ANTIBODY VALIDATION FOR CHIPCYTOMETRY™

HIGHLIGHTS

- **ChipCytometry™ is an all-in-one high-plex imaging platform for spatially resolved single-cell phenotyping**
- **ChipCytometry antibodies leverage decades of histopathology tradition, using clones validated for flow cytometry and IHC**
- **Commercially available, fluorescently conjugated antibodies are part of a flexible and practical solution**
- **ChipCytometry antibodies are fully validated in both single-marker and combination assays for specificity, sensitivity, and reproducibility**

BACKGROUND

Spatial biology has exploded as scientists recognize the need to observe cells in the tissue microenvironment to better understand mechanisms of disease and healthy state. Immunofluorescence techniques depend on thoroughly validated antibodies to resolve antigens in the tissue (Du et al., 2019). Although they are some of the most commonly used research tools, there are no universally accepted guidelines for antibody validation (Bordeaux et al., 2010). Furthermore, many spatial biology techniques do not provide transparent information on reagent validation.

ChipCytometry™ addresses these challenges by employing fluorescently conjugated antibodies from a variety of vendors (Figure 1). ChipCytometry leverages antibodies used for decades in clinical histology and validates them for use in ChipCytometry assays.

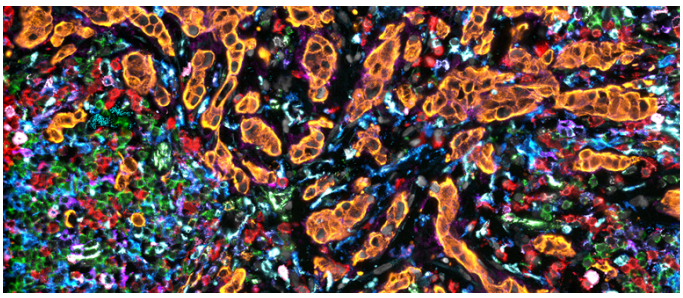

Figure 1. *A human breast cancer tissue stained with 16 fluorescently conjugated antibodies in a ChipCytometry assay.*

INTRODUCTION TO CHIPCYTOMETRY

Deep profiling of the tissue microenvironment is essential for understanding the biology of disease and its progression. Investigation of therapeutically relevant cells in their native environment requires a spatially resolved high-plex imaging approach. ChipCytometry enables scientists to visualize and quantify cells in their native microenvironment.

ChipCytometry is based on iterative immunofluorescence using the ZellScannerONE™ instrument, a fully integrated automated imaging system (Figure 2). High-resolution, high-dynamic range imaging achieves true single-cell resolution, enabling researchers to collect accurate data on a range of high- and low-expressing proteins.

Figure 2. *The ChipCytometry workflow uses the ZellScannerONE, an all-in-one instrument that combines iterative rounds of immunofluorescence imaging with advanced image analysis software for cell phenotyping and quantification.*

KEY FEATURES OF CHIPCYTOMETRY

ChipCytometry is a powerful platform for spatially resolved multiplexing that offers:

- Ability to profile dozens of biomarkers
- Compatible with FFPE and fresh frozen tissue as well as cell suspensions
- HDR imaging for accurate quantification of high- and low-expressing proteins
- Single-cell quantification and spatial analysis with custom software
- Sample preservation for up to 2 years on proprietary ZellSafe™ chips
- Whole slide imaging or ROI selection

TYPES OF CHIPCYTOMETRY ANTIBODIES

ChipCytometry offers the flexibility to mix and match pre-validated antibodies to create unique panels for immunology, oncology, and neurobiology applications. ChipCytometry antibodies have been fully validated for precise and consistent performance in cell suspensions and both FFPE and fresh frozen tissue sections. Antibodies are selected for their scientific application in relevant species, including human, non-human primate, and mouse samples (Figure 3).

Figure 3. *ChipCytometry antibodies detect protein expression levels for research applications for human, non-human primate, and mouse FFPE or fresh frozen tissue samples or cell suspensions.*

VALIDATION CRITERIA

Validating antibodies for use in ChipCytometry assays (Figure 4) is a multi-stage and iterative process that involves initial antibody screening, single-plex development, and multiplex optimization, throughout which antibodies are continually evaluated for:

1. **Specificity:** Antibodies bind their intended targets and do not demonstrate off-target effects.
2. **Sensitivity:** Antibodies detect low quantity antigens in order to differentiate positive cells from background fluorescence.
3. **Reproducibility:** Antibodies produce consistent results in similar or the same specimens over time.

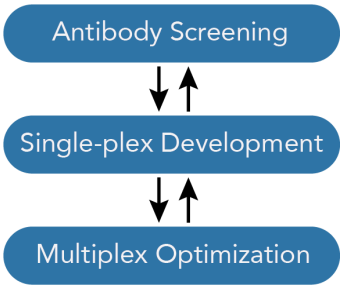

Figure 4. *Schematic of ChipCytometry antibody and assay validation: antibody screening, single-plex development, and multiplex optimization.*

CLONE TESTING

ChipCytometry antibodies leverage decades of histopathology experience, sourcing clones from a variety of vendors and previously validated for flow cytometry or IHC assays. New clones are selected based on the compatibility of the conjugated fluorophore with ChipCytometry instruments. Up to three clones are tested on fresh samples in the first cycle of immuno-staining. Figure 5 and Figure 6 show the results of clone screening for antibodies that target CD56. Each clone is evaluated to identify the strongest and most specific signal intensity with minimal off-target staining. The samples for antibody screening are selected based on where the target is expected to show positive staining.

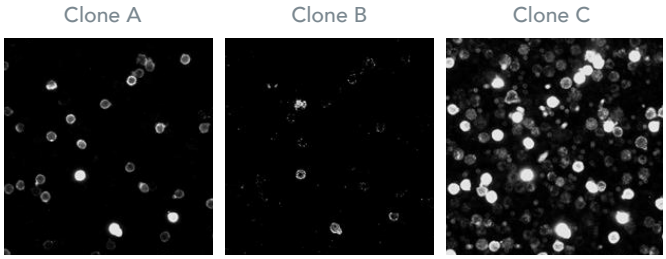

Figure 5. *Human PBMCs stained with 3 different clones that target CD56. Clone A passed validation tests for specificity, sensitivity, and reproducibility. Clone B failed due to weak, but specific, staining and Clone C failed due to free dye and non-specific staining of thrombocytes.*

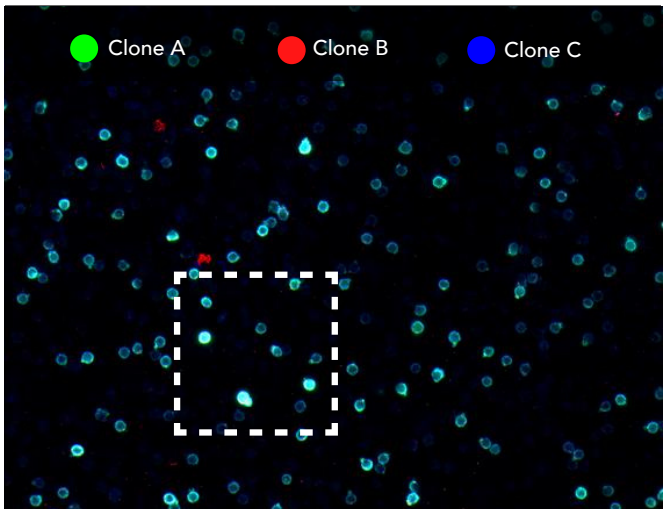

Figure 6. *Image overlay of the three antibodies for CD56. Clone A (green) and Clone C (blue) both demonstrate sensitive and specific staining of NK cells, but Clone C additionally stains cells expected to be CD56 negative. Clone B gives nonspecific signal that neither Clone A or Clone C shows.*

ANTIBODY TITRATION

Each antibody is titrated to select the correct concentration to optimally resolve cell populations. Too low of a dilution yields a weak signal, while too high of a dilution increases off-target binding effects and background fluorescence. Therefore, each antibody is titrated prior to experiment start to optimize the signal-to-noise ratio to ensure reliable analyses can be performed. Figure 7 and Figure 8 show the results of titration experiments for antibodies to pan-cytokeratin, CD45RA, and CD8.

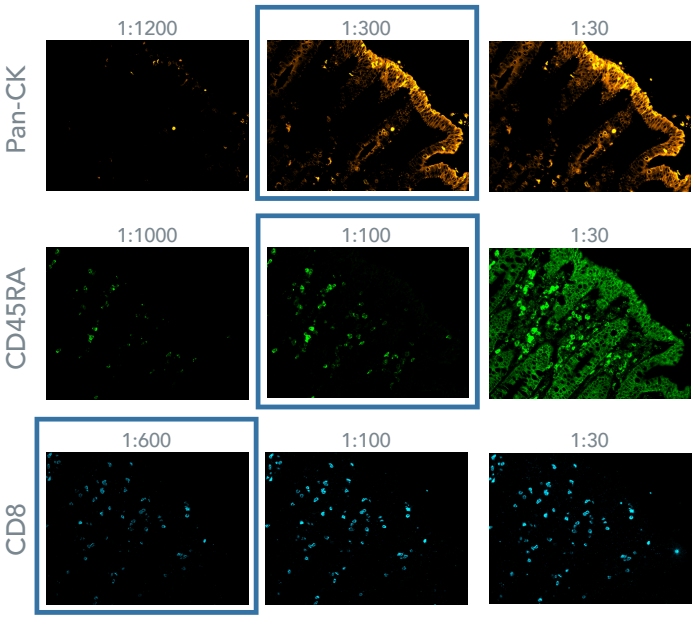

Figure 7. *A human FFPE colorectal cancer tissue stained with pan-cytokeratin, CD45RA, and CD8 antibodies at low, medium, and high concentrations.*

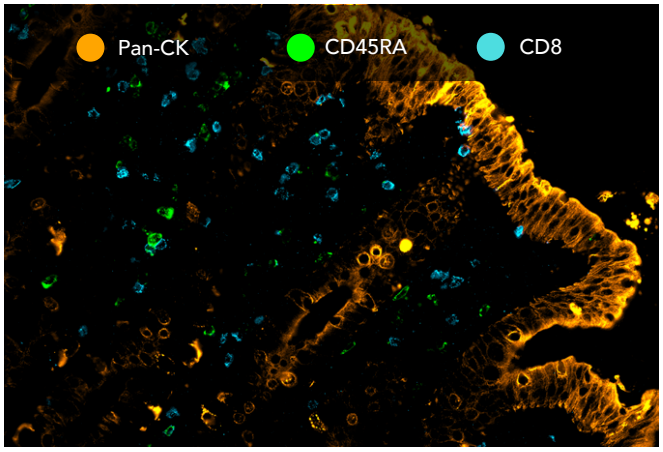

Figure 8. *Composite image of optimal dilutions for each antibodies to pan-cytokeratin (orange), CD45RA (green), and CD8 (cyan).*

LOCALIZATION CHECK

ChipCytometry antibodies must stain the intended tissue structures (e.g. epithelial tissue, stromal region, lymphoid follicles). In addition, antibodies must localize to the expected subcellular region. Figure 9 shows examples of surface, intracellular, and nuclear markers in tissue samples and cell suspensions. Current literature is reviewed to verify the expected expression pattern of the target in specific cell and tissue types.

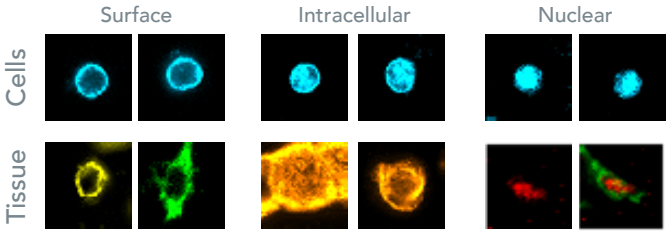

Figure 9. *ChipCytometry antibodies are evaluated for expected surface, intracellular, or nuclear staining.*

Table 1 shows examples of common surface, intracellular, and nuclear markers for ChipCytometry assays. ChipCytometry antibodies that target cluster of differentiation (CD) molecules and some structural components stain the cell surface, while other targets are found within and throughout the cell (e.g. CASP-3) or within the nucleus (e.g. FoxP3).

Surface		Intracellular
CD3	PD-1	CASP-3
CD4	PD-L1	Granzyme B
CD8	EpCAM	IL-10
CD11	Pan-CK	IL-12
CD20	SMA	IL-17A
CD56	Vimentin	INFγ

Nuclear		
FoxP3	p-Histone H3	p-Stat1
Ki-67	T-bet	p-Stat2

Table 1. *A number of common surface, intracellular, and nuclear markers for ChipCytometry assays.*

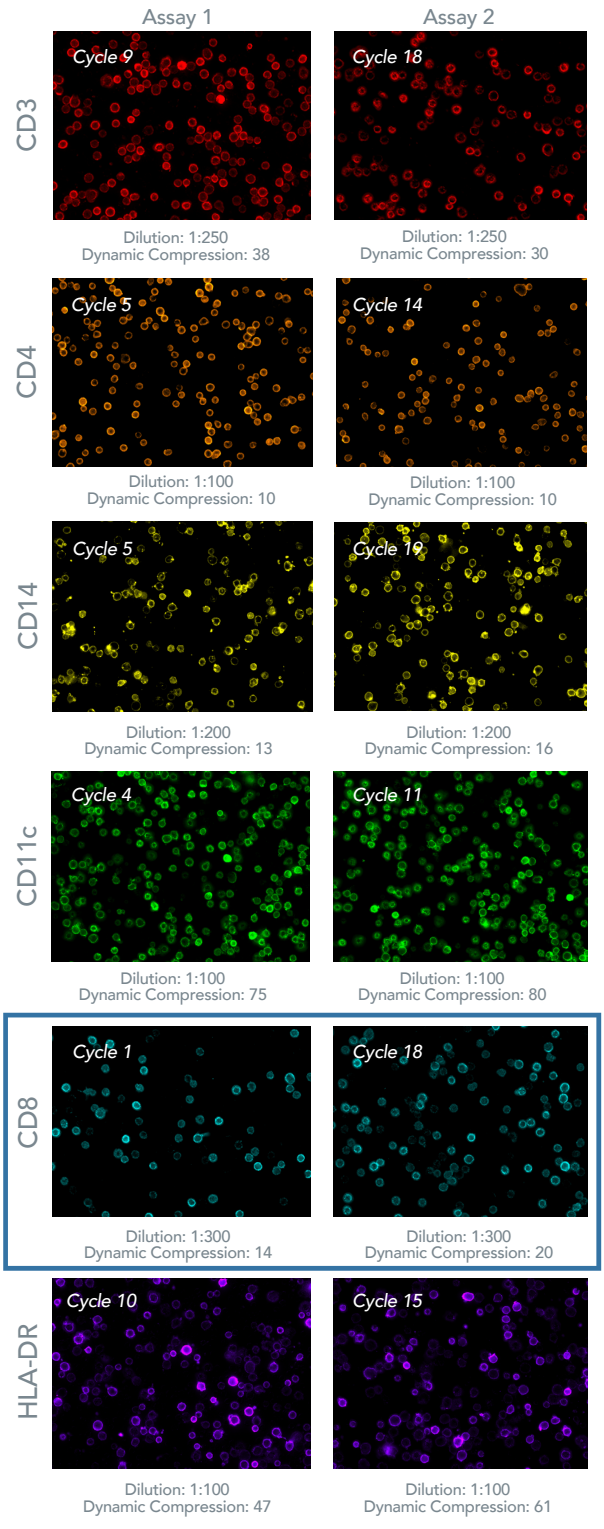

Figure 10. *Human PBMCs stained in either early (Assay 1) or later cycle (Assay 2).*

STERIC HINDERANCE ASSAY

Two experiments were designed where an antibody was either added in an earlier cycle (Assay 1) or a later cycle (Assay 2) to rule out effects of steric hinderance or cross-reactivity of antibodies. For each test, the same dilution and exposure time was used.

Qualitatively, fluorescence signal was the same regardless of if the antibody added in an earlier or later cycle (Figure 10). These observations were confirmed quantitatively by applying gates to density plots to positively select populations. Figure 11 shows the quantitative results for a CD8 antibody, where there is a high degree of concordance in the CD8 positive cells in Assay 1 and Assay 2. Taken together, these results indicate that the order of staining of the antibodies selected here has minimal effect on resolving cell populations.

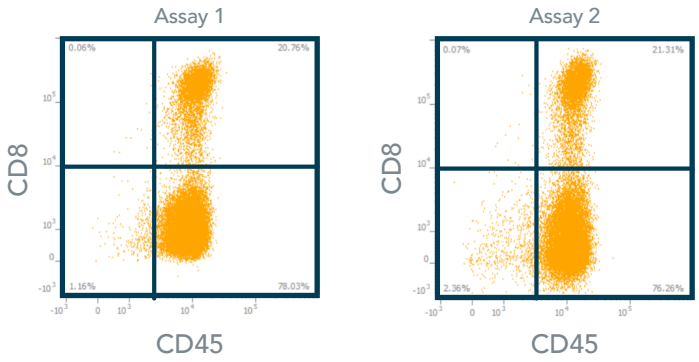

Figure 11. *Quantitative results of CD8 antibody staining in early cycle (Assay 1) and later cycle (Assay 2). Each dot on the plot represents a single cell.*

CONCLUSION

Spatial biology techniques require highly validated reagents for resolving antigens to accurately elucidate the tissue microenvironment. ChipCytometry employs commercially available antibodies as part of a practical solution for use in spatial biology research. ChipCytometry antibodies are evaluated in single and combination assays for specificity, sensitivity, and reproducibility.

In conclusion, ChipCytometry antibodies are fully validated to support deep characterization of the tissue microenvironment with ChipCytometry assays.

For more information on ChipCytometry, contact us at hello.canopy@bruker.com or visit canopybiosciences.com

References

1. Bordeaux et al. (2010). BioTechniques, 48(3), 197–209.
2. Du, Lin, Rashid et al. (2019). Nature Protocols, 14(10), 2900–2930.