A Comprehensive Analytical Validation Framework for Antibodies Used in the CellScape Multiplex Immunofluorescence Assay

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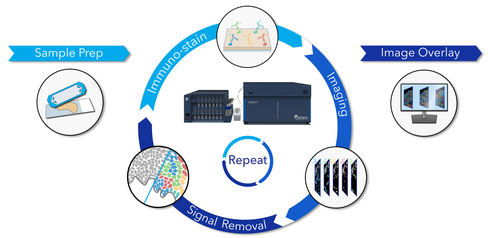
Summary

- The CellScape™ Precise Spatial Multiplexing platform enables automated, high plex and flexible cyclic multiplex immunofluorescence (mIF) with fluorophore-conjugated antibodies on fresh frozen and FFPE tissues.
- Carefully validated and optimized mIF antibody panels ensure specificity, sensitivity and reproducibility of imaging data, and they are crucial to achieving assay repeatability and standardization.
- High dynamic range (HDR) imaging improves quantitative phenotyping, while excellent digital sampling (182 nm/px) provides single-cell and subcellular resolution for exceptional image quality of mIF antibody panel data.
- We describe our validation procedure for multiplex assay panels in which signal intensity, inter-run reproducibility, and intra-run precision were evaluated in a multi-site testing matrix.
- The method described here was used to validate the VistaPlex™ Spatial Immune Profiling Assay Kit, compatible with human FFPE and used on the CellScape platform.

Our data demonstrate that the VistaPlex Spatial Immune Profiling Assay Kit is optimized for straightforward and reliable phenotyping of immune cells in human FFPE tissue.

Validation Workflow



VistaPlex Spatial Immune Profiling Biomarkers		
CD3	CD68	Ki-67
CD4	CD45	PD-L1
CD8	CD45RA	Pan-CK
CD20	CD45RO	Granzyme B
FoxP3	PD-1	DNA

Figure 1: The CellScape workflow for iterative on-instrument staining, imaging and signal removal.

Multiplex assay panels are validated as follows:

1. **Specificity**: Antibodies bind only to their intended targets.
2. **Sensitivity**: Signal is strong enough to differentiate positive cells from background and/or autofluorescence.
3. **Reproducibility**: Consistent staining in technical replicates

Method: Nine serial sections (SS) were cut from a single block of human tonsil FFPE tissue. Sets of three adjacent sections were scanned together on the same instrument to monitor intra-run precision. Inter-run precision experiments were performed by a different operator on different instruments across two sites.

Five regions of interest (ROIs), aligned across serial sections, were annotated for each analysis. Each ROI contained ≥ 1 germinal center. Stain patterns, intensity and phenotype quantitation were used to compare performance & reproducibility.

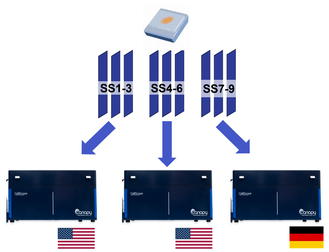


Figure 2: Schematic of the validation test plan illustrating intra-run comparisons (SS1-3) and inter-run comparisons (two different sites).

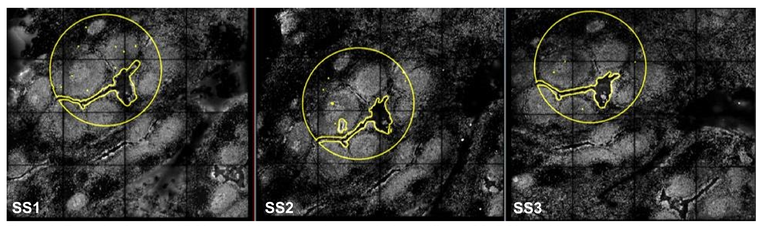


Figure 3: Example of matched ROIs containing germinal centers in three different SS; ROI matching ensured robust comparisons across samples.

Qualitative **Specificity** Assessment

Antibodies bind only their intended targets

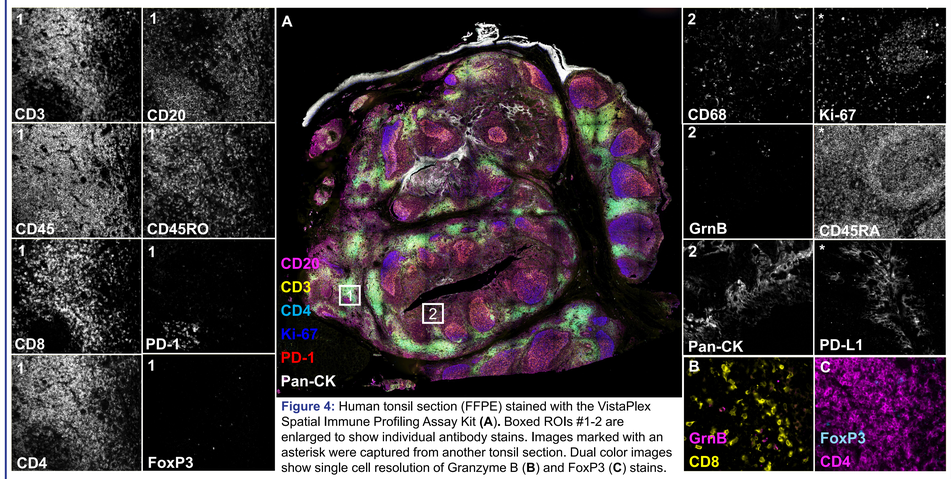


Figure 4: Human tonsil section (FFPE) stained with the VistaPlex Spatial Immune Profiling Assay Kit (**A**). Boxed ROIs #1-2 are enlarged to show individual antibody stains. Images marked with an asterisk were captured from another tonsil section. Dual color images show single cell resolution of Granzyme B (**B**) and FoxP3 (**C**) stains.

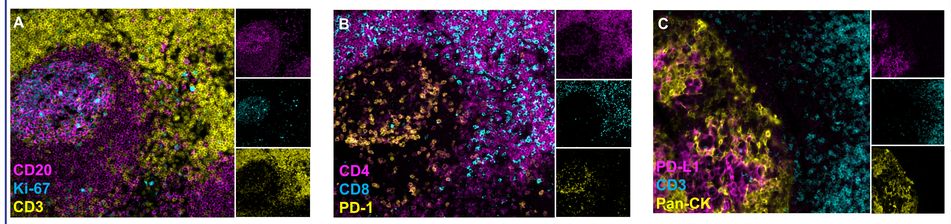


Figure 5: Colocalization assessment of eight antibodies. Distinct staining patterns confirm specificity of each antibody. CD3 and CD20 are expressed by T cells and B cells, respectively. Ki-67 is expressed by proliferating B cells (**A**). CD3-positive cells express either CD8 or CD4. CD4-positive follicular T cells express PD-1 (**B**). PD-L1 colocalizes with cytokeratin-positive tonsillar epithelia (**C**).

Quantitative **Sensitivity** Assessment

Signal is strong enough to differentiate positive cells from background fluorescence

Intensity ratios were calculated to index brightness of the antibody stains. Most antibodies exhibit stain intensities ≥ 4-fold above background. An intensity ratio ≥ 2 characterizes sufficiently sensitive antibodies.

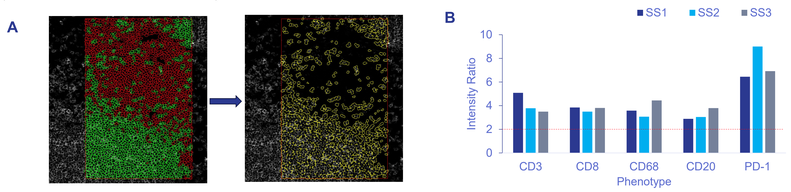


Figure 6: ROI annotation regions and single cell segmentation masks define positive cells. Inverse segmentation masks define background (**A**). The mean pixel intensity of each mask was determined and used to calculate the ratio of positive antibody signal vs. background. The intensity ratios were compared across serial sections from the same run (**B**).

Quantitative **Reproducibility** Assessment

Antibodies produce consistent results in technical replicates within runs and across sites

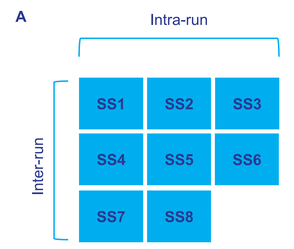
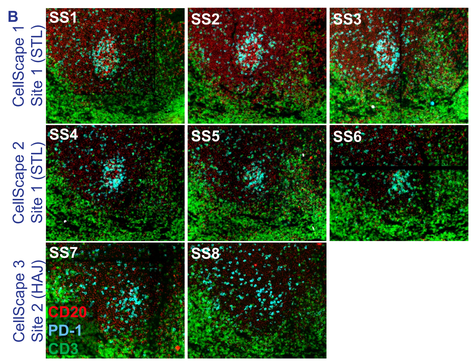


Figure 7: Schematic of intra-run and inter-run comparisons (**A**). Images comparing stains in matching ROIs across all serial sections; the anatomy of the germinal center changes noticeably between SS1 and SS8 (**B**).



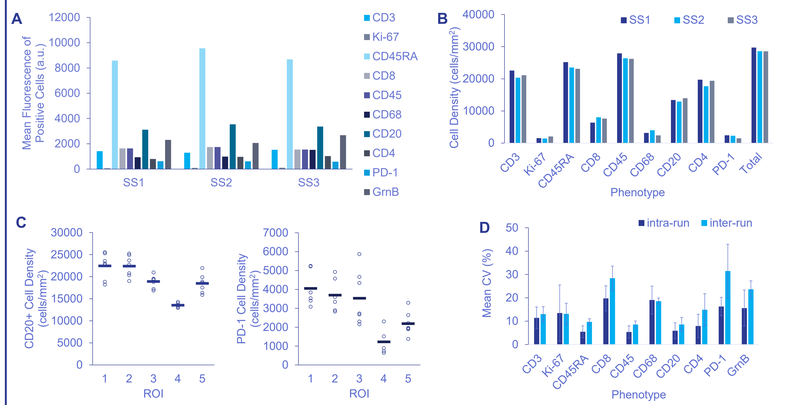


Figure 8: Mean fluorescence intensity (**A**) and raw cell density (**B**) of positive cells in serial sections stained in the same run. Inter-run cell density is shown in **C**. Points indicate counts for each serial section. Bar indicates the mean. Inter-run variance in cell density (**D**). Mean, standard deviation and coefficient of variation (CV) were determined for each ROI and the mean CV was calculated across serial sections. Error bars = standard deviation

Conclusions

- We developed a robust validation protocol for immune cell phenotyping in FFPE tissues using the VistaPlex Spatial Immune Profiling Assay Kt and deployed on the CellScape platform for Precise Spatial Multiplexing.
- Validation demonstrates consistent performance of the multiplex immunofluorescence VistaPlex antibody panel in both intra and inter run testing.
- Using pre-validated VistaPlex assay kits ensures reliable assay execution, streamlining assay development and validation.

Contact

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