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

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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

High-dimensional phenotyping with mass cytometry has revealed new cell populations and functions as well as immunological networks [1] but its use to measure transcription factors, which are difficult to detect due to intranuclear localization and DNA association, has been limited. High and stable expression of the transcription factor FOXP3 defines regulatory T cells (Tregs) [2] so robust methodology to detect FOXP3 is essential for mass cytometry-based analysis of Tregs in health, disease and/or in response to therapy.

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

To develop an optimal protocol to detect FOXP3 by mass cytometry, we aimed for clear resolution of $FOXP3^+$ Tregs, minimal staining of conventional T cells (Tconvs), and uncompromised detection of cell surface proteins necessary to identify Tregs/or and other cell types. We tested three buffer sets: the FOXP3 mass cytometry staining kit from Fluidigm; the commonly-used eBioscience FOXP3 fix/perm buffer set developed for flow cytometry [9], and a custom buffer system using paraformaldehyde (PFA)-based fixation and saponin-based membrane permeabilization (termed PFA/saponin; see Supporting Information). For all samples and buffers, FOXP3 gates were set on the basis of staining in live $CD4^{neg}$ 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 Treg-defining cell surface molecules in humans (Figure 1A, Supporting Figure 1A) [2, 10, 11]. These data were confirmed by determining the proportion of $CD25^+CD127^-$ cells within the $CD4^+FOXP3^+$ gate (Supporting Figure 1B). In the majority of samples, a proportion of Tconvs ($CD4^+CD25^-CD127^+$) displayed background FOXP3 staining using the eBioscience, but not Fluidigm or PFA/saponin, protocol (Figure 1A).

Since fixation methods can destroy or alter epitopes [12, 13], we determined the effect of each FOXP3 staining protocol on detection of a range of T cell surface markers. The 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 $CD25^+CD127^-$ cells (Figure 1B). In contrast, PFA/saponin 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 Treg-defining cell surface molecules. 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 to fluorescence flow cytometry. Since human immune cells are often analyzed after cryopreservation, we compared ex vivo or cryopreserved cells on both platforms. We found that cell surface marker detection was similar on both platforms for most antigens tested (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 due to differential CCR4 clone sensitivity (Supporting Figure 3F), highlighting the need for careful antibody clone selection to achieve optimal resolution in mass (and 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 via flow cytometry (Figure 1C, Supporting Figure 4). However, within each platform, there were no significant differences in the proportion of $FOXP3^+$ cells detected in ex vivo versus cryopreserved samples (Figure 1D). To determine if reduced detection of FOXP3 in mass cytometry was a limitation of the PCH101 clone, the experiment was repeated with the FOXP3 clone 236A/E7, with similar results (Figure 1E). This reduced sensitivity may be due to chemical properties of fluorophores versus metals, possibly resulting in a differential ability of metal- versus fluorochrome-conjugated antibodies to pass through the nuclear membrane. Additionally, 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 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 induced co-expression of CD25 and OX40 [16] by flow or mass cytometry using optimal methods (see Supporting Information and Supporting Figure 5). The proportion of antigen-specific $CD4^+$ responder cells detected by mass cytometry was similar to flow cytometry and, since Tregs comprise a substantial proportion of recall responses [17], both mass cytometry and flow cytometry detected a

clear population of antigen-specific FOXP3$^+$ cells. Notably, in contrast to ex vivo cells, the proportion of FOXP3$^+$ cells detected by mass cytometry was similar to that detected with flow cytometry.

We next tested the applicability of PFA/saponin-based FOXP3 staining to detect CD4$^+$CD25$^+$CD127$^-$ Tregs in samples other than peripheral blood via mass cytometry. 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 the 25 ACCENSE-defined populations, as well as their relative abundance within each tissue, was determined (Figure 2B). Strikingly, Tregs preferentially clustered into ACCENSE populations by tissue source. For example, Treg populations 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 and enriched for high PD-1. Despite strong tissue-specific segregation, a few populations were shared between tissues. For instance, a subset of CD45RA$^+$ Tregs (population 4) was found in 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 tissue-specific phenotypes and that phenotypes in peripheral blood may not reflect those of tissue-resident cells [19, 20].

In conclusion, we developed an optimal protocol to detect FOXP3 expression by mass cytometry, tested its suitability for ex vivo and cryopreserved samples, and showed its utility across a broad range of immune cell sources. We have further shown that antigen-specific FOXP3$^+$ Tregs can be detected by mass cytometry in whole blood. An important consideration is that, at least in ex vivo peripheral blood samples, mass cytometry is significantly less sensitive than fluorescence flow cytometry at detecting FOXP3 expression. Overall, our optimized PFA/saponin protocol is the best-validated method described to date to detect FOXP3 expression by mass cytometry without compromising

detection of cell surface markers. This method will enhance high-dimensional studies of Treg phenotype and function in the context of complex cellular networks.

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

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Figure Legends

Figure 1. ***Development of a FOXP3 staining protocol for mass cytometry and comparison to flow cytometry.*** (**A**) Fresh or thawed PBMCs (n=7) were stained using the Fluidigm or eBioscience FOXP3 Staining Kit or fixed in PFA and stained in saponin. Cisplatin$^-$bead$^-$ DNA1$^+$DNA2$^+$CD3$^+$CD4$^+$ single cells were gated as CD25$^+$CD127$^-$ or CD25^{--}CD127^{++} cells and analyzed for FOXP3 (gating in Figure S1A). **(B)** Cell surface molecules important for Treg identification were analyzed by mass cytometry; shown are mean counts within cisplatin$^-$bead$^-$DNA1$^+$DNA2$^+$CD3$^+$CD4$^+$ single cells for each method (n=7). **(C&D)** PBMCs (n=4-8) were analyzed by flow or mass cytometry (PFA/saponin fixation) before/after cryopreservation. The PCH101 anti-FOXP3 gate was set on cisplatin$^-$bead$^-$ DNA1$^+$DNA2$^+$CD3$^+$CD4$^-$ single cells. **(E)** Cryopreserved PBMCs (n=4) were stained as in (C&D) using 236A/E7 anti-FOXP3 or a fluorescence/metal minus one (FMO/MMO) control. Each line is data from one individual with data collected in (A-D) at least three or (E) one independent experiment. Statistical significance was determined for (A-B) with a one-way ANOVA with a Tukey multiple comparisons post-test or (C-E) with two-tailed paired t tests. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$

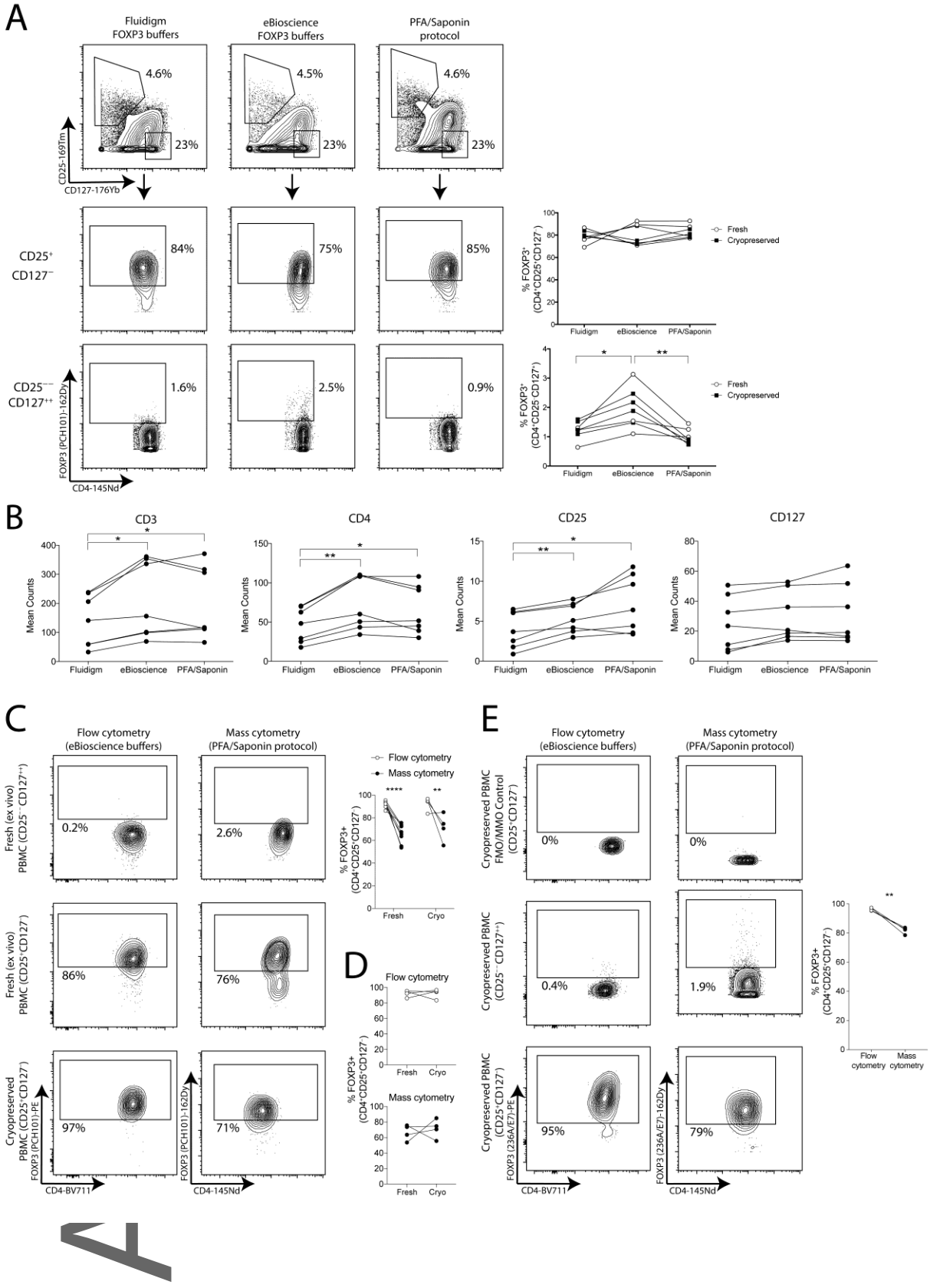
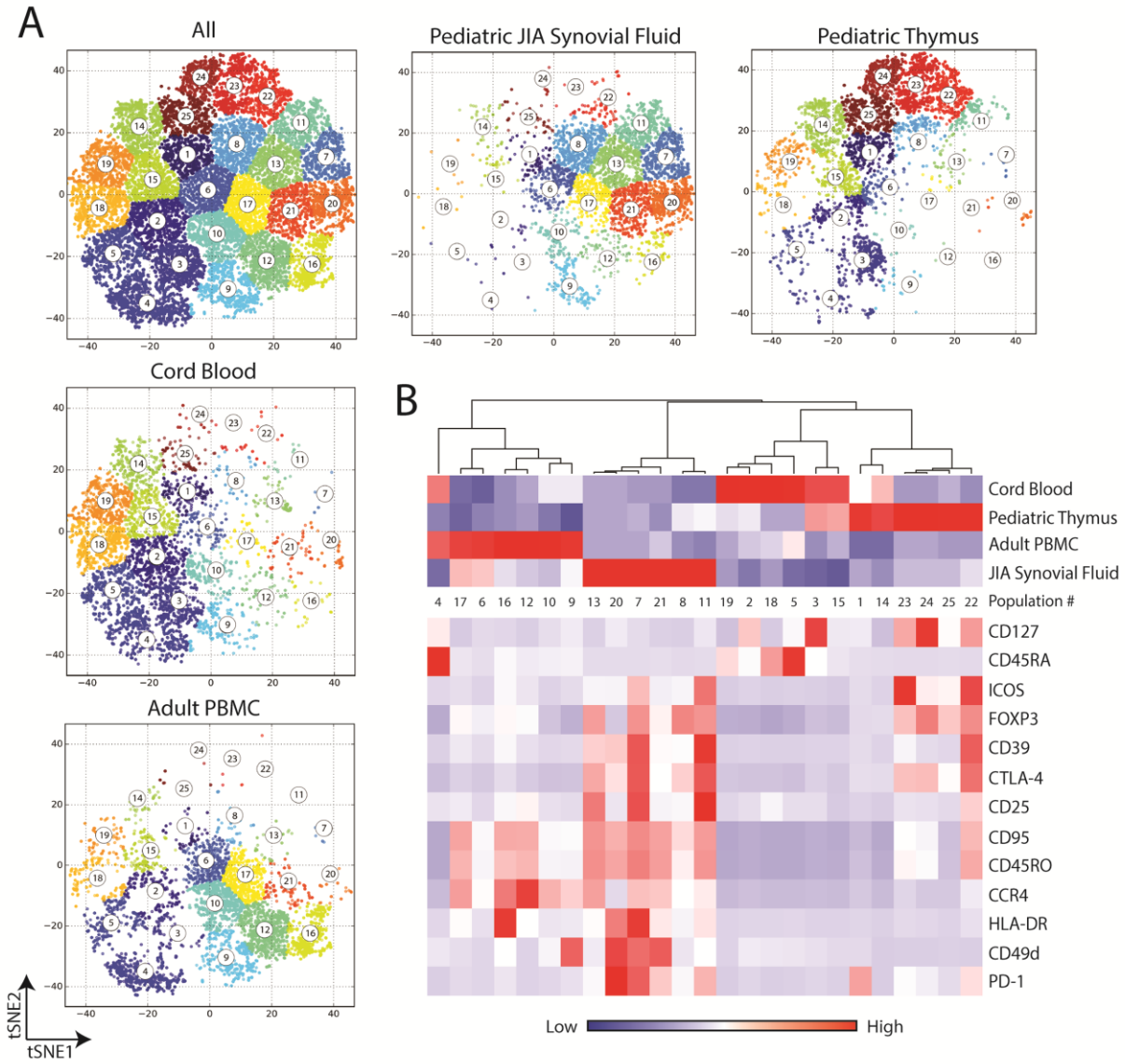
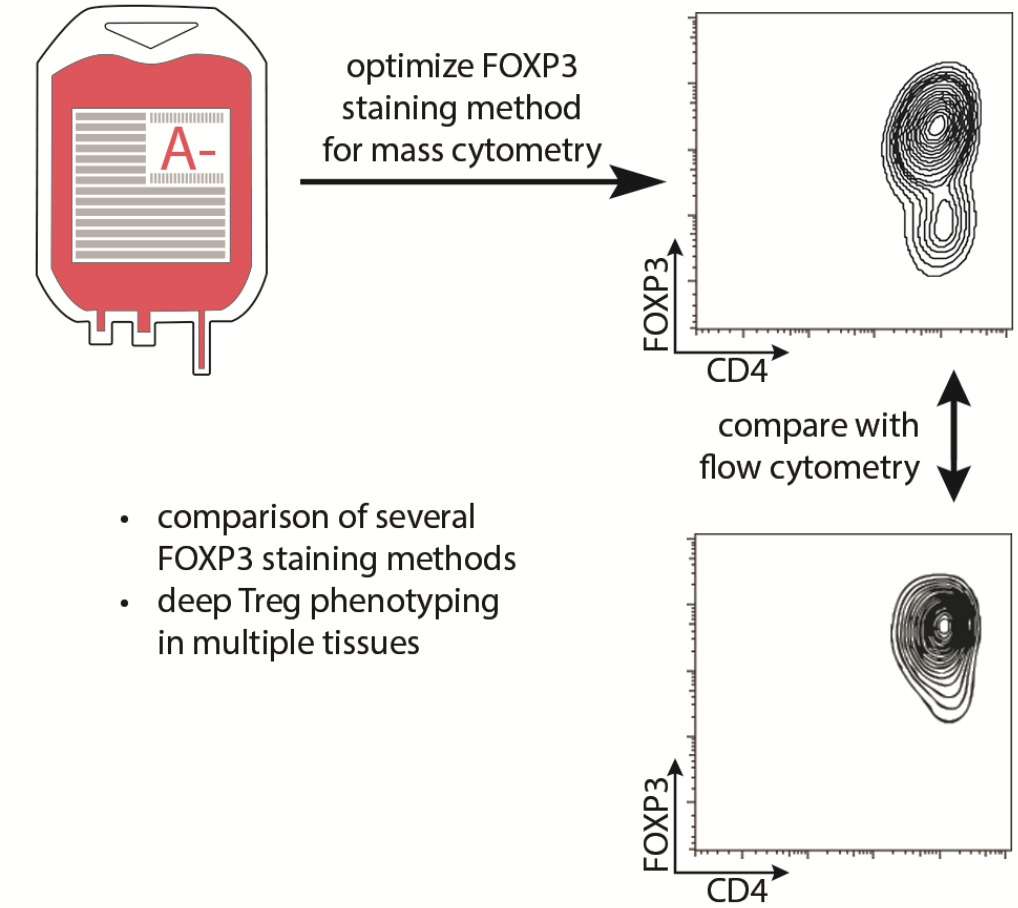


Figure 2. ***ACCENSE analysis of Tregs in peripheral blood, cord blood, thymus, and JIA synovial fluid.*** **(A)** Cryopreserved mononuclear cells from the indicated tissues were stained with the PFA/saponin method and analyzed by mass cytometry (n=3-4 per tissue). Data from cisplatin$^-$bead$^-$ DNA1$^+$DNA2$^+$CD3$^+$CD4$^+$CD25$^+$CD127$^-$ 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; populations were clustered by tissue frequency. Average marker expression and population frequencies were normalized using Z-score analysis to highlight the range of markers expressed in different populations and emphasize which tissues were enriched for each ACCENSE population. Data represent two independent experiments.



Graphic Abstract

We optimized a method to detect FOXP3 by mass cytometry and compared the resulting data to conventional flow cytometry. We also demonstrated the utility of the protocol to profile antigen-specific Tregs from whole blood, or Tregs from tissues such as cord blood, thymus and synovial fluid.



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