An optimized method to measure human FOXP3^{+} regulatory T cells from multiple tissue types using mass cytometry

Nicholas A. J. Dawson[1,2], Avery J. Lam[2,3], Laura Cook[1,2], Romy E. Hoeppli[2,3], Raewyn Broady[1,2], Anne M. Pesenacker[2,3], Megan K. Levings[2,3]

Author affiliations:
1. Department of Medicine, University of British Columbia Vancouver, BC, Canada
2. BC Children's Hospital Research Institute, Vancouver, BC, Canada
3. Department of Surgery, University of British Columbia, Vancouver, BC, Canada

Keywords: FOXP3, mass cytometry, regulatory T cells, human, flow cytometry, CyTOF

Corresponding Author: Megan K. Levings, Department of Surgery, University of British Columbia, A4-186, 950 West 28th Ave., Vancouver, B.C., Canada, V5Z 4H4

mlevings@bcchr.ca

Abbreviations

ACCENSE	automatic classification of cellular expression by nonlinear stochastic embedding
CSB	Cell Staining Buffer
CMV	cytomegalovirus
FBS	fetal bovine serum
FMO	fluorescence minus one
IMDM	Iscove's Modified Dulbecco's Medium
JIA	juvenile idiopathic arthritis
MMO	metal minus one
PBMC	peripheral blood mononuclear cell
PFA	paraformaldehyde
RT	room temperature
SEB	Staphylococcal enterotoxin B
Tconv	conventional T cell
Treg	regulatory T cell

link to latest version: DOI: 10.1002/eji.201747407

High-dimensional phenotyping with mass cytometry has allowed immunologists to discover new cell populations and functions, as well as immunological networks [1]. However, there has been limited use of this technology to measure transcription factors, which are often difficult to detect due to their intranuclear localization and association with DNA. High and stable expression of the transcription factor FOXP3 is the defining characteristic of regulatory T cells (Tregs) [2]. Therefore, robust methodology to detect this protein is essential to realize the benefit of mass cytometry-based analysis of Tregs in health, during the course of disease and/or in response to therapy.

Several studies have reported analysis of human Tregs using mass cytometry. Specifically, Mason et al analyzed pre-sorted $CD4^+CD25^+CD127^-$ Tregs but without FOXP3 staining in their mass cytometry panel [3]. In unfractionated peripheral blood, Hirakawa et al analyzed changes in FOXP3 expression in graft-versus-host disease following low-dose IL-2 therapy [4], Kordasti et al studied $FOXP3^+$ Tregs in aplastic anemia [5], and Bengsch et al studied cytotoxic protein co-expression patterns [6]. In tissue, Lowther et al used mass cytometry to phenotype circulating versus tumor-infiltrating $FOXP3^+$ Tregs from patients with glioblastoma multiforme [7] as did Chew et al in the context of hepatocellular carcinoma [8]. However, none of these studies reported validation of the FOXP3 staining protocol or compared results to "gold-standard" data obtained with flow cytometry. Using data from conventional flow cytometry as a benchmark, we sought to optimize FOXP3 staining protocols for use in mass cytometry for optimal detection of polyclonal and antigen-specific human Tregs from peripheral blood and other tissues.

When developing an optimal protocol to detect FOXP3 by mass cytometry, we aimed to achieve clear resolution of FOXP3$^+$ Tregs, minimal non-specific staining of conventional T cells (Tconvs), and uncompromised detection of cell surface proteins necessary to identify Tregs and define other cell types and function. We tested three buffer sets: (i) the recommended FOXP3 mass cytometry staining kit from Fluidigm (#201319); (ii) the commonly used and effective eBioscience FOXP3 fix/perm buffer set (ThermoFisher #00-5523-00) which was developed for conventional flow cytometry [9], and (iii) a custom buffer system which used paraformaldehyde (PFA)-based fixation and saponin-based membrane permeabilization (termed PFA/saponin; see Supporting Information). For all samples and buffers, the FOXP3 gates were set on the basis of staining in live CD4-negative cells (Supporting Figure 1A). We found that all three buffer systems resulted in similar proportions of FOXP3$^+$ cells within the CD4$^+$CD25$^+$CD127$^-$ gate, which is the widely accepted combination of cell surface molecules used to define Tregs (Figure 1A, Supporting Figure 1A) [2, 10, 11]. This finding was confirmed using a reverse gating strategy in which the proportion of CD25$^+$CD127$^-$ cells within the CD4$^+$FOXP3$^+$ gate was determined (Supporting Figure 1B). Notably, in the majority of samples, a small proportion of Tconvs (CD4$^+$CD25$^-$ CD127$^+$) displayed background staining of FOXP3 using the eBioscience staining protocol, but not with the Fluidigm or PFA/saponin protocols (Figure 1A).

Since fixation methods can destroy or alter epitopes, negatively affecting monoclonal antibody recognition [12, 13], we next determined the effect of each FOXP3 staining protocol on detection of a range of T cell surface markers. We found that Fluidigm buffers had the greatest negative effect on several Treg-defining cell surface markers including CD3, CD4, and CD25, resulting in poor resolution of CD3$^+$CD4$^+$ cells (Supporting Figure 1A) as well as CD25^{hi}CD127^{lo} cells

(Figure 1B). In contrast, the custom PFA/saponin protocol and eBioscience buffers showed equivalent and optimal detection of CD3, CD4, and CD25, supporting their use for detection of $CD4^+CD25^+CD127^-$ Tregs. Evaluation of other Treg-associated cell surface markers revealed that no single fixation method was optimal for detection of all markers tested. Although each method had its respective limitations (Supporting Figure 2A&B), the PFA/saponin protocol was optimal for detection of Tregs by mass cytometry because it neither caused background staining of FOXP3 in Tconvs, nor diminished detection of the most important Treg-defining cell surface molecules, namely CD4, CD25, and CD127. These data highlight the importance of determining the impact of fixation methods used to measure nuclear proteins on detection of other cytoplasmic or cell surface markers of interest.

Having identified an optimal method for staining FOXP3 via mass cytometry, we next compared mass cytometry data obtained using the PFA/saponin protocol and mass cytometry to conventional fluorescence flow cytometry. Since human immune cells are often analyzed after cryopreservation, we also compared data from ex vivo or cryopreserved cells on both platforms. We found that cell surface marker detection was similar for most antigens tested on both platforms (Supporting Figure 3A-E), with the notable exception of significantly lower proportions of CCR4-expressing $CD3^+CD4^+$ T cells in mass cytometry. This difference was likely at least partially due to differential CCR4 clone sensitivity (Supporting Figure 3F), highlighting the need to for careful antibody clone selection for optimal resolution in mass (as well as flow) cytometry.

For both ex vivo and cryopreserved samples, the proportion of FOXP3-expressing cells (PCH101 clone) detected via mass cytometry was significantly lower than that detected via flow cytometry (Figure 1C, Supporting Figure 4). This difference may be due to fundamental differences related to the chemical properties of fluorophores and metals, possibly resulting in a differential ability of metal- versus fluorochrome-conjugated antibodies to pass through the nuclear membrane. However, within each platform, there was no significant difference in the proportion of $FOXP3^+$ cells detected in matched ex vivo versus cryopreserved samples (Figure 1D).

To determine if this reduced detection of FOXP3 in mass cytometry may be a limitation of the PCH101 clone, we repeated the experiment using the FOXP3 clone 236A/E7 and found similar results (Figure 1E). This difference between flow and mass cytometry FOXP3 staining may, in part, be related to the fact that even the most sensitive metals (eg. 162Dy) are less sensitive than the brightest fluorophores (eg. PE) [14, 15].

It is often desirable to detect antigen-specific Tregs, so we next assessed the ability of the PFA/saponin protocol to enumerate $FOXP3^+$ Tregs within a population of antigen-specific $CD4^+$ T cells. Blood was stimulated with the indicated antigen for 44 hours and antigen-specific $CD4^+$ T cells were detected by measuring induced co-expression of CD25 and OX40 [16] by flow or mass cytometry. Optimal methods to detect FOXP3 in whole blood by flow or mass cytometry were used (see Supporting Information). The proportion of FOXP3-expressing cells within the $CD4^+CD25^+OX40^+$ gate was determined (Supporting Figure 5A). Consistent with the fact that Tregs comprise a substantial proportion of recall responses [17], we found that both mass

cytometry and flow cytometry detected a clear population of FOXP3$^+$ cells within the antigen-specific cell gate. The proportion of antigen-specific CD4$^+$ responder cells detected by mass cytometry was similar to flow cytometry (Supporting Figure 5B). Notably, in contrast to ex vivo cells, the proportion of FOXP3$^+$ cells detected by mass cytometry was similar to that detected with flow cytometry (Supporting Figure 5A).

We next tested the applicability of the PFA/saponin-based FOXP3 staining protocol to detect CD4$^+$CD25$^+$CD127$^-$ Tregs in samples other than peripheral blood via mass cytometry. Specifically, mononuclear cells from umbilical cord blood, CD8-depleted thymocytes, or synovial fluid from juvenile idiopathic arthritis (JIA) patients were stained with a panel of T cell and Treg-related markers, including FOXP3. Data were analyzed with ACCENSE [18], which compares cells on a two-dimensional plot while maintaining single cell resolution and complexity, and then further identifies statistically significant subpopulations (Figure 2A). The relative expression of each marker within each of the 25 ACCENSE-defined populations, as well as their relative abundance within each tissue, was determined and plotted on heat maps (Figure 2B). Strikingly, Tregs preferentially clustered into ACCENSE populations by tissue source. For example, Treg populations of in cord blood were uniquely identified by high CD45RA, whereas Treg populations in JIA synovial fluid were defined by high expression of multiple activation/effector molecules, with a specific enrichment for high PD-1 expression. Despite strong tissue-specific segregation, a few populations were shared between tissues. For instance, a subset of CD45RA$^+$ Tregs (population 4) was found in both peripheral blood and cord blood, and populations 3 (CD127$^+$) and 15 (low for all markers) were present in cord blood and thymus. Overall, these results support a growing body of evidence indicating that Tregs acquire unique

147 tissue-specific phenotypes and that phenotypes in peripheral blood may not reflect those of
148 tissue-resident cells [19, 20].
149
150 In conclusion, we have developed an optimal protocol to detect FOXP3 by mass cytometry,
151 tested its suitability in ex vivo and cryopreserved samples, and shown its utility in a broad range
152 of immune cell sources. We have further shown that antigen-specific FOXP3^{+} Tregs can be
153 detected by mass cytometry in whole blood. An important consideration is that, at least in ex
154 vivo peripheral blood samples, mass cytometry is significantly less sensitive than traditional
155 fluorescence flow cytometry at detecting FOXP3 expression. Overall, our optimized
156 PFA/saponin protocol is the best-validated method described to date to detect FOXP3 expression
157 by mass cytometry without compromising detection of cell surface markers. This method will
158 enhance high-dimensional studies of Treg phenotype and function in the context of complex
159 cellular networks.

Acknowledgements. This work was supported by a grant from the Leukemia Lymphoma Society of Canada (to RB and MKL). NAJD and AJL hold CIHR Doctoral Research Awards; LC holds a BC Children's Hospital Research Institute postdoctoral fellowship; AMP holds a JDRF postdoctoral fellowship; REH and MKL hold salary awards from BC Children's Hospital Research Institute. We thank Mike Williams at the University of British Columbia Antibody Lab, Andy Johnson and David Ko at the University of British Columbia and BC Cancer Agency Flow Cores, respectively, and Fluidigm for helpful discussions, technical support and custom antibody conjugation. The authors thank volunteers, patients and their parents for contribution of samples, as well as BC Children's Hospital staff who made this study possible.

Conflict of Interest: The authors declare no commercial or financial conflict of interest.

Author contributions: NAJD designed and conducted experiments, analyzed data, and wrote the manuscript. AJL designed and conducted experiments, analyzed data, and critically reviewed the manuscript. LC designed and conducted experiments, critically reviewed the manuscript, and provided experimental design guidance. REH and AMP contributed to experiment design, reviewed results, and critically reviewed the manuscript. RB secured funding and contributed to experimental design. MKL secured funding, provided overall guidance for experimental design and interpretation, and wrote the manuscript.

References

1 **Spitzer, M. H. et al.** *Cell* 2016. **165**: 780-791.
2 **Seddiki, N. et al.** *J Exp Med* 2006. **203**: 1693-1700.
3 **Mason, G. M. et al.** *J Immunol* 2015. **195**: 2030-2037.
4 **Hirakawa, M. et al.** *JCI Insight* 2016. **1**: e89278.
5 **Kordasti, S. et al.** *Blood* 2016. **128**: 1193-1205.
6 **Bengsch, B. et al.** *J Immunol Methods* 2018. **453**: 3-10.
7 **Lowther, D. E. et al.** *JCI Insight* 2016. **1**: e85935.
8 **Chew, V. et al.** *Proc Natl Acad Sci U S A* 2017. **114**: E5900-E5909.
9 **Law, J. P. et al.** *Cytometry A* 2009. **75**: 1040-1050.
10 **Liu, W. et al.** *J Exp Med* 2006. **203**: 1701-1711.
11 **Baecher-Allan, C. et al.** *J Immunol* 2001. **167**: 1245-1253.
12 **Elghetany, M. T. et al.** *Cytometry B Clin Cytom* 2005. **65**: 1-5.
13 **Turac, G. et al.** *PLoS One* 2013. **8**: e68519.
14 **Bendall, S. C. et al.** *Science* 2011. **332**: 687-696.
15 **Tricot, S. et al.** *Cytometry A* 2015. **87**: 357-368.
16 **Zaunders, J. J. et al.** *J Immunol* 2009. **183**: 2827-2836.
17 **Seddiki, N. et al.** *Eur J Immunol* 2014. **44**: 1644-1661.
18 **Shekhar, K. et al.** *Proc Natl Acad Sci U S A* 2014. **111**: 202-207.
19 **Thome, J. J. et al.** *Trends Immunol* 2015. **36**: 428-435.
20 **Bjornson, Z. B. et al.** *Curr Opin Immunol* 2013. **25**: 484-494.

Figure Legends

Figure 1. ***Development of a FOXP3 staining protocol for mass cytometry and comparison to flow cytometry.*** (**A**) Fresh or thawed, cryopreserved PBMCs were stained with the indicated monoclonal antibodies using the Fluidigm FOXP3 or eBioscience FOXP3 Staining Kit, or fixed in paraformaldehyde (PFA) and stained in the presence of saponin (PFA/saponin). Samples were analyzed by mass cytometry after an overnight DNA intercalation step. Viable (cisplatin$^-$) bead$^-$ DNA1$^+$DNA2$^+$CD3$^+$CD4$^+$ single cells were divided into CD25$^+$CD127$^-$ or CD25^{--}CD127^{++} fractions, then analyzed for FOXP3 expression (see Figure S1A for example gating). Representative (left) and summarized (n=7) data (right). **(B)** PBMCs were prepared as in (A) and analyzed on a mass cytometer. Shown are the mean counts of each target within the viable (cisplatin$^-$) bead$^-$DNA1$^+$DNA2$^+$CD3$^+$CD4$^+$ single cell gate for each fixation method. Data from n=7 individual donors are shown. Shown are cell surface molecules important for Treg lineage identification. **(C&D)** PBMCs (n=8) were divided and either cryopreserved or analyzed immediately using eBioscience FOXP3 staining buffers and flow cytometry or PFA/saponin fixation and metal-tagged antibodies for mass cytometry. The FOXP3 (PCH101 clone) gate was set on the basis of a viable (cisplatin$^-$) bead$^-$DNA1$^+$DNA2$^+$CD3$^+$CD4$^-$ single cell gate. Representative (left) and individual (n=4-8) data (right). Each line is data from one individual with data collected in at least three independent experiments. **(E)** Cryopreserved PBMCs (n=4) were stained as in (C-D) using the 236A/E7 FOXP3 clone or a fluorescence/metal minus one (FMO/MMO) control. Representative (left) and individual (n=4) data (right). Each line is data from one individual with data collected in one independent experiment. For (A-B), statistical significance was determined with a one-way ANOVA with a Tukey multiple comparisons post-

test. For (C-E), statistical significance was determined with two-tailed paired t tests. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$

Figure 2. ***ACCENSE analysis of Tregs in peripheral blood, cord blood, thymus, and JIA synovial fluid.*** **(A)** Cryopreserved mononuclear cells from the indicated tissues were stained with the PFA/saponin method and analyzed by mass cytometry (n=3-4 per tissue). Data from viable (cisplatin$^-$) bead$^-$DNA1$^+$DNA2$^+$CD3$^+$CD4$^+$CD25$^+$CD127$^-$ single cells from each tissue were pooled and analyzed using ACCENSE v0.5.0-beta (Barnes-Hut-SNE dimension reduction and k-means significance of 10^{-8}). **(B)** Heat map analysis of mean count expression of each protein included in the ACCENSE analysis was completed using FlowJo v10.3 and R v3.3.2 statistical software. ACCENSE populations were clustered by tissue frequency. Average marker expression was normalized across all populations using Z-score analysis to highlight the range of individual marker expression in different populations. Average population frequency was normalized within each population using Z-score analysis to emphasize which tissues were enriched for each ACCENSE population. Data shown are representative of two independent experiments.

Figure 1

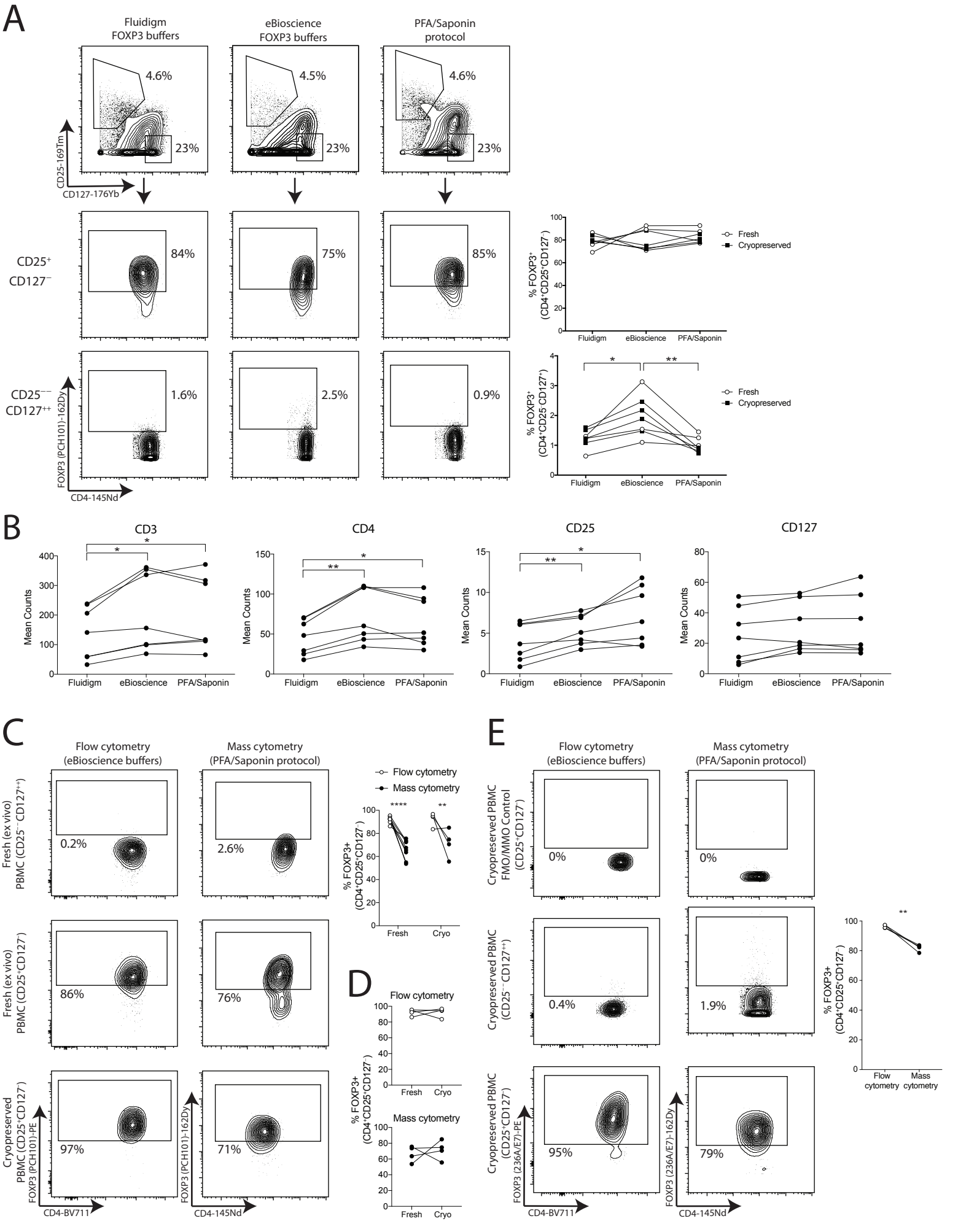
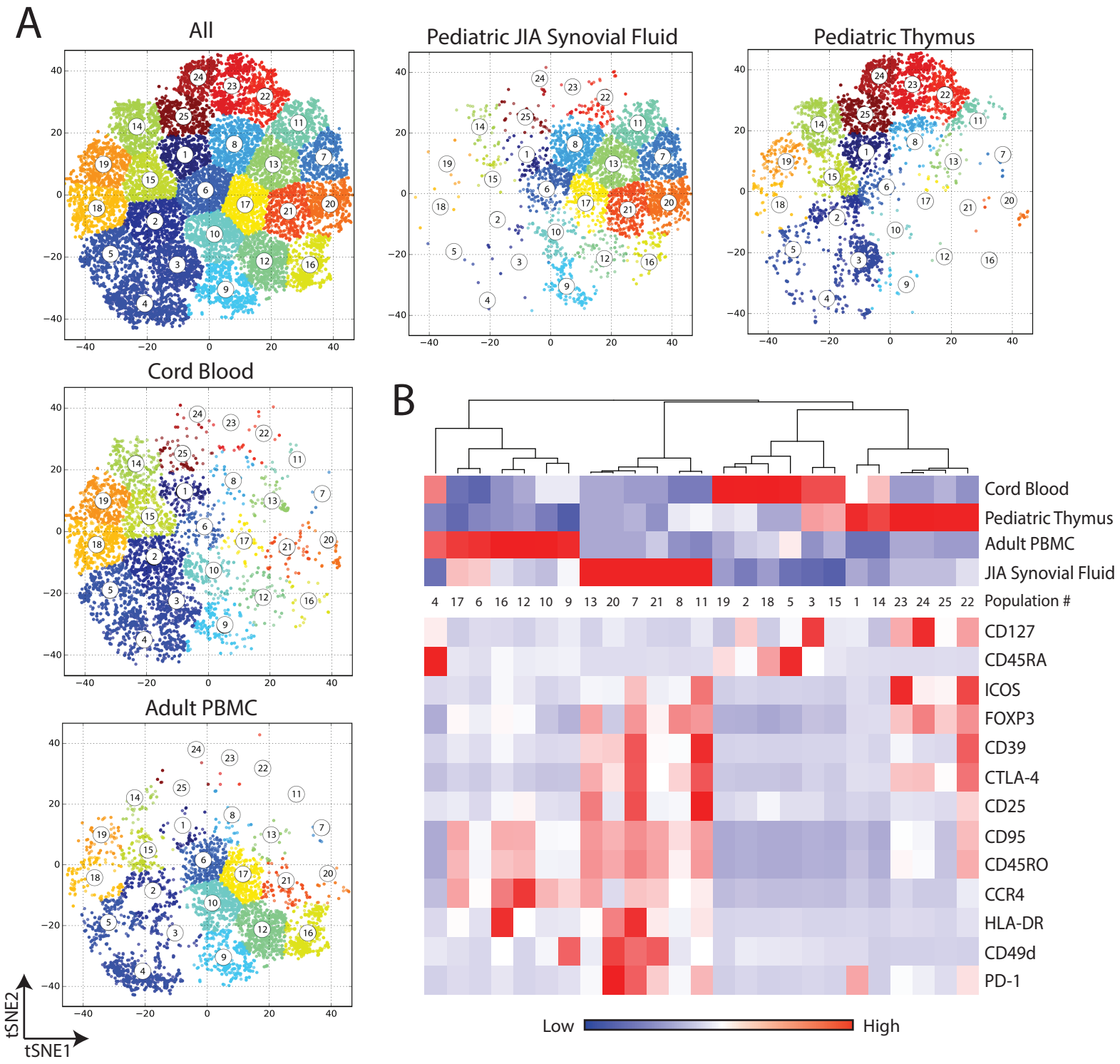

A
Fluidigm FOXP3 buffers
eBioscience FOXP3 buffers
PFA/Saponin protocol
4.6%
4.5%
4.6%
23%
23%
23%
CD25-169Tm
CD127-176Yb
CD25+ CD127−
84%
75%
85%
CD25−− CD127++
1.6%
2.5%
0.9%
FOXP3 (PCH101)-162Dy
CD4-145Nd
% FOXP3+ (CD4+CD25+CD127−)
Fresh
Cryopreserved
Fluidigm
eBioscience
PFA/Saponin
B
CD3
CD4
CD25
CD127
Mean Counts
C
Flow cytometry (eBioscience buffers)
Mass cytometry (PFA/Saponin protocol)
Fresh (ex vivo) PBMC (CD25−− CD127++)
0.2%
2.6%
Fresh (ex vivo) PBMC (CD25+CD127−)
86%
76%
Cryopreserved PBMC (CD25+CD127−)
FOXP3 (PCH101)-PE
97%
71%
CD4-BV711
CD4-145Nd
% FOXP3+ (CD4+CD25+CD127−)
Flow cytometry
Mass cytometry
Fresh
Cryo
D
E
Cryopreserved PBMC FMO/MMO Control (CD25+CD127−)
0%
0%
Cryopreserved PBMC (CD25−− CD127++)
0.4%
1.9%
Cryopreserved PBMC (CD25+CD127−)
FOXP3 (236A/E7)-PE
95%
79%
FOXP3 (236A/E7)-162Dy
Flow cytometry
Mass cytometry

Figure 2


A
All
Pediatric JIA Synovial Fluid
Pediatric Thymus
Cord Blood
Adult PBMC
tSNE2
tSNE1
B
Cord Blood
Pediatric Thymus
Adult PBMC
JIA Synovial Fluid
4 17 6 16 12 10 9 13 20 7 21 8 11 19 2 18 5 3 15 1 14 23 24 25 22
Population #
CD127
CD45RA
ICOS
FOXP3
CD39
CTLA-4
CD25
CD95
CD45RO
CCR4
HLA-DR
CD49d
PD-1
Low
High