APPLICATION NOTE

Simultaneous Detection of Cell Surface Phenotype and Intracellular Functions with Superior Signal Resolution by CyTOF Technology

Robust and reproducible intracellular staining with mass cytometry identifies critical functional markers undetectable with spectral flow cytometry

Introduction

In both basic and clinical research, accuracy and reproducibility in intracellular analysis are crucial for studying cellular mechanisms. For example, detecting and quantifying cytokines can advance the understanding of inflammatory diseases and provide important insight into novel therapeutic targets. CyTOF™ technology enables the single-cell analysis of 50-plus surface and intracellular markers simultaneously, resulting in multiplexed, unbiased information about the functional activity and patterns of a cell vs. merely its expression of surface markers. This enables identification of more diverse functional subsets, providing a rich source for discovering biomarkers, capturing mechanisms of disease and understanding immune responses to therapeutics.

Because mass cytometry uses metal-tagged antibodies detected by a time-of-flight mass analyzer, the technology inherently generates high signal resolution of intracellular markers regardless of panel size. This overcomes limitations of signal overlap and subsequent loss of resolution that can occur with fluorescence-based flow cytometry. Here, we highlight evidence across multiple research areas demonstrating CyTOF technology as the gold standard for high-dimensional functional profiling, superior to that of conventional or spectral flow cytometry.

This application note outlines:

- Limitations in functional marker detection with spectral flow cytometry that can be addressed using CyTOF technology
- A direct comparison of spectral flow cytometry and CyTOF intracellular detection
- Case studies demonstrating robust CyTOF analysis of the major type 2 cytokine IL-13 and the immunoregulatory cytokine IL-10 in human cells

An in-depth view of critical functions inside cells: CyTOF technology delivers precise intracellular target detection of cytokines

Analysis of intracellular molecules can offer important information about cell function, providing a more dynamic picture of the inner workings of cells, including changes in metabolic pathways and phosphorylation states across healthy and diseased samples. This type of functional data can help determine cell and tissue status in conditions such as diabetes, cancer and neurodegenerative disorders.

There is so much to be learned about intracellular functions as conventional technologies have not been able to reliably and accurately detect intended targets. Fluorescence-based flow cytometry, for example, falls short in intracellular detection likely due to the mechanics of cell and associated fluorescent marker acquisition and processing. In contrast, CyTOF technology excels at high-dimensional functional analysis, offering unmatched consistency, reliability and accuracy in intracellular detection. This benefit is based in the technology itself and its ability to better detect markers inside the cell – detecting signals that fluorescence flow cytometry just cannot "see."

Additionally, fluorescence-based flow cytometry requires extensive panel design and iterative optimization of reagent concentrations and staining conditions, as well as routine use of reference controls to achieve simultaneous detection of intracellular and surface markers for functional analyses. Of note, the limitation of commercially available reagents in multiple colors restricts building a panel in a way that may be needed to accommodate a higher number of specific targets. This potential for improper panel design can result in spectral unmixing algorithms failing to reliably detect critical functional markers such as type 2 immunity IL-13 and the immunoregulatory cytokine IL-10[1].

The published works below describe the capabilities of CyTOF technology to clearly detect these markers and the impact that an increased understanding of intracellular markers can have on disease research and therapeutic development.

Table 1. The biological significance of IL-10 and IL-13 is highlighted through recent examples of CyTOF intracellular staining.
These publications are further detailed in case studies below.

Therapeutic Application	Purpose	Significance
Asthma, bronchoalveolar lavage (BAL) cells	CyTOF intracellular staining of IL-10 identified distinct macrophage subsets enriched only in healthy controls and asthma patients with mild disease[4].	This case highlights the importance of capturing functional markers, including IL-10 and IL-13, to reveal novel macrophage subsets associated with disease severity in patients with asthma. The authors were able to use this information to **stratify asthma patients based on their distinct immune profiles and disease severity.**
Non-small-cell lung cancer (NSCLC)	Identifying IL-10 generated critical insight into immune associations with high-grade immune-related adverse events in 46 NSCLC patients post pembrolizumab treatment[5].	Accurate and consistent intracellular detection of IL-10 with CyTOF technology enabled the identification of circulating IL-10+ B cells and their role as a **predictive biomarker for immune checkpoint toxicity.**
Parkinson's disease (PD)	CyTOF staining of functional markers enabled the characterization of a peripheral blood immune cytokine expression profile in early- and late-onset patients with PD[6].	Robust intracellular staining was crucial for identifying key cytokines, such as IL-10 and IL-13, to characterize the **altered peripheral immune subsets in early- and late-onset PD patients.**

CASE STUDY: Technology comparison

Intracellular staining with CyTOF technology demonstrates superior signal resolution of cytokines compared with spectral flow cytometry

To more accurately design a technology comparison study between mass cytometry and spectral flow cytometry, researchers at Boston University used small panels, each comprised of surface and intracellular markers, in their experimental workflow. While mass cytometry does not have challenges with spectral spillover, designing smaller panels in fluorescence flow cytometry reduces spectral overlap and unmixing steps, thus offering a more balanced and direct comparison of intracellular detection. However, while smaller panels can be simpler to work with, they are less suitable for complex research questions that require simultaneous analysis of multiple markers.

The group developed 3 small clone-matched panels covering 3 sets of intracellular markers – cytokine production (12 parameters), phosphorylation events (11 parameters, data shown in Appendix) and T cell lineage transcription factors (12 parameters, data shown in Appendix)[1]. Each panel was designed to minimize the potential impact of spectral overlap on the resolution of spectral data.

Identical antibody clones were used for all panels, generating data for peripheral blood mononuclear cells (PBMC) collected from human donors that were cultured, stimulated and evenly split for staining with each cytometry platform[2]. Across all 3 panels, CyTOF technology was strikingly superior in detecting intracellular and intranuclear events.

Several important findings were discovered:

- The CyTOF platform identified distinct populations of CD4+ T cells expressing IL-10 in a stimulation-specific manner, while only background was observed for IL-10 with spectral flow cytometry in unstimulated and stimulated subsets (Figures 1 and 2)
- The IL-13+ population detected by spectral flow cytometry was indistinguishable from the negative population and notably lower in frequency than what was detected using CyTOF technology, which demonstrated superior detection power and lower frequency measurement by resolving a clear stimulation- specific positive population of IL-13+ cells[3] (Figure 1)

CyTOF technology clearly detected cytokine signals that spectral flow cytometry could not

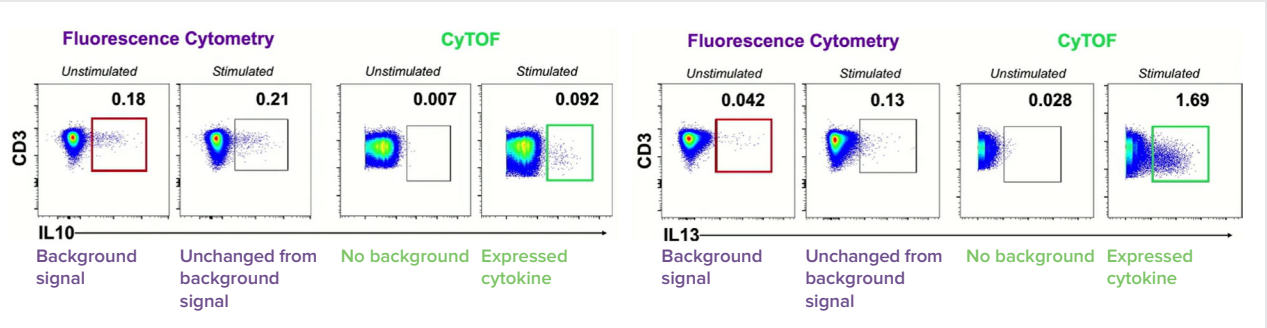

Figure 1. CyTOF data detected IL-10 and IL-13 expressing cells, where spectral flow cytometry data shows high background signal instead. Cells confirmed to be expressing IL-10 and IL-13 with Elispot data. Left: FSFC gating of IL-10 and IL-13 expression. Boxes show only background for unstimulated and stimulated populations. Right: CyTOF gates of IL-10 and IL-13 expression. Boxes highlight population with positive cytokine expression.

Rare IL-10 and IFNγ co-expressing cell subset identified only by CyTOF

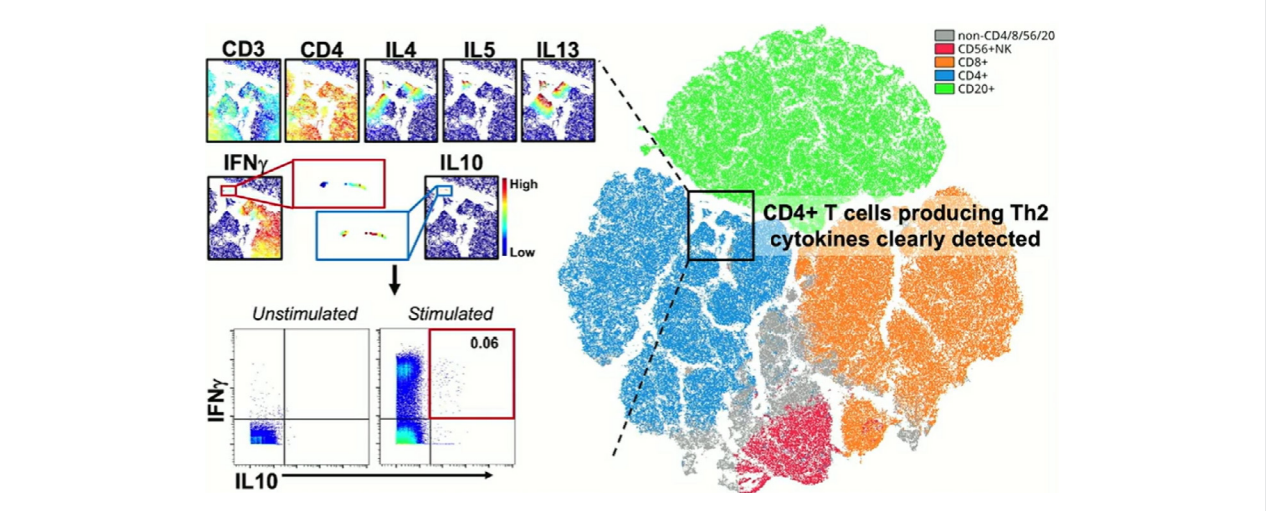

Figure 2. Cytokine panel data analysis using opt-SNE, a variation of t-SNE plots that allows improved visualization of high-parameter data, shows all T cells including the IL-10 rare cell population. It is remarkable to be able to see such low-frequency cells – 0.06% of cells showing IL-10 – and see them so clearly.

Discussion

Given the identical protocols and sample-processing steps, differences in resolution and data analysis did not appear to be due to spectral overlap, autofluorescence or antibody conjugate size. It was hypothesized that the differing mechanics of cell interrogation between the platforms leads to different intracellular detection capabilities.

One possible mechanism contributing to the limited intracellular staining efficiency of fluorescence flow cytometry could be the poor penetration of excited fluorophores through cellular structures to reach the laser detector[3]. CyTOF technology, on the other hand, disintegrates cells into ion clouds in which heavy metal tags are then quantified based on their time-of-flight in a mass spectrometer, eliminating any physical interference from intact cellular structures. Overall, CyTOF technology demonstrates superior performance for intracellular functional analyses compared with fluorescence flow cytometry.

Study at a glance*:

- Direct comparison of CyTOF technology and spectral flow cytometry demonstrated superior intracellular signal with metal-tagged antibodies
- Spectral flow cytometry failed to detect the stimulation-specific signal for intracellular markers IL-10 and IL-13 that was cleanly achieved by CyTOF technology; intranuclear markers were detected with superior signal:noise by mass cytometry compared with fluorescence cytometry

HIGHLIGHTED CLINICAL RESEARCH STUDIES

The biological significance of IL-10 and IL-13 and how the intracellular detection capabilities of CyTOF technology impact immunology research

The ability of CyTOF technology to detect the intracellular markers IL-10 and IL-13 provides an important milestone in immunology research by enabling the true investigation of cell dynamics involved in inflammation and immune response in autoimmune disorders, cancer and neurodegenerative disease. Detailed here are some examples of how cytokine detection using CyTOF platforms directly advanced this research (summarized in Table 1).

CyTOF technology enables the detection of IL-10 and IL-13 to characterize bronchoalveolar (BAL) cells and stratify asthma patients

This case highlights the importance of capturing functional markers IL-10 and IL-13, revealing novel macrophage subsets that are associated with disease severity in patients with asthma. As a result, the authors were able to stratify asthma patients based on their distinct immune profiles and disease severity.

Study at a glance:

- Accurate staining of intracellular cytokines IL-10 and IL-13 was essential for identifying immune populations correlated with asthma severity
- An IL-10-producing CD206+CD11b+CCR4+ macrophage subset detected by CyTOF technology was enriched only in mild asthma patients and healthy controls (Figure 3)
- Distinct immune subsets associated with disease severity identified by CyTOF technology provide novel insights into asthma patient stratification in clinical settings

CyTOF intracellular staining of IL-10 reveals direct association with disease severity

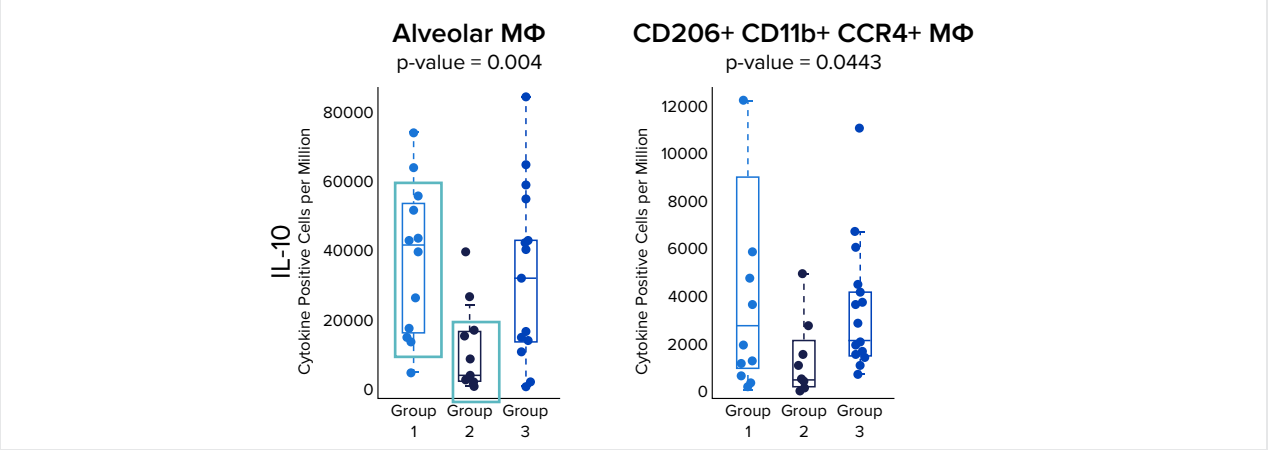

Figure 3. High-dimensional CyTOF profiling on BAL immune subsets. Intracellular cytokine expression in macrophages. Group 1: healthy control patients and mild disease; group 2: severe asthma patients; group 3: mixture of severe and mild asthma patients[4].

Robust intracellular CyTOF staining reveals a novel biomarker of a regulatory B cell (Breg) defect and its correlation to immune checkpoint blockade toxicity

Accurate CyTOF intracellular staining of IL-10 generated critical insight into immune associations with high-grade immune-related adverse events in 46 non-small-cell lung cancer (NSCLC) patients post pembrolizumab treatment[5].

Study at a glance:

- Intracellular staining with CyTOF technology ensured accuracy in detecting IL-10 along with 30-plus immune markers to capture IL-10+ regulatory B cells, which were associated with high-grade immune-related adverse events (Figure 4)
- The loss of circulating IL-10+ regulatory B cells is a potential predictive biomarker for immune checkpoint blockade-induced toxicity

Distinct differences in IL-10+ B cells associated with toxicity outcome identified by CyTOF technology

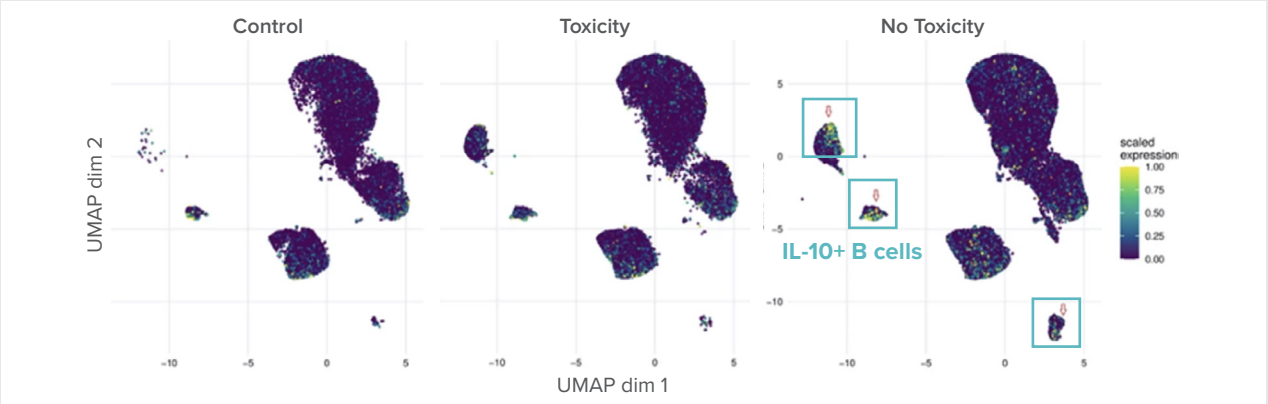

Figure 4. Dimensionality reduction analysis of CyTOF data revealed distinct B cell population deficiencies in patients with treatment toxicity. UMAP plots for the no toxicity group, toxicity group and control group showing IL-10 expression (heat map) in Breg clusters, highlighted with red circles.

CyTOF quantification of intracellular markers reveals a distinct peripheral immune landscape in Parkinson's disease

CyTOF intracellular staining was crucial for identifying key cytokines such as IL-10 and IL-13 to characterize the altered peripheral immune subsets in early- and late-onset PD patients.

Study at a glance:

- Intracellular functional profiling with CyTOF technology (27 surface and 15 intracellular markers) enabled the characterization of a peripheral blood immune cytokine expression profile in early- and late- onset patients with Parkinson's disease (PD)
- Downregulation of IFNγ, CCL17 and IL-10 was observed in CD8+ T cells in late-onset PD patients, while CCL17, IL-10 and IL-13 expression decreased in NK cells (Figure 5)
- An altered immune profile in PD patients was characterized by a decrease in pro-inflammatory cytokines and chemokines compared with healthy controls

CyTOF quantification identifies altered cytokine expression in NK cell clusters of patients with PD

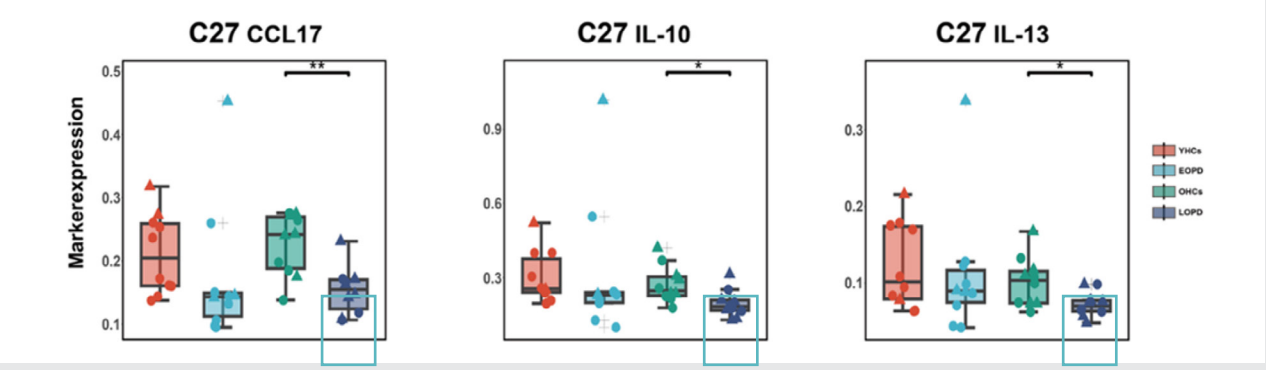

Figure 5. Impaired cytokine secretion by NK cells in patients with PD compared with that in healthy controls, especially in patients with late-onset PD. Comparisons of cytokine and chemokine marker expression within selected clusters. EOPD: early-onset Parkinson's disease; LOPD: late-onset Parkinson's disease; YHC: young healthy control; OHC: old healthy control. + for the outliers. ▲ = male; o = female. One-way ANOVA, Bonferroni correction. *p <0.05, ** p<0.01.

Summary

- Accurate and reproducible intracellular staining is critical for functional analyses in biomedical research
- Spectral flow cytometry could not detect intracellular cytokines (IL-10, IL-13) and showed less resolution for intranuclear targets such as pSTAT3 and T-bet
- CyTOF technology provides robust and unbiased intracellular protein detection while maintaining a higher panel capacity of 50-plus markers compared with fluorescence flow cytometry
- Robust staining of intracellular cytokines, such as IL-10 and IL-13, with CyTOF technology was demonstrated in multiple fields of research, including autoimmune asthma, immuno-oncology and neurodegenerative PD
- Mass cytometry uniquely and simultaneously measures a high number of markers with high resolving power, making it a reliable and precise technology to accurately characterize immune complexity

Appendix

Data shown from the phosphorylation events panel and the T cell lineage transcription factors panel developed by the Boston University team to compare spectral flow cytometry and CyTOF technology. Findings included:

- Robust detection by CyTOF technology of all intranuclear markers, including pSTAT1, pSTAT3, pSTAT5, p38 and pERK1/2, was observed, presenting an overwhelmingly clear signal:noise ratio, while spectral flow cytometry could not separate all the positive and negative populations[3] (Figure 6)
- The transcription factors T-bet and TOX were captured by CyTOF technology with higher resolution than spectral flow cytometry[3], with a very clear split in TOX vs. T-bet, enabling easy gating decisions. In spectral flow cytometry data, these 2 populations were difficult to distinguish (Figure 7)

Distinct differences in intracellular phosphorylation marker staining by spectral flow cytometry and CyTOF technology

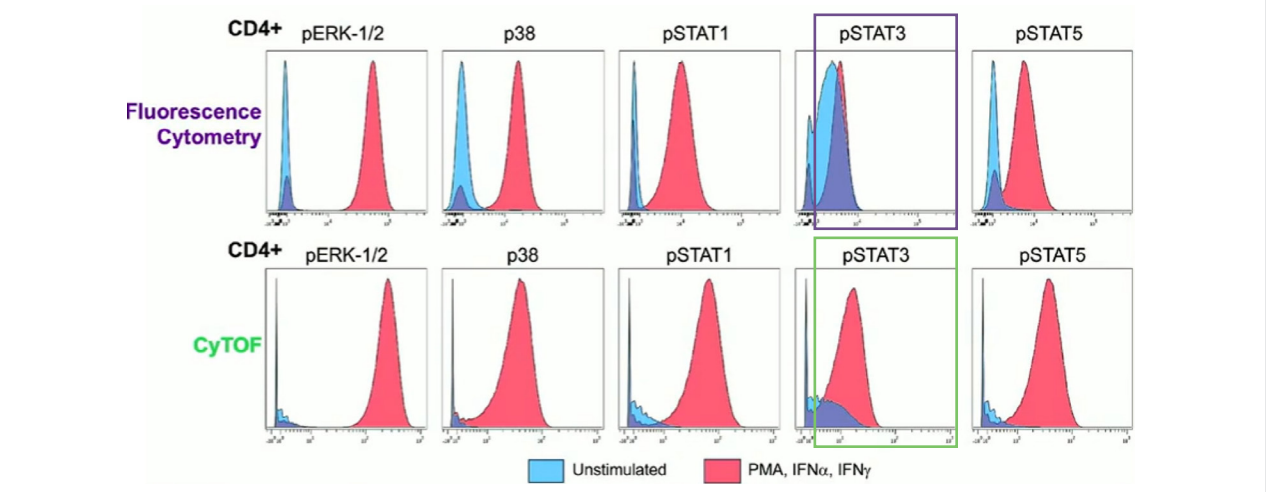

Figure 6. Histograms from the small phosphoprotein panel by spectral flow cytometry and CyTOF technology. Comparison of marker expression within the CD4+ population of stimulated and unstimulated samples shows robust detection of all markers in CyTOF data with a clear signal:noise ratio.

Higher resolution in CyTOF data enables easy gating decisions

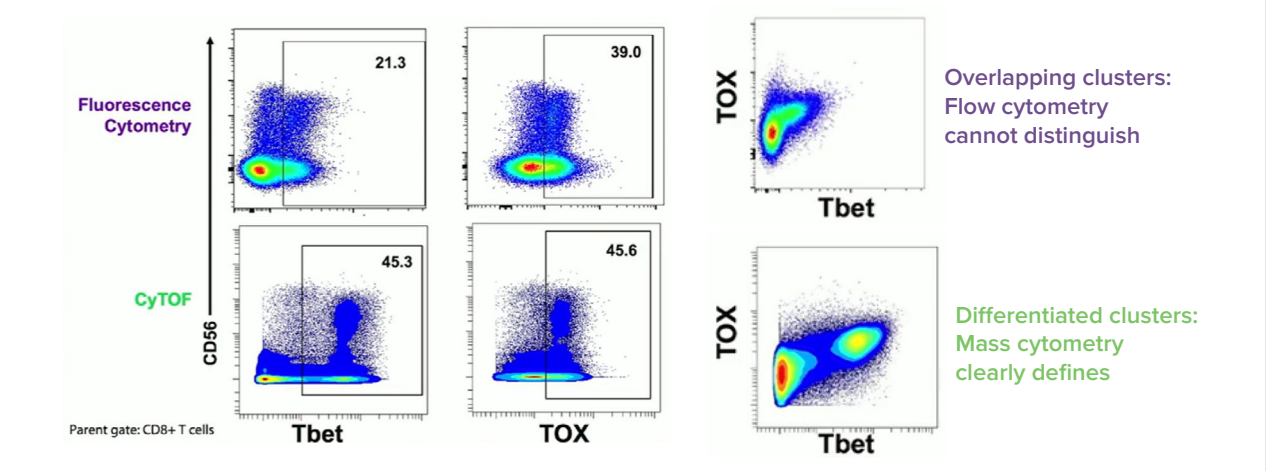

Figure 7. Data from the transcription factors panel highlights a very different signal:noise ratio seen with CyTOF technology, with twice as many positive cells for T-bet. On the right, plotting TOX vs. T-bet shows the importance of signal resolution. A clear view of cells that are expressing the transcription factors vs. the cells that are not means there is no ambiguity in gating decisions with CyTOF technology.

References

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* This ongoing study is part of a collaboration between Boston University and Standard BioTools.

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