SmartBioSurface® Slides

High-Plex Immunofluorescence of Non-Adherent Cells with the Quanterix Spatial PhenoCycler® Platform

The Challenge: High-Plex Imaging of Suspension-Derived Cells

High-plex immunofluorescence workflows, originally developed for tissue-based applications, are increasingly being applied to suspension-derived cells, including peripheral blood leukocytes, rare circulating cell populations, and other non-adherent cell types. Unlike cells embedded within tissue sections, suspension-derived cells lack intrinsic attachment to a substrate and may therefore be susceptible to detachment during sample preparation and staining procedures. Previous studies have shown that cell retention can vary depending on cell type and experimental conditions, highlighting the importance of effective immobilization strategies for suspension-cell immunofluorescence workflows [1].

These challenges become particularly relevant in high-plex cyclic immunofluorescence, where samples are exposed to multiple rounds of staining, washing, reporter exchange, and imaging. The Quanterix Spatial PhenoCycler® platform enables highly multiplexed molecular profiling through DNA-barcoded antibodies and sequential fluorescent reporter hybridization, allowing numerous markers to be evaluated within a single sample [2]. The PhenoCycler-Fusion platform further integrates automated fluidics and cyclic imaging with whole-slide analysis and downstream single-cell spatial profiling [3].

To support reliable high-plex analysis of suspension-derived cells, an immobilization substrate must maintain cell adherence throughout repeated processing steps while preserving cellular morphology and ensuring compatibility with fluorescence imaging.

The Solution: SmartBioSurface® Slides

SmartBioSurface® is a proprietary nanostructured titanium-based coating developed by Tethis S.p.A. to promote spontaneous adhesion of non-adherent and rare cells onto microscopy-grade glass while preserving cellular morphology and structural integrity [4,5].

Figure 1. SmartBioSurface® slides. Left image: SmartBioSurface® slides available in different formats. Right image: Atomic Force Microscopy (AFM) image of the nanostructured coating (50 nm thickness).

The coating consists of a nanostructured titanium dioxide (TiO_2) layer deposited onto optical-grade glass. Its ability to support spontaneous cell adhesion while maintaining compatibility with fluorescence microscopy makes SmartBioSurface® a suitable substrate for multiplex immunofluorescence applications involving suspension-derived cells and workflows requiring extensive sample processing.

Materials & Methods

1. Sample Preparation

PBMC Isolation from Peripheral Blood Samples

Peripheral blood samples collected from healthy donors [6] in EDTA tubes were processed to isolate leukocytes through red blood cell lysis.

Leukocyte counts were determined using an automated hematology analyzer (EmoCue). Whole blood was incubated with Red Blood Cell Lysis Buffer (RBL) for approximately 5 min at room temperature to selectively remove erythrocytes while preserving leukocytes. Samples were centrifuged at 249 × g for 5 min and the supernatant was discarded.

To ensure complete erythrocyte removal, two additional lysis cycles were performed. Following the final centrifugation step, leukocytes were resuspended in Dulbecco's Phosphate Buffered Saline (DPBS) 1× without Ca^{2+} and Mg^{2+} at a target density of approximately 250,000 cells/cm^2.

Cell Deposition and Fixation on SmartBioSurface®

To define a controlled cell deposition area compatible with PhenoCycler imaging and flow-cell requirements, a reusable CultureWell™ silicone gasket (Grace Bio-Labs) was positioned on the SmartBioSurface® slide according to the placement specifications reported in the Quanterix Spatial PhenoCycler-Fusion User Guide [7].

An 80 µL cell suspension containing approximately 250,000 peripheral blood leukocytes was dispensed within the gasket-defined area and incubated for 20 min at room temperature to allow cell adhesion to the nanostructured coating.

Following adhesion, samples were gently washed with DPBS and fixed with 4% paraformaldehyde (PFA) for 20 min at room temperature. Slides were subsequently rinsed with DPBS and allowed to dry overnight at room temperature.

The dried slides were shipped at room temperature to G-STeP (Gemelli Science and Technology Park), Fondazione Policlinico Universitario Agostino Gemelli IRCCS (Rome, Italy), where they were stored at −80 °C until analysis.

Figure2. Cell deposition on SmartBioSurface. Left: reusable CultureWell™ silicone gasket used to define the deposition area. Right: SmartBioSurface slide showing the gasket-defined deposition area (red arrow), where the enriched cell suspension was seeded, incubated for spontaneous cell adhesion and fixed with 4% PFA.

2. Thawing, Rehydration and Antigen Retrieval

Prior to the PhenoCycler staining workflow, the SmartBioSurface slides stored at −80 °C were removed from storage and allowed to equilibrate to room temperature. The slides were subsequently rehydrated with 1× DPBS through two consecutive 5-min incubations.

Antigen retrieval was then performed outside the PhenoCycler instrument as part of the pre-analytical workflow developed by Tethis. A 10 mM citrate buffer at pH 6.0 was preheated to 65 °C. The slides were immersed in the preheated buffer, heated to 94 °C and maintained at 94 °C for 30 min. Following retrieval, the slides were cooled to 65 °C and subsequently allowed to reach room temperature. Slides were then washed for 5 min in 1× DPBS before proceeding to the antibody-staining step.

3. DNA-Barcoded Antibody Cocktail

The PhenoCycler panel comprised 12 antibodies in total, including 11 inventoried Quanterix Spatial DNA-barcoded primary antibodies targeting CD45RO, CD4, CD20, CD8, CD68, CD79a, CD163, CD38, CD3e, CD44, and CD11c, and an anti-CXCR4 antibody conjugated with a DNA barcode at G-STeP (Fondazione Policlinico Universitario Agostino Gemelli IRCCS).

The antibodies were combined into a single staining cocktail and applied to the prepared SmartBioSurface slides prior to loading onto the PhenoCycler system.

The antibody cocktail was incubated for 3 h at room temperature. This incubation corresponds to the antibody-staining phase of the PhenoCycler workflow and is distinct from the subsequent cyclic reporter hybridization and imaging steps performed by the PhenoCycler system [7].

4. PhenoCycler Cyclic Imaging

Following antibody staining and washing, the prepared SmartBioSurface slide was loaded onto the Quanterix Spatial PhenoCycler system for cyclic multiplex imaging. The acquisition consisted of an initial DAPI imaging cycle followed by five sequential marker cycles, during which fluorescent DNA reporters were hybridized to the corresponding DNA-barcoded antibodies and the selected fluorescence channels were acquired [7]. The reporters were subsequently removed before the next imaging cycle, while the DNA-barcoded antibodies remained associated with their respective cellular targets throughout the workflow.

5. Positive Control

FFPE human tonsil tissue was included as a positive control to assess antibody performance and confirm target-specific staining. The tissue control was processed according to the standard FFPE workflow described in the Quanterix Spatial PhenoCycler-Fusion User Guide, including the recommended antigen retrieval procedure prior to antibody staining [7]. Representative tonsil images were used to assess the expected staining patterns of the evaluated markers and to support interpretation of the corresponding signals observed in peripheral blood leukocytes.

Results

1. Cell Retention Throughout the PhenoCycler Workflow

SmartBioSurface® slides maintained qualitative cell retention throughout the complete PhenoCycler workflow. Cells remained adherent to the substrate following antibody staining, repeated washing steps, reporter exchange, and cyclic fluorescence imaging, with no appreciable cell loss observed by qualitative assessment.

These observations demonstrate that SmartBioSurface® supports cell immobilization under the processing conditions required for cyclic multiplex immunofluorescence and is compatible with PhenoCycler analysis of suspension-derived peripheral blood leukocytes.

2. Multiplex Immunofluorescence Performance

A 12-marker panel was evaluated to assess compatibility between SmartBioSurface® and the PhenoCycler workflow. Specific staining patterns were observed for CD45RO, CD4, CD8, CD3e, CD11c, CD44, and CD68. CD68 displayed a predominantly granular/perinuclear staining pattern that was consistent with the signal distribution observed in the FFPE tonsil control.

These findings demonstrate the feasibility of performing multiplex immunofluorescence on SmartBioSurface® slides using the Quanterix Spatial PhenoCycler® platform.

3. Marker-Specific Staining Optimization

Although most evaluated antibodies generated interpretable staining patterns, marker performance varied under the tested conditions.

Signals corresponding to CD20, CD79a, and CD38 were not detected in the peripheral blood samples analyzed. CD163 staining was not observed, consistent with its predominant expression in tissue-resident macrophage populations rather than circulating peripheral blood leukocytes. CXCR4 exhibited non-specific staining in both peripheral blood and tonsil control samples.

These observations suggest that additional marker-specific optimization may further improve assay performance for selected targets and sample types.

The FFPE tonsil control provided an independent reference for evaluating antibody performance and facilitated interpretation of the observed staining patterns.

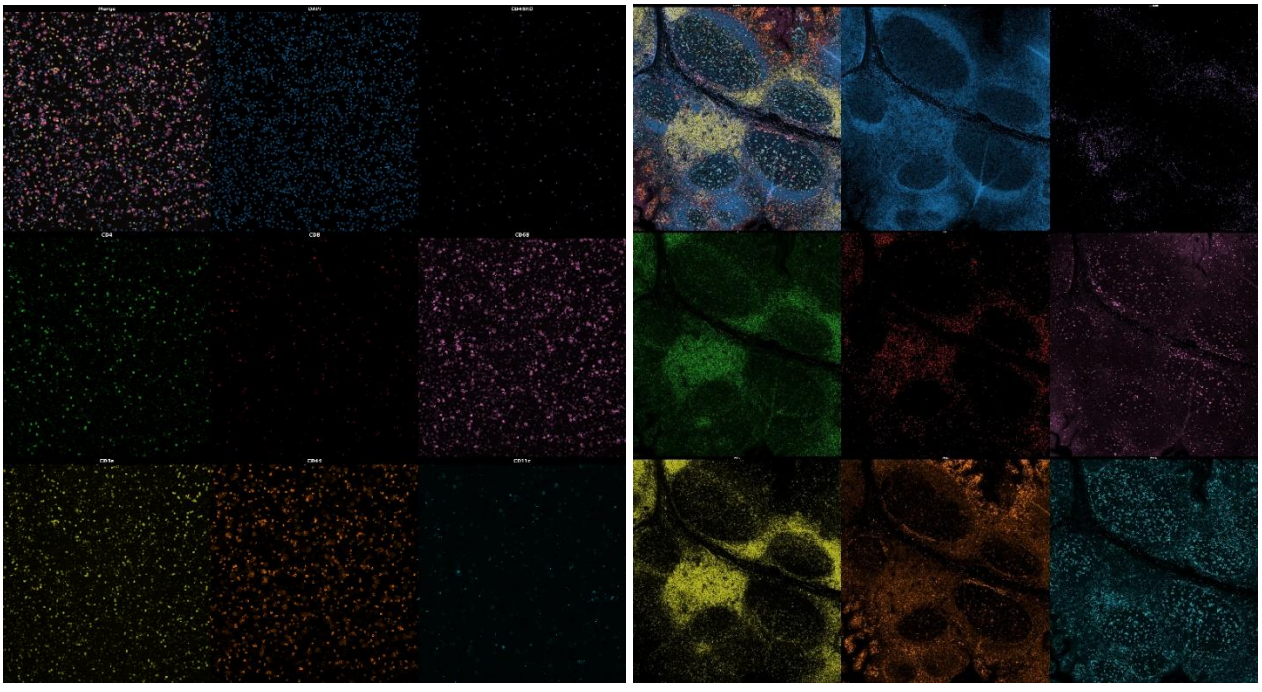

Figure 3. Multiplex immunofluorescence staining using the PhenoCycler platform. Representative images of peripheral blood leukocytes on SmartBioSurface® slides (left) and FFPE human tonsil tissue used as a positive control (right). For each sample, individual fluorescence channels and the corresponding composite merge are shown for the evaluated markers. DAPI was used for nuclear visualization. Images were acquired following completion of the cyclic PhenoCycler workflow.

Conclusions

This study demonstrates the compatibility of SmartBioSurface® slides with the Quanterix Spatial PhenoCycler® platform for high-plex cyclic immunofluorescence of suspension-derived peripheral blood leukocytes.

Qualitative cell retention was maintained throughout repeated staining, washing, reporter exchange, and imaging cycles, supporting robust sample processing under PhenoCycler workflow conditions. In addition, multiplex detection of several immune-cell markers was achieved with specific and reproducible staining patterns for multiple targets.

The successful integration of the Tethis pre-analytical workflow with the PhenoCycler staining and imaging workflow supports the use of SmartBioSurface® as a suitable substrate for high-dimensional characterization of suspension-derived cells.

Further optimization of selected antibodies may expand panel performance and facilitate broader application of high-plex immune profiling in non-adherent cellular samples.

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