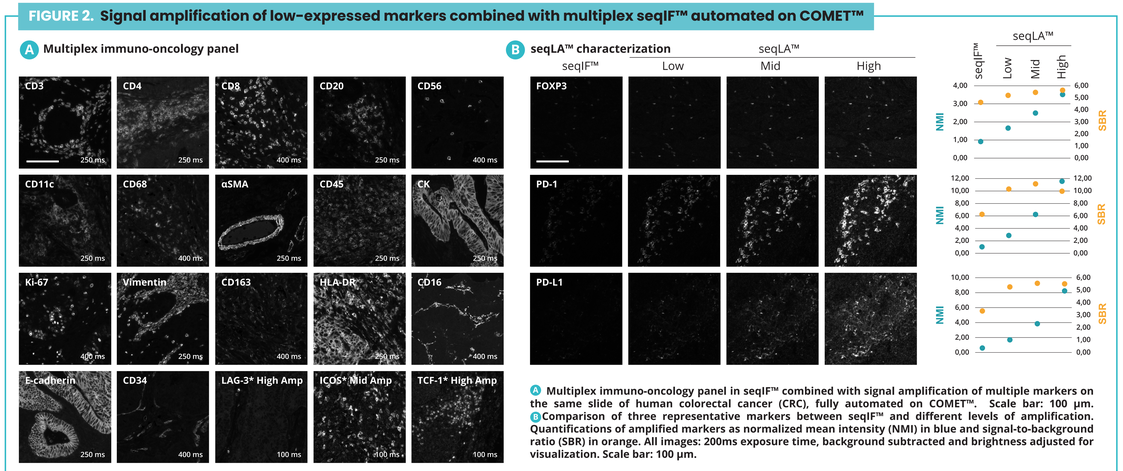


A COMET™ is an automated platform providing a slide-in, data-out workflow for spatial biology on up to 4 slides simultaneously. B Lunaphore microfluidic FFeX™ technology allows fast and uniform delivery of reagents to a closed staining chamber. C Schematic of an amplification (seqLA™) cycle. Layers of linker molecules and fluorophore conjugated antibodies are added in a cyclic manner to amplify the signal. The whole scaffold can then be eluted for consecutive seqIF™ or seqLA™ cycles.

Results and Discussion

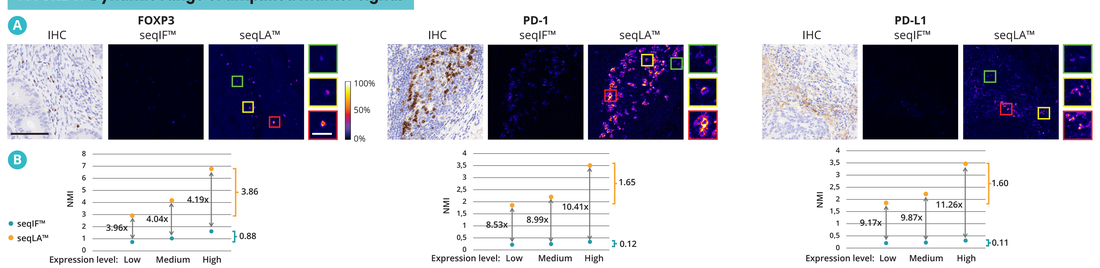
Our study demonstrated the successful integration of markers with amplified signal within a multiplex seqIF™ panel using the seqLA™ technique automated on COMET™ instrument (**Fig.1 and 2**). Amplified signal from low-expressed markers could be reliably detected and their amplification produced a controllable signal intensity increase between low- and high-expressing cell populations, providing a higher dynamic range when compared to the unamplified staining (**Fig.3**). Furthermore, the staining patterns of amplified markers shows good correlation with chromogenic IHC staining. Amplification enabled the detection of the full spectrum of six immune marker expression intensity and identification of several low-expressing subtypes of regulatory immune cells such as Treg and macrophages within the TME (**Fig.4**). Combining amplified markers within a larger panel enabled an enhanced analysis of the TME and complex immune cell classification within tertiary lymphoid structures (TLS). HORIZON™ image analysis software was used for unsupervised clustering, scatter plot and matrix visualization to identify different cell clusters and phenotypes (**Fig.5**).

FIGURE 2. Signal amplification of low-expressed markers combined with multiplex seqIF™ automated on COMET™



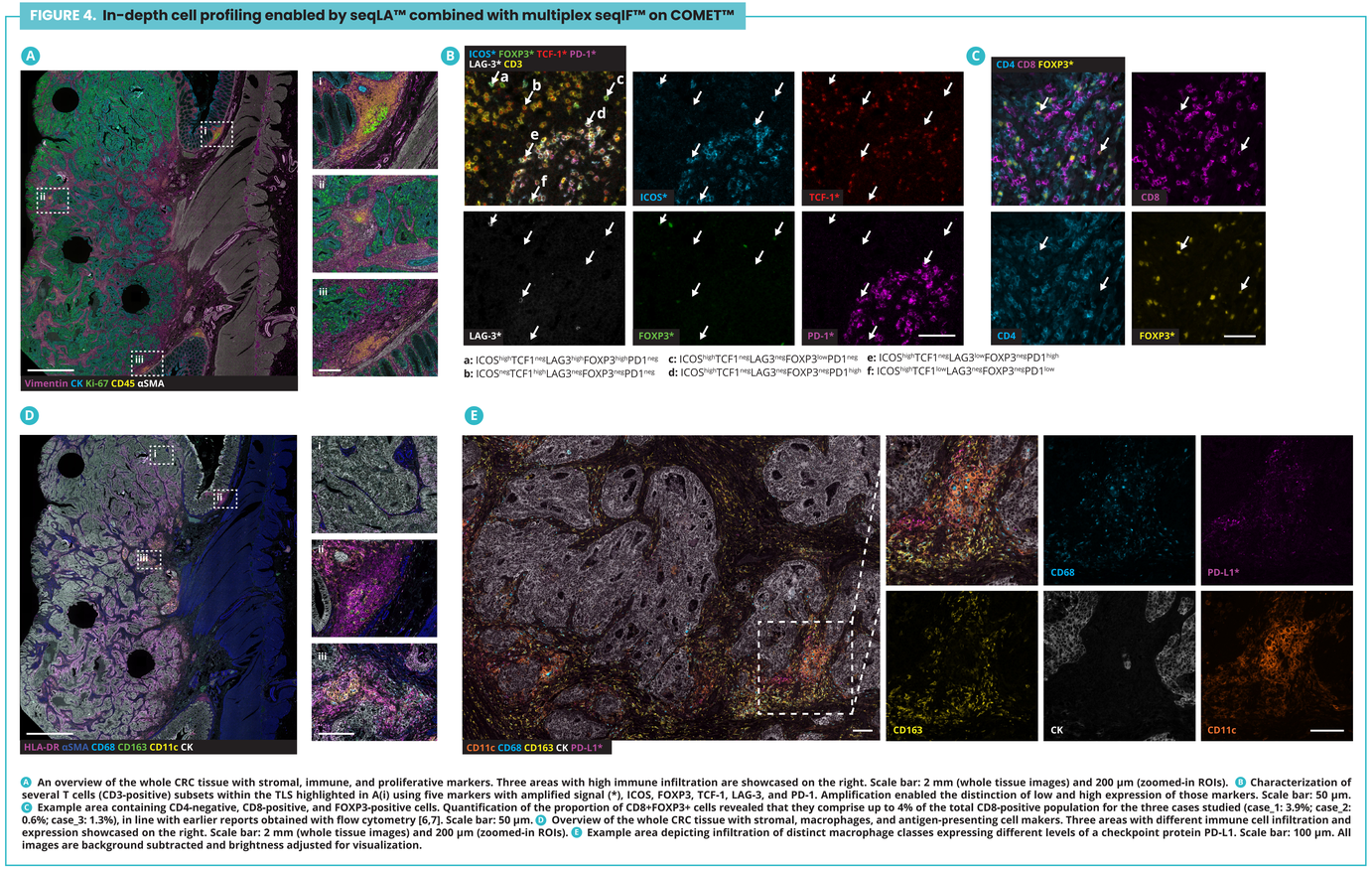
A Multiplex immuno-oncology panel in seqIF™ combined with signal amplification of multiple markers on the same slide of human colorectal cancer (CRC), fully automated on COMET™. Scale bar: 100 µm. B Comparison of three representative markers between seqIF™ and different levels of amplification. Quantifications of amplified markers as normalized mean intensity (NMI) in blue and signal-to-background ratio (SBR) in orange. All images: 200ms exposure time, background subtracted and brightness adjusted for visualization. Scale bar: 100 µm.

FIGURE 3. Dynamic range of amplified marker signal



A Comparison of a dynamic range of signal between seqIF™ and seqLA™ staining to detect FOXP3, PD-1, and PD-L1 markers on human colorectal cancer FFPE tissue with corresponding IHC images. Cells expressing low, medium, and high levels of each marker are shown on the right in green, yellow and red rectangles, respectively. The autofluorescent signal was subtracted. Scale bar: 100 µm (larger images) and 20 µm (small images). B Quantifications displayed as NMI for the seqIF™ (blue) and amplified seqLA™ signal (orange) of the cells expressing low, medium, and high levels of FOXP3, PD-1, and PD-L1. The degree of signal amplification (DSA) calculated as a ratio of NMI of amplified signal and seqIF™ for each group of cells is shown next to the arrows. DSA shows a high degree of linearity of signal increase in the three populations of cells. The difference in NMI between populations of cells expressing low and high levels of FOXP3, PD-1, and PD-L1, shown on the right side of each graph, indicates an increased dynamic range with seqLA™ technique compared to the standard seqIF™ staining.

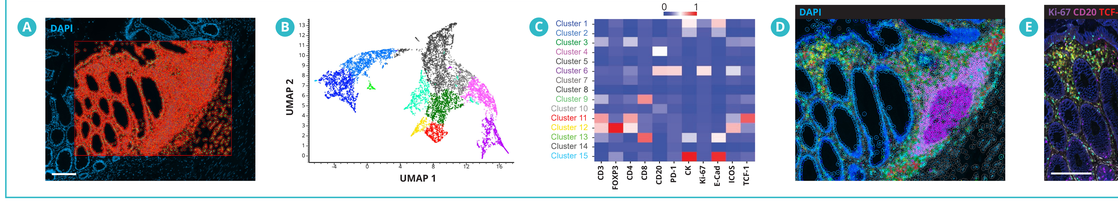
FIGURE 4. In-depth cell profiling enabled by seqLA™ combined with multiplex seqIF™ on COMET™



a: ICOS^{high}TCF1^{neg}LAG3^{high}FOXP3^{high}PD1^{neg} **b:** ICOS^{neg}TCF1^{high}LAG3^{neg}FOXP3^{neg}PD1^{neg} **c:** ICOS^{high}TCF1^{neg}LAG3^{neg}FOXP3^{low}PD1^{neg} **d:** ICOS^{high}TCF1^{neg}LAG3^{neg}FOXP3^{neg}PD1^{high} **e:** ICOS^{high}TCF1^{neg}LAG3^{low}FOXP3^{neg}PD1^{high} **f:** ICOS^{high}TCF1^{low}LAG3^{neg}FOXP3^{neg}PD1^{low}

A An overview of the whole CRC tissue with stromal, immune, and proliferative markers. Three areas with high immune infiltration are showcased on the right. Scale bar: 2 mm (whole tissue images) and 200 µm (zoomed-in ROIs). B Characterization of several T cells (CD3-positive) subsets within the TLS highlighted in A(i) using five markers with amplified signal (*), ICOS, FOXP3, TCF-1, LAG-3, and PD-1. Amplification enabled the distinction of low and high expression of those markers. Scale bar: 50 µm. C Example area containing CD4-negative, CD8-positive, and FOXP3-positive cells. Quantification of the proportion of CD8+FOXP3+ cells revealed that they comprise up to 4% of the total CD8-positive population for the three cases studied (case_1: 3.9%; case_2: 0.6%; case_3: 1.3%), in line with earlier reports obtained with flow cytometry [6,7]. Scale bar: 50 µm. D Overview of the whole CRC tissue with stromal, macrophages, and antigen-presenting cell makers. Three areas with different immune cell infiltration and expression showcased on the right. Scale bar: 2 mm (whole tissue images) and 200 µm (zoomed-in ROIs). E Example area depicting infiltration of distinct macrophage classes expressing different levels of a checkpoint protein PD-L1. Scale bar: 100 µm. All images are background subtracted and brightness adjusted for visualization.

FIGURE 5. Streamlined cell segmentation, classification, and phenotyping using HORIZON™



Analysis of the first TLS from Figure 4, A(i), using HORIZON™ image analysis software. A Region of interest with nuclear segmentation highlighted in yellow and cell approximation in red. B Scatter plot visualization of Leiden clusters in Uniform Manifold Approximation and Projection (UMAP) coordinates. C Matrix visualization of the clusters with the markers used for analysis and normalized intensity. D Overlay of the respective cluster colors onto the cell segmentation. E Composite tissue image with markers' colors matching their respective most relevant cluster. Background subtracted and brightness adjusted for visualization. Scale bar: 200 µm.

Conclusions

Hyperplex seqIF™ panels integrating this novel seqLA™ technique enables the detection of markers that are expressed at low levels, which could not be robustly captured by non-amplified mIF, such as the full expression range of critical immune checkpoint markers. Analyzing a high number of markers simultaneously while allowing selective amplification improves the profiling of immune and tumoral cells within their environment. HORIZON™ image analysis software facilitates the image post processing, cell segmentation, classification, and phenotyping.

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