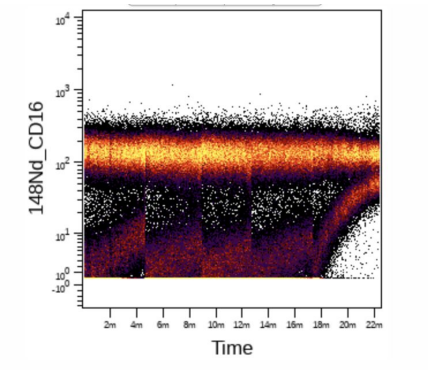

Example 1: Utilizing Fluidigm's Maxpar Direct Immune Profiling Assay to Phenotype COVID-19+ Patients

Solving CyTOF Signal Drift Artifact -- Daniel Geanon 01/03/2021

Abstract: Mass cytometry (CyTOF) is a powerful high-dimensional immunophenotyping tool, but it is not without its data artifacts. Background signal increase within a given acquisition -- unresolved by routine EQ bead based normalization -- has been reported by many users with many explanations proposed. We propose that this artifact is multivariate, resulting from sample overstaining, poor sample fixation, and prolonged exposure to pressure from CyTOF PSI, as well as prolonged room temperature exposure. This artifact can be eliminated by properly titrating antibodies, more aggressive fixation techniques, and by reducing samples acquisition time (<2m cells, <1hr).

Experiments: We hypothesized that this data artifact was antibody (and not metal) dependent, and that it could be recreated across the entire CyTOF mass range. Thus, in our first experiment, we single-stained cells with CD45-metal conjugates in 11 different channels and pooled the single-stained samples prior to acquisition. The samples were stained with either a titrated amount of CD45, or an oversaturating amount (5x titrated concentration). The "titrated" sample and the "overstained" sample were each subjected to a range of fixation conditions (2.4%PFA, 4%PFA, 4%PFA + 2% GA). Each sample was then washed in CAS and acquired for 2hrs at an event rate <400 events/second. After EQ bead based file normalization and routine data clean up (EQ bead removal and Gaussian doublet removal) the intensity of each CD45_metal channel was evaluated on the positive and negative populations, both at the beginning and end of acquisition. We observed signal loss on the positive populations (Figure 1) and subsequent signal gain in the negative populations (Figure 2) across each of the 11 channels, suggesting that this data artifact is not specific to certain CyTOF metals. Staining with a titrated amount of antibody significantly reduced this artifact (Figure 3), while more aggressive sample fixation mitigated the artifact even in the context of sample overstaining (Figure 1 & 2).

To further interrogate this artifact, we selected the CD45 conjugate that most drastically recreated this artifact (CD45_169Tm, Figure 4) and subjected it to intensified instances of overstaining/poor fixation. PBMCs were stained with CD45_169Tm at 20x the titrated amount and fixed with 0.24%PFA. In this experiment, the sample was pooled with a CD45-89Y barcoded population at a 3:1 ratio and exposed to a range of conditions prior to acquisition. These conditions include resuspending cells in CAS and running them through the CyTOF PSI for 60 minutes (cells were collected in a FACS tube on the other end of the sample line after pressure exposure), 2 hour CAS exposure at room temperature, 2 hour

CAS exposure on ice, and 2 hour CSM exposure on ice. Our data (Figure 5) suggests that exposure to pressure is the biggest variable that causes this artifact, while temperature was a minor contributing variable.

Lastly, we hypothesized that the frequency of overstained cells was an important variable in this data artifact. Thus, the cells stained with CD45_169Tm at 20x and fixed with 0.24% PFA were pooled with the reference CD45_89Y cells at either a 3:1 or 1:3 ratio. Each pool was resuspended in CAS, acquired at an event rate < 400 events/second, and normalized via routine EQ bead based file normalization. As expected, the pool with majority overstained CD45_169Tm cells displayed a much more significant signal drift than the pool with majority CD45_89Y cells (Figure 6). Therefore, high abundance markers on high frequency immune cell subsets are very prone to displaying this artifact (CD3, for example).

Discussion: In sum, we propose that this CyTOF specific intra-acquisition artifact *is observed* when samples are overstained/poorly fixed and *is caused* by pressure from the CyTOF PSI shearing off metal labeled polymers from positive populations and the subsequent non-specific binding of these metal labeled polymers to negative populations. Of note, we report this to be a unique artifact, not previously described by other CyTOF signal drift over time issues, such as those resulting from changes in plasma ionization efficiency or detector sensitivity that result in lower marker intensities over the course of many acquisitions (which can be resolved by EQ bead based normalization), or by the shearing off of metal labeled polymers caused by acidic wash solutions such as nitric acid or hydrofluoric acid (which cannot be resolved by EQ bead based normalization). Users have reported this intra-acquisition signal drift artifact most frequently in the context of high-abundance markers conjugated to low-sensitive channels (141Pr-148Nd) channels, which aligns with our theory. In this mass range, high abundance antigens must be completely saturated with CyTOF antibody (and metal labeled polymer) in order to visualize positive from negative, creating a likely scenario for sample-overstaining (Figure 7). Additionally, we propose that the canonical CyTOF fixation method (1.6% PFA for 10 minutes at room temperature) is not sufficient to fix CyTOF antibodies and metal labeled polymers to cells, especially when final cell pools can easily be in the tens of millions with novel CyTOF two-tier barcoding techniques (Figure 8). In sum, we emphasize the importance of properly titrating CyTOF antibodies (especially for high abundance markers conjugated in the low-mass range) and suggest a more aggressive fixation method such as 4%PFA or 4%PFA + 2%GA. We have found that these more aggressive fixation methods actually improve staining quality and do not alter cell subset frequencies (Figures 9 & 10). Thus, we have adopted 4%PFA fixation as the standard fixation technique for CyTOF staining, and titer down CyTOF antibodies when this signal drift artifact is observed.

Figures:

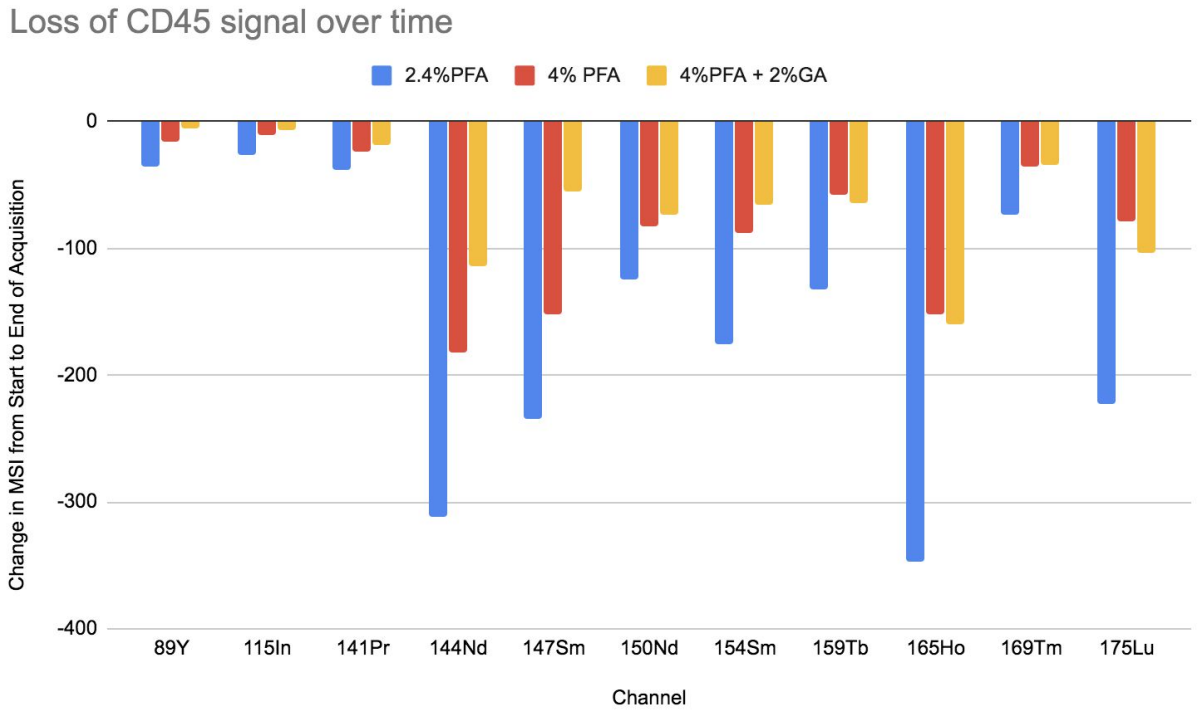

Figure 1a: Cells were single-stained with 11 CD45-metal conjugates at 5x the titrated amount, washed, pooled, and subjected to 3 fixation conditions. Each of the 3 pools were washed in CAS and acquired individually for t=2hrs. Each positive population was manually gated, and the loss of positive signal was evaluated for each CD45-metal conjugate during the course of acquisition. More aggressive fixation significantly reduced signal drift overtime, suggesting that stronger fixation prevents metal labeled polymers from unbinding to their target cells when exposed to pressure during acquisition.

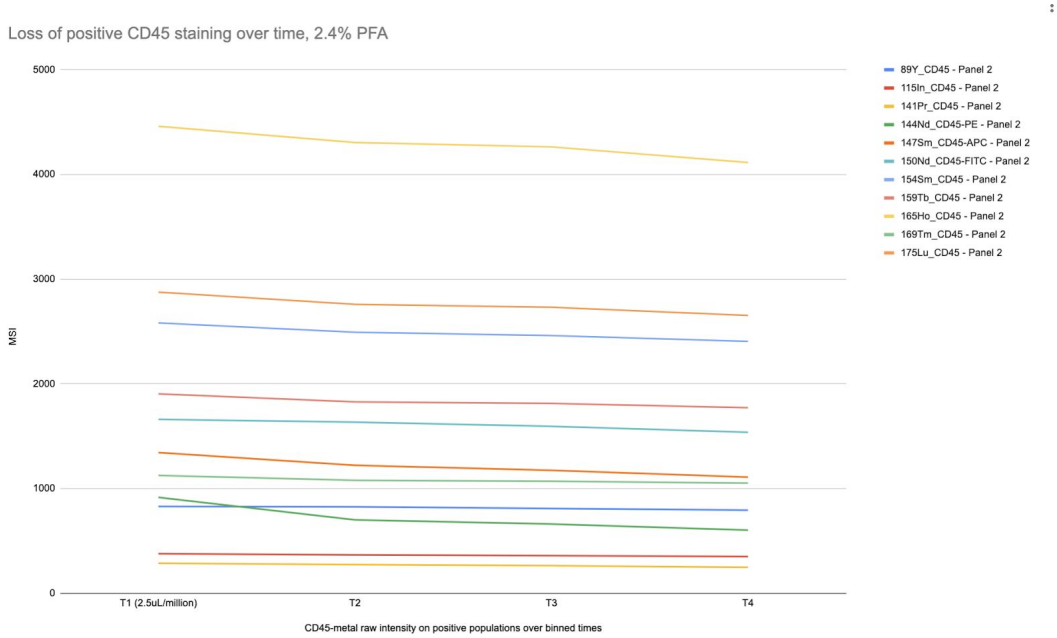

Figure 1b: Raw intensities on each manually gated positive population evaluated for MSI over the course of acquisition to illustrate the signal loss in each metal channel (only data from 2.4% PFA fixation shown).

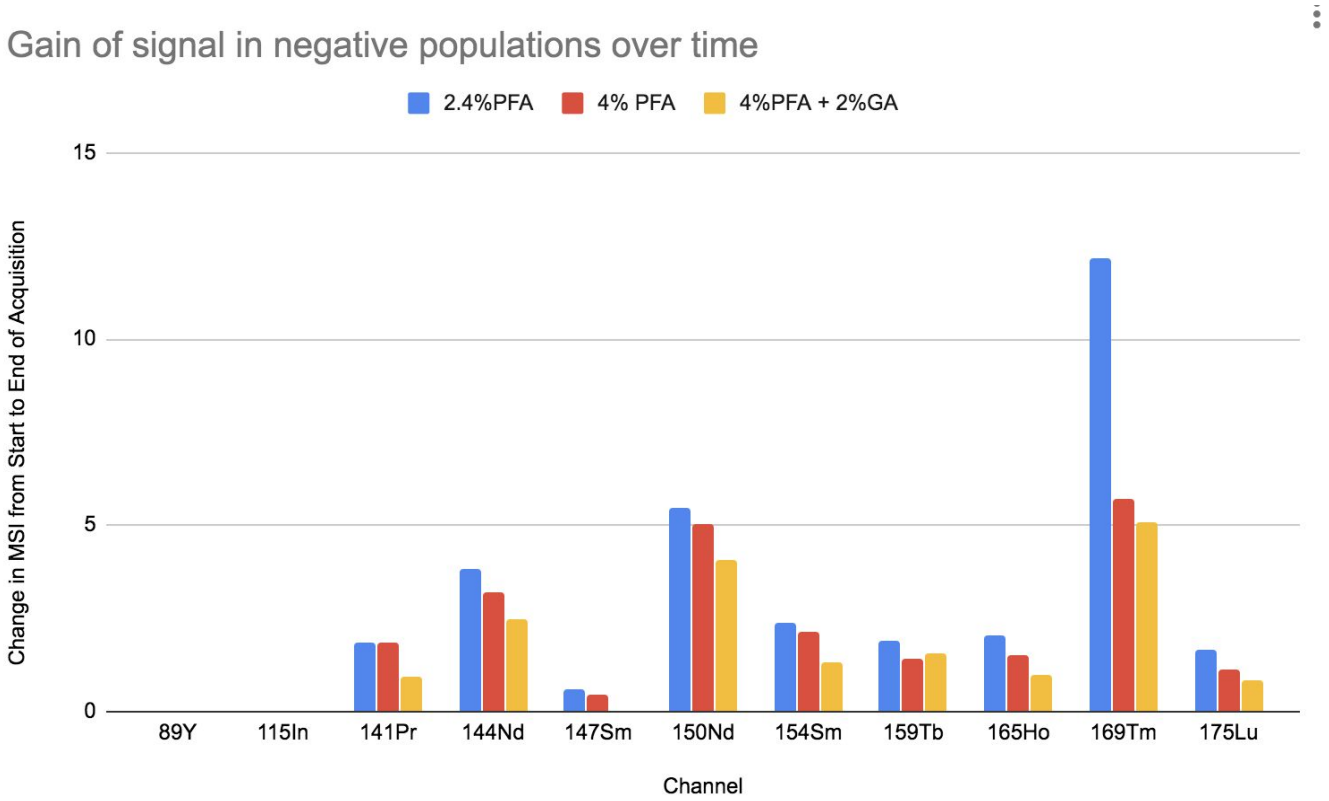

Figure 2: The gain of signal on the negative populations was evaluated in the same experiment as described above (Figure 1). More aggressive fixation mitigated increase in background overtime, supporting the hypothesis described in Figure 1. Of note, decrease in positive signal intensity did not necessarily correlate with increase in MSI on the manually gated negative populations (see 169Tm).

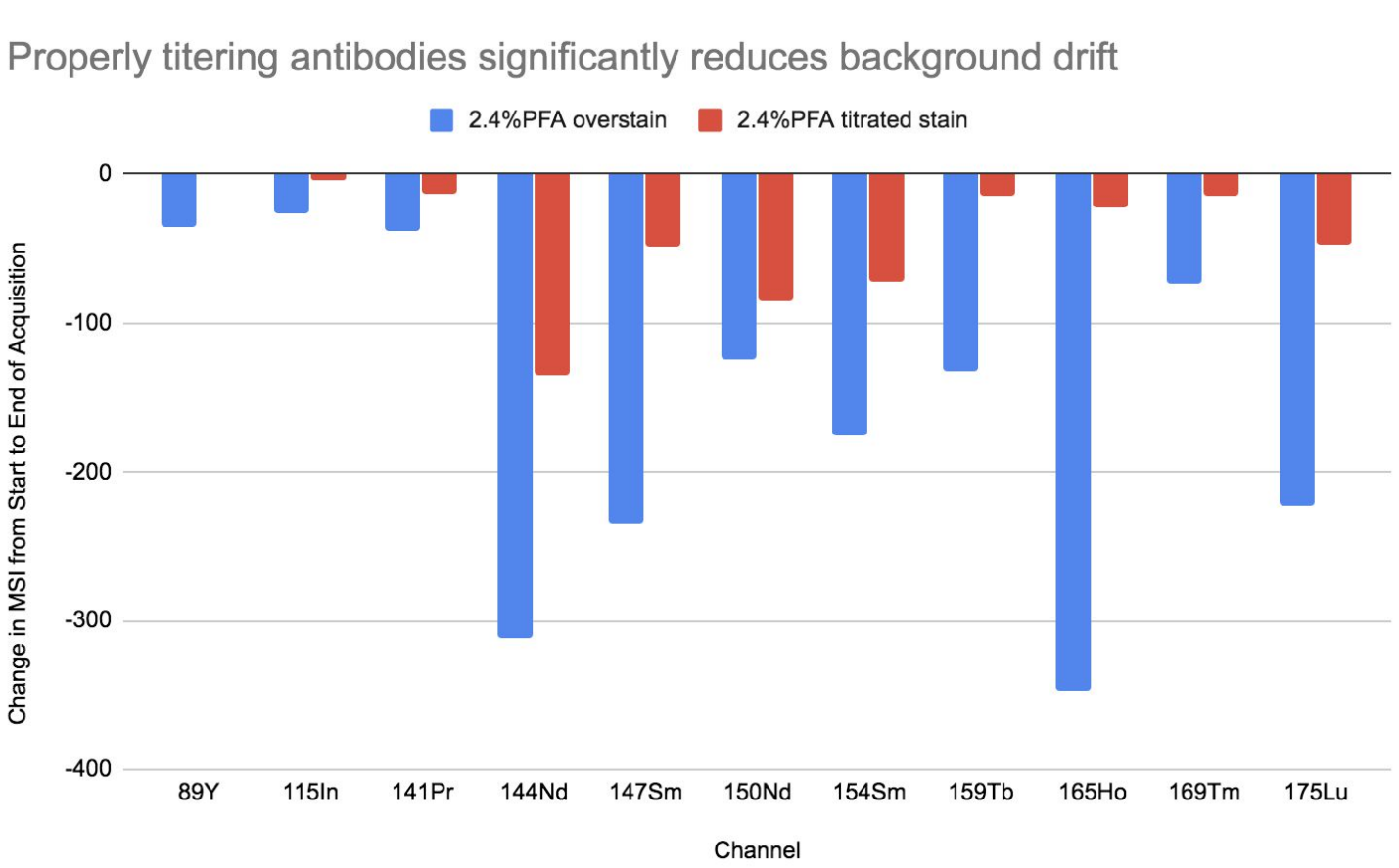

Figure 3: The signal drift from the experiment described in Figure 1 (in which “overstained” cells were stained with 5x the “properly titrated” CyTOF antibody concentration) was compared to signal drift on cells stained with “properly titrated” antibodies, fixed with 2.4%PFA, washed in CAS, and acquired for t=2hrs. When samples are overstained, there exists a large reservoir of metal labeled polymer on target cells that can be sheared off during the course of acquisition. When stained in a titrated amount, less metal labeled polymer is available on the positive population to unbind and subsequently bind to the negative during acquisition. In sum, staining with a titrated amount of antibody is the best way to mitigate this signal drift artifact.

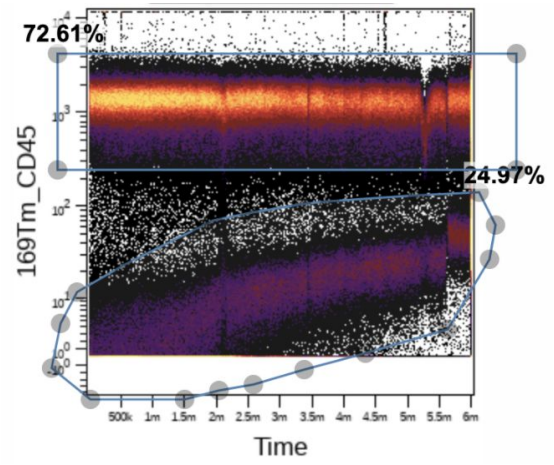

Figure 4: Biaxial representation of CD45_169Tm signal drift during the course of acquisition (2.4%PFA fixed, corresponding to the 169Tm data from Figures 1 & 2), after EQ bead based normalization.

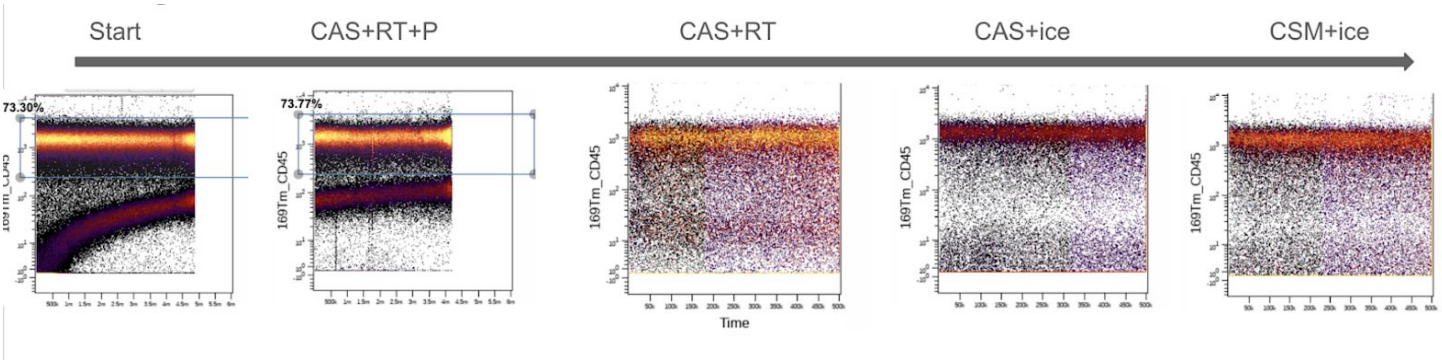

Figure 5: Samples were stained with 20x titrated amount of CD45_169Tm, fixed with 0.24%PFA, and subjected to a range of conditions prior to acquisition (sample was pooled with reference CD45-89Y population in 3:1 ratio). A) Sample was washed in CAS and acquired for t=2hrs B) Sample was run through CyTOF PSI for t=1hr in CAS, collected on other end, and acquired in CAS. C) Sample was left in CAS at RT for t=2hrs and acquired. D) Sample was left in CAS on ice for t=2hrs and acquired. E) Sample was left in CSM on ice for t=2hrs then acquired. This data suggests that pressure is the main force that shears poorly fixed/overstained metal labeled polymers off of cells. This artifact also manifested (much less intensely) from exposure to room temperature, but did not manifest when cells were left on ice.

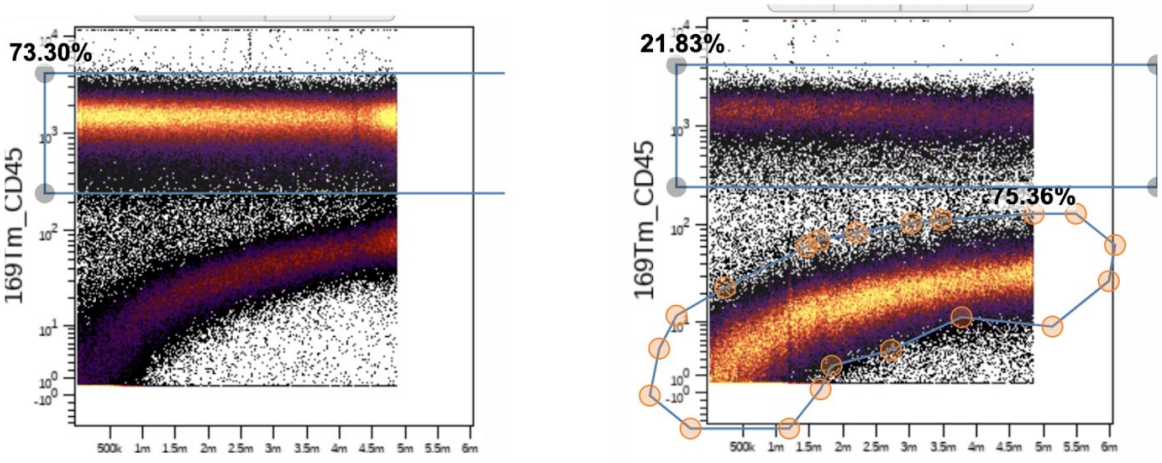

Figure 6: A) Same as Figure 5A, cells overstained with CD45_169Tm were pooled with CD45_89Y reference cells in a 3:1 fashion and acquired. B) Cells overstained with CD45_169Tm were pooled with reference CD45_89Y cells in a 1:3 fashion and acquired. As expected, signal drift manifested more intensely in the 3:1 pool, as a larger reservoir of 169Tm labeled polymer was available to bind to a smaller number of CD45_89Y cells, resulting in higher 169Tm intensity on the 89Y+ cells.

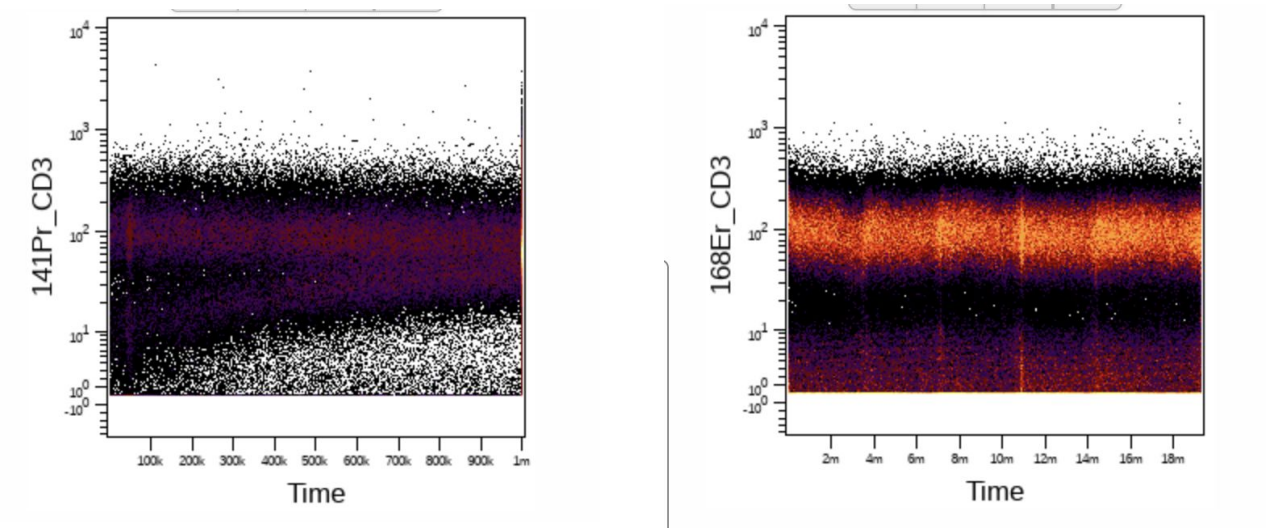

Figure 7: PBMCs were stained with two unique CD3 CyTOF conjugates and subjected to the same fixation conditions (2.4%PFA). We hypothesize that CD3_168Er stains the target epitope in a non-saturating fashion as the metal is in the high-sensitive range. Therefore, there is not a sufficient reservoir of metal labeled polymer to fall off of cells and cause signal drift. However, we propose that the CD3_141Pr conjugate is staining in an oversaturating manner, as 141Pr is on the low-sensitive range. Thus, a sufficient reservoir of metal labeled polymer exists for polymer to be sheared off of cells during acquisition and cause this signal drift phenomenon.

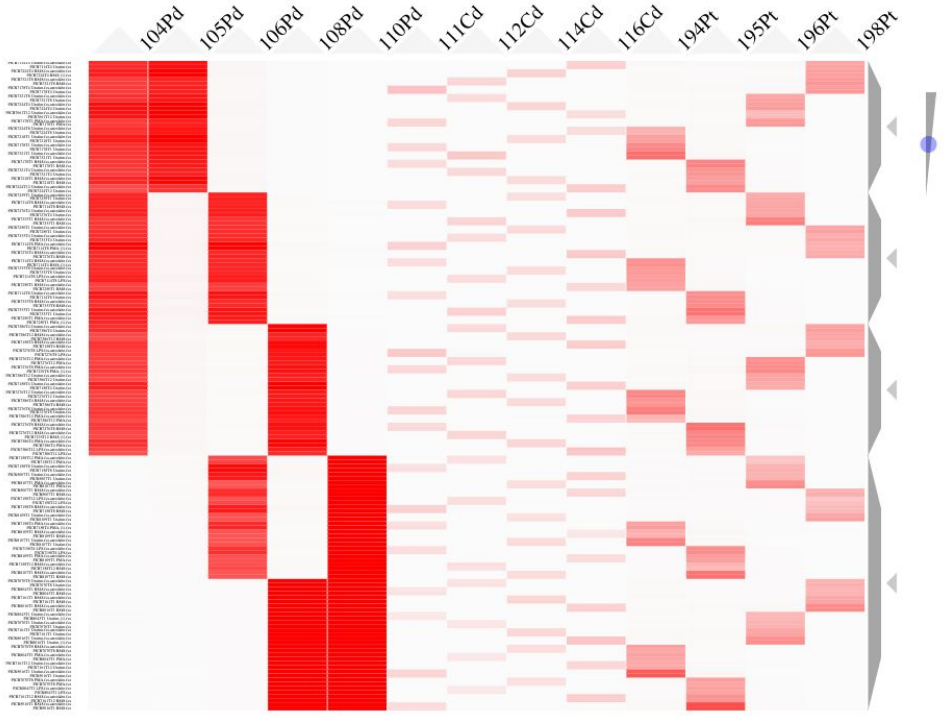

Figure 8: Two-tier barcoding schematic, in which 80 biological samples were acquired simultaneously via CyTOF, Live-cell barcoding was performed utilizing CD298/B2M antibodies conjugated to cadmium and platinum isotopes in an 8 choose 2 fashion (such that each biological sample is positive for one cadmium and one platinum isotope). Although palladium based barcoding was performed simultaneously with sample fixation, each live-cell barcoded pool had 16 biological samples and approximately 20M cells, and we propose that Fluidigm's recommended 1.6%PFA fixation technique does not scale appropriately for massively barcoded large cell pools.

Figure 9: Astrolabe Diagnostics Differential Expression Analysis comparing marker staining intensity on each automatically identified cell subset (semi-supervised FlowSOM clustering) across three fixation conditions (2.4%PFA, 4%PFA. 4%PFA + 2%GA). Red signifies increased marker staining intensity with stronger fixation relative to 2.4%PFA fixation.

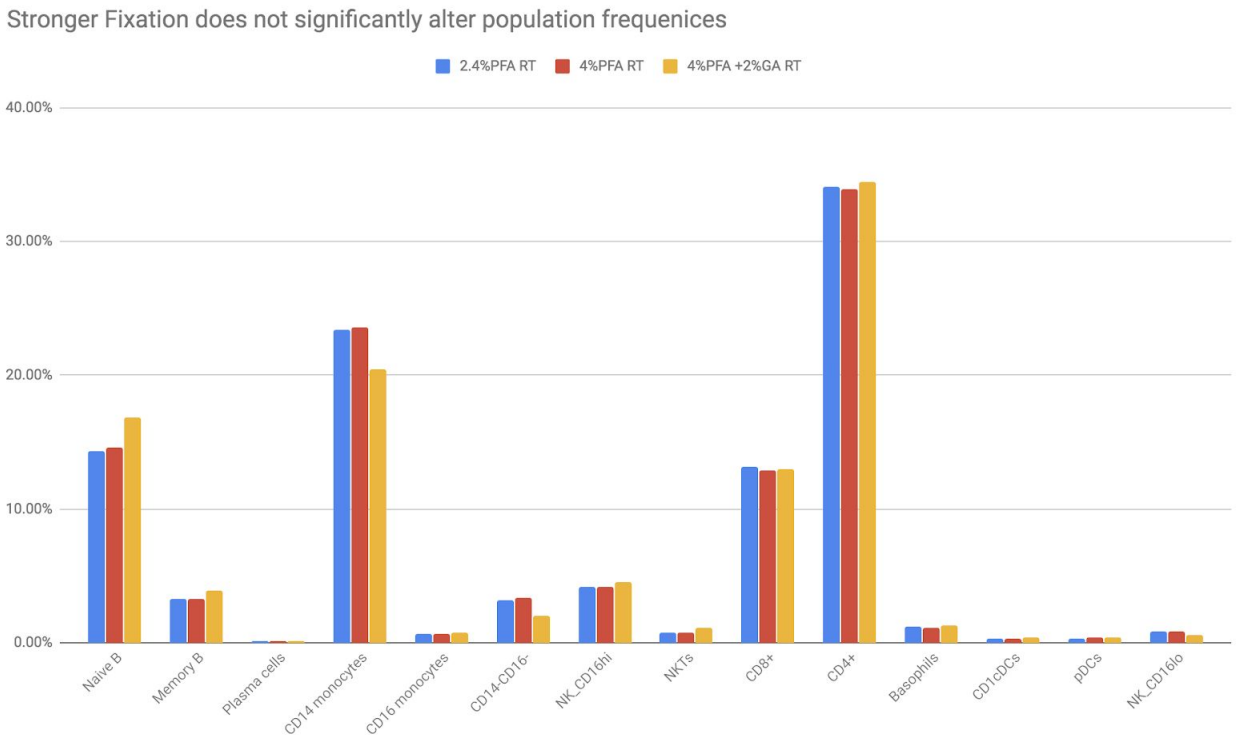

Figure 10a: PBMCs were stained with a 14-core marker lyophilized CyTOF antibody cocktail, subjected to three fixation conditions (2.4%PFA, 4%PFA, 4%PFA + 2% GA) and acquired individually via CyTOF. Manual gating was utilized to identify major immune subsets, and the frequency of each subset was compared across fixation conditions. Stronger cell fixation has no appreciable impact on cell subset frequencies.

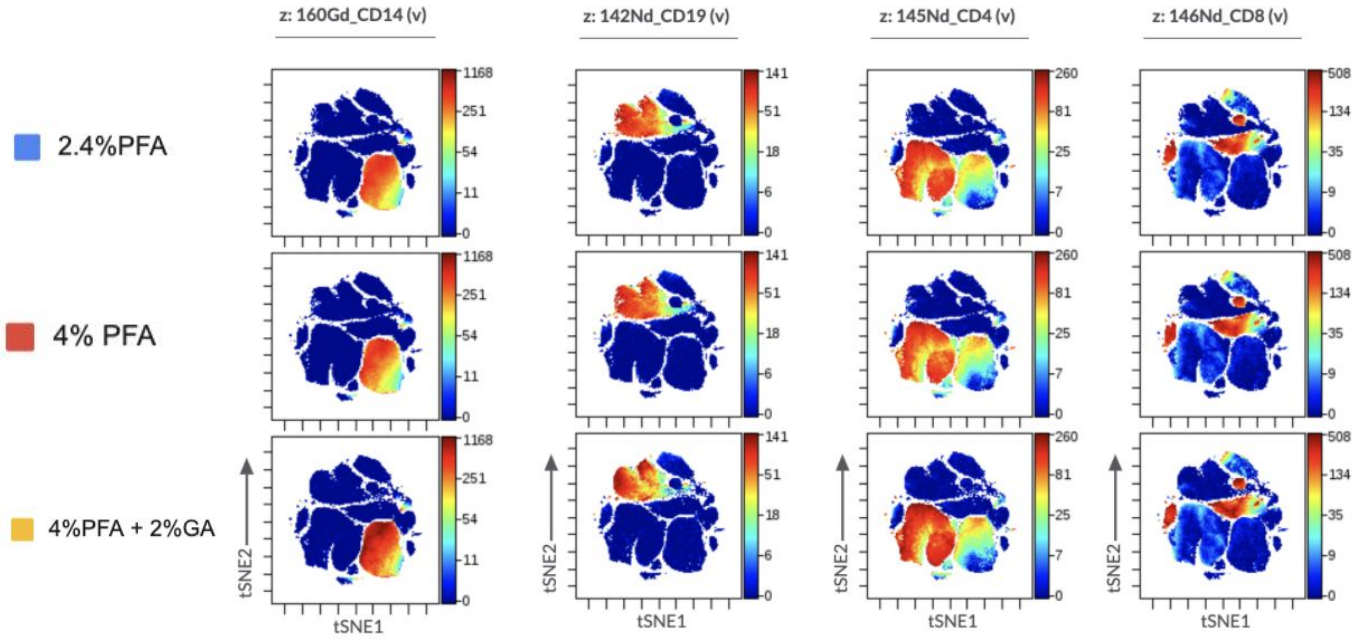

Figure 10b: Visne Representation of data presented in Figure 10a (2.4% PFA top row, 4%PFA second row, 4% PFA + 2%GA bottom row)

Table 2. Core module markers and major immune lineage identification

High +
Low -
Int/subset heterogenity v

		CD4+ T cells	CD8+ T cells	B cells	NK cells	NKT cels	Monocytes	pDCs	CD1c DCs	Basophils	Neutrophils	Eosinophils
168 Er	CD3	+	+	-	-	+	-	-	-	-	-	-
145 Nd	CD4	+	-	-	-	-	v	v	-	-	-	-
146 Nd	CD8	-	+	-	v	v	-	-	-	-	-	-
143 Nd	CD45RA	v	v	+	v	v	v	v	-	-	-	-
142 Nd	CD19	-	-	+	-	-	-	-	-	-	-	-
170 Er	CD38	v	v	v	v	v	+	+	+	+	-	-
155 Gd	CD27	v	v	v	v	v	-	-	-	-	-	-
161 Dy	CD56	-	-	-	+	+	-	-	-	-	-	-
148 Nd	CD16	-	-	-	v	v	v	-	-	-	+	-
160 Gd	CD14	-	-	-	-	-	v	-	-	-	-	-
151 Eu	CD123	-	-	-	-	-	v	+	-	+	-	-
150 Nd	CD1c	-	-	v	-	-	-	-	+	-	-	-
174 Yb	HLA-DR	v	v	+	-	-	v	+	+	-	-	-
152 Sm	CD66b	-	-	-	-	-	-	-	-	-	+	+

Figure 11: Schematic of Mount Sinai HIMC 14-marker CyTOF lyosphere, utilized to rapidly and reproducibly label all major circulating immune cell subsets in PBMC samples.