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Running head: The data scientist's primer to CyTOF®

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Abstract

Mass cytometry enables the measurement of up to 50 features on single cell. This has catalyzed a shift towards multidimensional data analysis methods, rather than the manual gating strategies as traditionally for in flow cytometry data. This shift means that data scientists are involved in the analysis process to an increasing degree. As the data is analyzed in a more unbiased fashion, where noisy or uninformative observations are not easily excluded, a deeper knowledge of the origin, noise, and modalities of the data is therefore needed to embark on useful data analysis. In this primer, we introduce the idiosyncrasies of mass cytometry data with a focus on the technical properties of how data generated with the CyTOF® system, and the characteristics of protein expression in the cells of the hematopoietic continuum, specifically targeted towards data scientists. We also provide a comprehensive online repository of scripts, tutorials, and example data.

Keywords: CyTOF; Bioinformatics; Immunophenotyping; Data processing; Mass cytometry

Introduction

Single cell cytometry as a scientific discipline is almost half a century old. Flow cytometry has been a staple of the cytometry field for decades and data handling and analysis is heavily influenced by historical practices. Regardless of whether one works with fluorescence cytometry, mass cytometry, or sequence-based cytometry, measurement outputs are not the actual quantity of the feature of interest, but rather the physical entities (light emitting molecules, metal isotopes, or sequence reads) used to tag probes are measured as a proxy. Therefore, data scientists are not dealing with exact feature counts, but density-based estimates - each subject to technical peculiarities that are necessary to understand when analyzing the data. Taken together, historical practices and the features of the data render data analysis in the field of single cell cytometry nontrivial to data scientists entering the field.

As with all bioinformatics, in-depth understanding of the nature of the data is essential for meaningful data analysis. The complex mixture of cells in tissues, and the nature of these cells, greatly influence the data and the results one aims to find. For example, cells are not independent entities - rather, they exist in a continuum of development and dynamically switch between states and communicate and modulate each other's functions through receptor-ligand interactions (1). Clusters of functionally similar cells are therefore likely to overlap in their expression profiles, and unlikely to be as distinct as one could expect. The same goes for proteins: expression levels of individual proteins cannot be considered independent of other (measured and unmeasured) features. This leaves ample room for improving the methodology, and last, but not least, building algorithms that take expression and cell abundance dependence into account.

Despite the liberal use of the term “proteomics” in conjunction with cytometry, CyTOF® and other antibody-based methods are not actually dealing with a proteome-wide profiling the cells, but instead a carefully selected panel of proteins (limited to proteins for which monoclonal antibodies exist) and physicochemical features such as phosphorylation state that are believed to either define the cell subsets that one expects to find in the tissue, or provide insights about these cells under different conditions (eg, disease state or stimulation condition). The data acquisition is in this regard highly rational, but also heavily biased.

The target audience of this article are data scientists either planning to start analysis of CyTOF® data already generated by their bench scientist and clinician colleagues, or aiming to delve deeper into analysis algorithms or preprocessing tools development on published datasets. In the following, we introduce the most important sources of biological and technical noise and discuss key points required for data scientists to start working with mass cytometry data, such as data formats, pre-processing, batch correction, etc. The actual downstream data analysis tasks are not the main focus of this article, but these are briefly summarized and references to comprehensive resources are provided. For more in-depth discussion, visualizations, and data examples, please visit our data and methodology catalogue at http://cytof.biosurf.org.

The diverse nature of single cell data

The vast majority of cytometry studies revolve around immune cells. These cells can be divided into discrete lineages based on canonical cell-surface markers, for example, CD19 and/or CD20 for B cells, CD3 and CD4 and/or CD8 for major T cell types, and CD14 for monocytes. In some cases, this type of logical subdivision by lineage markers need to be applied sequentially; for example, CD56 can define NK cells, but is typically applied after gating out CD3+ T cells, since

some of these latter also express CD56. Once major cell lineages are defined, there are many minor subpopulations that can be defined depending upon the combinations of additional markers included. The deeper the subdivision, the less consensus there is about the definitions of subpopulations, essential markers, and how discrete versus fluid these subpopulations are. Their biological relevance is potentially even more controversial. This lack of consensus impacts the use of clustering algorithms, as there is little ground truth to reference against, beyond the major cell lineage divisions.

When dealing with data that is both complex and potentially controversial, it can be helpful to consider the kinds of research questions being asked. Often, these questions are about changes in the cell populations over time or with disease state, in terms of their frequencies and functions. These changes need to be cast against the normal fluctuations in these populations over time within an individual, or between individuals. As a general rule, fluctuations within a single healthy individual over time tend to be lower than the differences between unrelated individuals (2,3).

Additionally, there are a number of experimental factors that may impact analyses. These can result from sample isolation and storage; whether samples are fixed prior to staining; or whether cells are stimulated during the assay (Table 1). In order to compare data between experiments, it is important to have verified that the assays themselves are comparable. Please note that many of the experimental factors mentioned in Table 1 are not unique to mass cytometry assays; they equally apply to standard fluorescence flow cytometry.

Running the CyTOF® system

Mass cytometry has recently been developed as an alternative to fluorescence flow cytometry (65). Instead of fluorophores, mass cytometry uses metal ions as the labeling reagent, with inductively-coupled plasma mass spectrometry (ICP-MS) used for detection and quantification instead of photomultiplier tubes. This eliminates the issue of spectral overlap and theoretically allows 100 or more features to be measured simultaneously. Practically speaking, the majority of experiments are performed with less than 50 channels, due to limitations both in isotopically pure elements with suitable valence and atomic mass, and stable chelation for certain sizes or valences of the metal ions. Most commonly, the metal ions are bound by a polymer with pendant chelating groups and then the metal-loaded polymer is used to label antibodies or other specific reagents. Before running on the mass cytometer, the cells are incubated with the metal-containing reagents such as antibodies against various surface markers. Currently, mass cytometry does not have a parameter analogous to side scatter in fluorescence flow cytometry. Recent work using osmium tetroxide or wheat germ agglutinin has begun to offer a parameter more directly proportional to cell size and therefore more analogous to forward scatter (66). Additionally, cells are typically also incubated with metal-containing reagents such as iridium-containing DNA intercalators to allow cell detection regardless of whether any metal-labeled antibodies bind.

Currently, the primary application of mass cytometry is to label antibodies or other probes with stable metal isotopes to differentiate different types of cells. These cells can originate from a variety of sample types, including cultured cell lines, whole blood or peripheral blood

mononuclear cells (PBMCs) isolated from whole blood, or disaggregated tissue such as pancreas, tonsil, or bone marrow (43–45). These can be from healthy subjects, or clinical patients undergoing cancer biopsy or organ transplantation (67,68). Most of the mass cytometry literature has been focused on human subjects; however, other organisms such as bacteria, mice, and macaques have also been studied (27–29).

Fluidigm Corporation is currently the only vendor of mass cytometry instruments with their CyTOF® systems. The first CyTOF® was released in 2009, followed by the CyTOF2® in 2013, and most recently the Helios™ in 2015. Many specifications, instrument metrics, and software properties differ between versions of the CyTOF®. In the following, we are referring to the Helios™ system unless otherwise specified.

Sample preparation

Preparing samples for the CyTOF® requires multiple steps. The different steps are summarized in Figure 1 and elaborated on below. Please note that the specific order of the steps may vary depending on the assay type. For example, in phosphoflow assays, fixation (Step I) is performed immediately after stimulation (Step E) but before surface staining (Step H) (69). However, in intracellular cytokine staining, the order is stimulation, surface staining, and then fixation (70).

Probe selection

The first step in sample preparation is the choice of probes for the sample of interest. In many cases, these probes are antibodies that bind to biomolecules of interest. However, other probes such as carbohydrate-binding proteins (28), small molecules such as tellurium-based oxygen

sensors (71), or modified nucleotides for cell-cycle analysis (72) have also been used. The degree of specificity of the reagent is critical: some probes may have a primary affinity for the target but a cross-reactivity (secondary affinity) toward some other molecule (see Section 7.3.1 in (73)).

Panel design

Once the probes are chosen, panel design is the next step. This involves the matching of probe and elemental isotope to achieve the desired signal intensity in the assay readout. Variables to consider include sensitivity of the instrument to the isotopic mass (typically, the instrument is most sensitive to masses in the AW 159-170 range; lanthanide masses outside this range may have up to 4 fold lower signal intensity and up to 6 fold for ^{89}Y), signal spillover from isotopic and elemental impurities in the metal stock (74), typical expression level of the target biomolecule on all marker-expressing cell types within the sample, and modality of marker expression level (eg, unimodal vs. bimodal). Next, there is a potential for oxide formation, which adds 16 mass units (^{16}O) to an element's mass. Oxide formation is primarily a problem for La, Pr, Nd, and Sm-labeled probes, and can never be completely eliminated (74). However, the standard daily tuning of the instrument uses the signal from the tuning solution, optimizing machine parameters to limit the ^{139}La+^{16}O signal to less than 3% of the ^{139}La signal. Since ^{139}La is the most easily oxidized lanthanide, this also limits all other oxides to under 3% of their corresponding non-oxide signal. Finally, the last noteworthy contributor to spillover is "abundance sensitivity", which has to do with the uniformity of the distribution of ion arrival times at the detector (effectively, ion peak width in Figure 2A). This distribution is narrower for low-mass ions (e.g., ^{133}Cs in the tuning solution) and broader for higher-mass ions (eg, ^{193}Ir). As ion optics have improved, this has decreased from ~2% for the first CyTOF® model to ~0.3% for CyTOF2™

and Helios™ for ^{159}Tb (75). Typically, the resolution of the instrument is sufficient to achieve baseline separation in time-of-flight ion peak between adjacent mass channels. However, since peak width and height are linked, extremely high signal intensity (typically >10^4 Dual) can cause spillover of the right and left peak legs into the M+1 and M-1 mass channels, respectively. Fundamentally, if there is a question about whether a signal comes from a specific probe signal or spillover from another channel or probe, control experiments analogous to flow cytometry "fluorescence-minus-one" (FMO) can be performed, where probes are left out one by one to allow the user to see residual, nonspecific signal (76). Finally, recent work by Chevrier et al has begun the process of building compensation tools for mass cytometry (77).

Staining

After panel design, any unlabeled probe must be conjugated to detectable ions. This is typically done by covalently attaching a metal-chelating polymer loaded with specific metal ions onto the probe. The polymers are assumed to be loaded to saturation (typically 16-22 metal ions per polymer with current reagents). In most cases, there is variation in the number of metal-loaded polymers that attach to a particular probe. This is usually driven by the amino acid sequence of proteinaceous probes such as antibodies and lectins during the conjugation process (78–80). However, for a given specific antibody clone, the number of polymers (and therefore probe-related metal signal) is reproducible.

Washing

During the staining process, it is important to have all buffers and probes be of high purity to avoid contamination from metals such as barium in laboratory dish soap, lead from piping, etc. Cells are typically stained to saturation, and multiple washes are done at each step to remove

unbound reagents and therefore reduce associated background. Once stained, the cells are washed in high-purity water (typically 18 MΩ MIlliQ water) to remove residual buffer salts that can build up and cause instrument tuning drift. Once the water washes are complete, the cells are resuspended in more MilliQ water (typically containing diluted normalization reference beads) to an appropriate concentration that balances the need for speed of acquisition against the needs of cell-to-cell algorithmic resolution and to avoid clogging the instrument. For a relatively simple sample type such as unstimulated healthy control PBMCs, a concentration of around 800,000 cells/mL is common. Whole-blood or bone marrow samples may require dilution to 600,000 cell/mL, and disaggregated tissue samples may even require further dilution for minimal clogging and good resolution of cells vs debris.

Choosing number of cells

The required starting sample size depends on a number of factors. Poor initial cell viability or robustness of the target cells will result in a lower yield of Live Intact Singlets after gating out debris, doublets, and dead cells. The average frequency of the rarest population of interest is important. For example, in peripheral blood or PBMCs, looking at only total CD4+ cells (~30% of total Live Intact Singlets in a typical donor) would require fewer cells (81), while phenotyping plasmablasts (~0.01% of total Live Intact Singlets in a typical Day 0 donor) or antigen-specific T cells would require substantially more to even detect them, let alone have enough events for statistically robust analysis (82). There are two main factors affecting total sample acquisition. First, total starting amount of sample: for example, there are substantially fewer cells resulting from a fine-needle tissue biopsy than from a standard blood draw, even before losses during processing. Second, length of sample acquisition: at a typical cell acquisition rate of 200-500 events per second (where typically only 40-60% will be Live Intact Singlets after gating out

debris, doublets, and dead cells), it may be logistically prohibitive to run exceptionally large samples to look for extremely rare cell populations. In some cases, it may be possible to enrich for your overall cell population of interest using affinity selection methods, but this will necessarily distort the frequencies of other populations of potential interest. The number of samples required to achieve appropriate statistical power of course depends on the question being asked. For comparison of relatively heterogeneous sample groups (e.g. healthy vs. cancer), at least 8-10 samples in each group should be included (83). For comparison of more homogeneous groups (e.g. studies of age-related effects on immunophenotype) sample numbers in the hundreds may be required to achieve a reasonable signal to noise ratio (30,31)).

Mass-tag cellular barcoding

Mass-tag cellular barcoding (MCB) is a method to covalently stain cells within a sample with a unique combination of pre-selected isotopes dedicated to barcoding (84). This allows for sample multiplexing, i.e. running multiple samples concurrently as one tube on the CyTOF®. For example, using six palladium (Pd) isotopes of different mass, 20 different combinations of three Pd isotopes can be made. While resulting in fewer possible barcode combinations, the strength of the "n-choose-k" combinations (e.g., choosing specifically three isotopes out of six possibilities), is that most cell-cell doublets across samples can be filtered out, as they will result in nonexistent barcodes. This is in contrast to "exhaustive" barcoding using all possible combinations (84) of N barcode agents, which yields more possible barcodes but has the major weakness of miscoding a sample if one or more reagents fail.

There are currently two methods for barcoding. The first mass cytometry barcoding was published by Bodenmiller et al. (84) using small molecules. This was later expanded by Zunder

et al. (85) or the undisclosed proprietary system of Fluidigm. In these cases, the cells in each sample are first fixed, then permeabilized and intracellularly stained using one of the different barcode combinations. If surface epitopes are sensitive to harsh permeabilizing agents, transient partially permeabilizing cell membranes using saponin can also be used (86). The second class of mass cytometry barcoding agents are based on antibodies such as CD45 (87–89) and can be used on both live cells or fixed cells. As well as saving time and reagents, barcoding to combine samples helps minimize technical variations such as tube-to-tube antibody cocktail volume and instrument variation when running samples individually (86). However, one pitfall of barcoding is that including one or more low-quality samples (poor viability, high amount of debris, etc.) in the barcoded sample can result in background increases, reagent titer problems, and reduced recovery due to sample clumping for all individual samples contained in the combined barcoded sample.

Data acquisition

After washing in MilliQ water to finish removing buffer salts, the sample cell pellet is resuspended in MilliQ containing calibration beads. The sample should be diluted to an appropriate concentration to minimize both instrument clogging as well as formation of cell doublets (see Pre-gating below). The diluted cells are introduced into the mass cytometer through a nebulizer, in which the suspension is aerosolized to droplets containing single cells. These droplets are carried through a heated spray chamber and into the mass cytometer by a flow of make-up gas. In this process, the droplets shrink by evaporation and enter the ICP torch, burning at approximately 7,000 Kelvin, where they are vaporized, atomized, and the metal ions are ionized. The resulting ion clouds consist of positively charged ions in close proximity to each other. The resulting charge repulsion expands the clouds to approximately 1-2 mm in diameter.

These clouds are passed through a series of three vacuum interface cones. Passing through the cone interface, the ions cool down and shrink the clouds to a focused beam of ions. The cell transmission efficiency of the introduction system means that 50-60% of the cells in the initial suspension are lost at this point (as high as 75% for CyTOF® v1 and v2), and the ion transmission efficiency means that the number of ions has been reduced by 5,000-10,000 fold depending on the instrument (this is the primary reason for labelling each antibody with approximately 100 metal ions) (79). Both cell loss and ion loss are stochastic.

The focused ion beams are passed through an electrostatic quadrupole ion deflector, which bends charged particles in the ion beam to enter the quadrupole at a 90-degree angle, while neutral elements and photons pass straight through and leave the measurement path. The pure ion beam consists largely of low mass ions stemming from organic molecules. These are removed by a radio frequency quadrupole, in which an alternating current causes lower mass ions to gyrate, thereby ejecting them out of the quadrupole, leaving only the ions of analytical interest to be passed forward to the TOF chamber.

The steady beam of reporter ions are introduced to the TOF chamber by voltage pulses at 76.8 kHz, corresponding to 13 microsecond “pushes” of ions. Due to the reflectron, the ions from each push leave the TOF chamber in mass-resolved bands of ions (from low to high mass). The mass ordered ion packets leaving the TOF chamber are detected by a discrete dynode electron multiplier, analogous to the photomultiplier tubes used for signal amplification in fluorescent flow cytometry. When an ion strikes the first dynode, a cascade of electron amplification is triggered, resulting in electron pulses that is captured by the anode of the detector. The signal is recorded as a digitized waveform generated by the detector, with each ion mass in each push detected

within known through mass calibration time window of 20-25 ns duration, and each ion-induced pulse spanning 2-3 ns. If an ion is present in low concentration (typically 2-3 pulses per the 20-25 ns mass channel) the probability of pulses overlapping is low, and each pulse generated by an ion can be counted. At higher concentrations, several pulses will be detected in a mass window during each measurement, and intensity distributions will overlap (Figure 2B). In this case, the signal intensity in the mass window is calculated by integration of digital values of voltage detected in each of the 20-25 1-ns bins; the resulting integral is referred to as analog signal. In order to convert analog signal into ion counts, dual detector coefficients are determined via calibration, as previously described (65). For the Helios™ model, the default setting is that one pulse per push is counted in counting mode, with the transition to analog signal starting when two or more pulses are detected per "push".

The dual instrument (Di) count coefficient is calculated by running the CyTOF® with a tuning solution containing six metal ions spanning the entire lanthanide mass range. Each of the ions are present at known concentrations, and runs are performed with varying quadrupole deflector voltage, gradually increasing the number of pulses per push. Plotting the analog intensity counts against the digital pulse counts, reveals a range in which digital and analog counts correlate (r^2>0.8). In this range of "dual validity", the slope of the regression is the dual count coefficient, which, if multiplied by the integrated intensity of pulses, gives an approximation of the pulse count even when a high concentration of pulses hit the detector at once (Figure 2C).

While Di (Dual Instrument) is the current data standard, Dd (Dual Data) was used used in the first few years of mass cytometry (65,90). Dd used the first 30 seconds of the CyTOF® data itself for each sample acquisition to calculate the Dual coefficients for each channel present in the

template. Although this had the potential advantage of calculating each channel's Dual coefficient directly rather than relying on Di's extrapolation based off the Tuning Solution isotopes, it relied on the assumption that each channel would be sufficiently represented in that initial time segment to calculate a valid Dual coefficient. If the cell density was low or a channel represented a marker present at very low frequency, this constraint might not be satisfied and usually resulted in overestimation of the Dual slope coefficient. As a result, Dd values were typically higher than Di values. Because of these errors in its calculation, Dd is no longer used; it is only mentioned here for historical reasons, but may still be relevant for analyses where validation or benchmarking is performed against legacy data. Reprocessing of the original IMD file is the only way to make a new Di FCS file when the original FCS file was acquired in Dd mode.

It is important to note that all ions within the detectable mass range that are present in the sample will strike the detector. For example, if the sample contains barium or iodine resulting from contaminated buffers, the barium and iodine ions will make it through the ion optics and strike the detector. This signal can be seen in the "TOF" view of the "rain plot", which shows the entire mass range of the instrument. However, only channels that are included in the marker template for the sample will actually be recorded and written to the resulting output files. This has three major implications: first, if a marker was included in the staining experiment but its channel was not included in the template, there will be no data recorded for that channel and there is no way to recover it. Second, when a channel with contamination is not recorded, it may have a spillover into a channel that is. For example, if a sample is contaminated with ^{139}La but ^{139}La was not in the template, it will not be obvious from the resulting FCS file that at least some of the background in the ^{155}Gd channel comes from lanthanum oxide (^{139}La+^{16}O = mass 155)

and not an off-titer antibody labeled with ^{155}Gd. To help deal with both of these issues, some operators are choosing to record the entire mass range in all experiments, but this has implications for the output file size. This poses no issue for total data acquisition, but because of the significantly increased number of channels, the total ion count is likely to be higher and therefore the Lower Convolution Threshold parameter (see below) may need to be increased.

Output data formats

The Integrated Mass Data (IMD) format

Both the number of electron pulses and the integrated electron intensity is recorded for each of the measured mass channels, for each 13 µs push. According to Fluidigm, the data rate for IMD files is 0.3 MB/sec/channel on the Helios™. This corresponds to 76,800 recorded lines with number of mass channels x 2 variables per second, so a run of 20 min (approximately 250,000-300,000 cells) will result in a ~15 GB raw text file and a binary IMD file that is approximately 2.5 fold smaller. The ion counts are recorded at a continuous rate, so data from individual cells must be distinguished from each other after the data acquisition. The beam of reporter ions stemming from an individually labelled cell are too long to fit a single push, so the beam is divided into a number of pushes referred to as the event length. Cells typically have an event length between 10 and 150, which is determined primarily by the total metal content of the parent cell, and does not necessarily correlate directly with the original size of the cell. The other parameter used to define a cell event is the Lower Convolution Threshold, which measures total signal intensity across all acquisition channels. In theory, this value can be decreased if the experiment uses fewer parameters or raised if the experiment has more channels. However, the Helios™ default lower convolution threshold of 400 Intensity units is sufficient for the majority of

experiments (often between 10 and 40 total acquisition parameters), and is therefore typically not changed. A true cell event is thus by the Helios™ software default defined as between 10 (lower limit) and 150 pushes (upper limit) with a sum of ion counts for all quantified channels above the lower convolution threshold. However, if there is a contamination or other kind of background in any channels (eg, ^{127}I or ^{138}Ba, or a reagent channel streak from debris or insufficient washing), this streaking background may be high enough to trigger a “cell event” reading from the above default settings and would get written to the daughter FCS file. These anomalous events can be removed during data analysis; however, if the operator is acquiring for a standard number of cell events (rather than standard total acquisition time), the background has the effect of lowering the fraction of true cell events in the resulting expression matrix. If significant background is present, increasing the sample dilution can help decrease the background signal intensity to alleviate this effect, but at the cost of increased acquisition time.

A final software parameter is the option to use Noise Reduction (default “on”). The noise reduction algorithm calculates background signal as the mean signal intensity per push between cell events (determined by event length and lower convolution threshold), and then subtracts that background signal from the signal measured during a proximal cell event.

After estimating ion counts, removing noise, and summing up ion counts for cell events, the data is ready to be exported to FCS format, for which the size is approximately 100-fold smaller than the binary IMD file, with an average size of around 150 MB for a standard single sample run. By default, non-zero ion counts are randomized to fall uniformly distributed in the interval]x-1,x] of a given count. This is essentially done for visualization reasons: count data will produce a “picket fence” histogram, whereas the randomized count data will produce a histogram

resembling a density plot. This aesthetic effect could also be achieved by simply plotting density distributions rather than histograms, but for historical reasons, histograms are used. Randomization can be turned off before exporting to FCS, and otherwise counts can be converted back to the original number by converting decimals to integers and adding 1.

The size of the raw IMD files often deter operators from storing these data after converting to FCS. However, it is always advisable to keep and organize raw data from experiments (91), and the files are highly compressible (usually >97% reduction in size). Additionally, the IMD file is typically required for reprocessing of the data. For example, the DNA intercalator channel is usually one of the strongest signals in true cell pushes, so if a sample is unusually weakly stained for the intercalator, many of these pushes will fall below the lower convolution threshold and be considered noise. As a result, a given cell may fail to reach the default minimum event length limit and therefore may not get written to the FCS file as a cell event. A similar case occurs when a cell is bound by only a few probes (either a small panel, or the cell is not well-characterized by a given panel) and therefore has little associated metal signal. In such situations, re-processing the IMD file to FCS with a more liberal lower event length limit or lower convolution threshold may help increase event recovery. While this will also allow more debris to satisfy the cell thresholds, true cell events can typically be resolved during gating analysis. Also, as mentioned above, the IMD would be required to reprocess an old Dd file into a more appropriate Di file.

The Flow Cytometry Standard (FCS) format

The FCS format is a binary data file standard originally developed for storage of flow cytometry data (92). The data rate for FCS files on the Helios™ is 12.4 bytes/event/channel. As well as a

segment containing the ion count matrix, the FCS file contains an additional text segment with information about the experimental setup, e.g. the antibodies, isotopes, and mass used for each channel. The ion count matrix can be extracted for analysis, manipulation, or plain-text conversion using, for example, the FlowCore package for R (93). This package also enables writing FCS files from a manipulated ion count matrix and the corresponding metadata if downstream analysis in FCS-centric software is desired. Additional operations, such as merging FCS files from disrupted CyTOF® runs can be performed using a function in FlowJo or using the cytofCore package for R (https://github.com/nolanlab/cytofCore).

It should be reiterated that the cells listed within an FCS file are determined by the software's use of the upper and lower event length and lower convolution threshold settings mentioned in the IMD section above. To write the FCS file, the push data is captured across all measured channels for the time window that satisfies those three parameters, then converted into the single measurement for each channel for each cell. Because the time window is taken, if there is background due to streaking (debris, mistitered probe, etc.), the signal from that background will be written to the FCS file. In such cases, it can be difficult to conclusively determine whether a dim signal in the FCS file resulted from streaking background vs. a dim but punctate cell-associated signal.

CyTOF® data preprocessing

Before informative data analysis can be done, a number of preprocessing steps must be completed in order to convert the estimated ion counts in the FCS file into a matrix of ion counts for live, intact, single cells that can be analyzed in any suitable statistical environment. Depending on experiment setup, additional tasks such as bead normalization and sample debarcoding must also be performed. The entire process is referred to as “pre-gating” and is typically done manually in workflows dependent on multiple steps of user decision making. Both the IMD and FCS formats are binary and must be read by a suitable software package in order to carry out the pre-gating.

Transformation of ion counts

The exported FCS file contains dual instrument or dual data counts, either raw integers or non-zero integers randomized between]x-1,x] for each channel, for each event. Since most cell events will not be bound by at least a few of the probes used in a given panel, a great number of the values will be equal to 0. The dynamic range of the channels means that values will be more densely located in the low end of the spectrum, so data is often transformed for visualization purposes. Historically, this was implemented to allow for precise manual gating by visual inspection of bi-axial expression plots (Figure 4A and 3B), but strictly speaking, these transformations are unnecessary for multidimensional data analysis if one uses appropriate algorithms with appropriate parameters (Figure 4C and 3D).

The convention is to ArcSinh transform the ion counts, as this transformation retains the linearity in the low end of the count spectrum, while resembling a log transformation in the upper end. The range of the ion counts where linearity is retained can be adjusted by dividing counts by a user-defined factor before ArcSinh transformation, thus emphasizing the signal in the lower end of the spectrum. For CyTOF® data, a co-factor around 5 is typically used – i.e. all counts are divided by 5 to deemphasize noise around 0. Alternatively, a Vlog transformation has been proposed, which, according to the authors, enables more or less direct comparison of mass cytometry and flow cytometry data if applied to both (94). Traditionally, flow cytometry data is transformed using a biexponential distribution (95), but ArcSinh with a co-factor of around 150 can also be used.

Normalization and batch correction

The rate of acquisition can vary between 2,000-20,000 cells per second in a standard flow cytometer. For CyTOF®, the rate is often closer to 200-300 cells per second, which means that the instrument will need to run for longer to acquire a useful number of data points - especially when running larger samples resulting from barcoding. During the course of a CyTOF® run, instrument sensitivity may decline due to cone contamination and mass calibration drift and cause overall signal intensity decay. To ameliorate these differences, polystyrene beads (Fluidigm EQ™) with a known composition and concentration of isotopes can be used as references to which reporter signals can be normalized. Briefly, the isotope signature of the beads is unique, and simultaneously running cells and beads through the CyTOF® enables correction of fluctuations in the signal. Commercial beads are synthesized to contain different metal isotopes that span the mass range used to label the proteins of interest, in order to normalize across the entire measured mass spectrum. Fluidigm provides software for

normalization and an additional algorithm has been implemented in MATLAB by Finck et al. (96) Additionally, periodic retuning of the instrument during very long acquisition runs (typically >6hr) will recalibrate the mass calibration, detector voltage, and current settings to help correct for instrument drift.

Like any assay type, batch effects arising from such things as different lots of reagent, runs on different days (96), or runs on different CyTOF® instruments (97) do exist and must be considered when making comparative analyses. These effects can be minimized with appropriate experimental design, such as having enough of a reagent lot for an entire experiment, running all samples for a project in a shorter amount of time, appropriate distribution of sample types such as cases and controls across each plate, including all timepoints for a given donor on the same plate, and the inclusion of a well-characterized healthy control with each batch to help identify problematic plates (98). Batch effects are a known problem for most omics data types, and has in many cases been addressed by statistically modeling the noise and removing it. Although highly successful for transcriptomics (99), mass cytometry is still at a stage where studies are primarily single-center efforts and batch effects are only now becoming an issue (100). Methods such as warping and range-based normalization has been used for flow cytometry (101), but these methods are not suitable for mass cytometry to the same degree, and address primarily instrument noise and not biological or assay noise (antibody staining inconsistencies, etc.). More recently, distribution matching methods have been developed for mass cytometry, but the utility has yet to stand the test of community scrutiny (102). Simpler workarounds include clustering of samples individually, followed by cluster matching and further downstream analyses.

Pre-gating live, intact, single cells

If the beads were kept after normalization, the first step in preparing the data for analysis is to remove beads, doublets (cell and bead/cell), and debris. This is traditionally done by biaxial comparison of a DNA channel and the respective bead isotope (Figure 5A). The current commercial normalization beads contain ^{140}Ce, which is a mass not typically used for antibody labeling, thus making it easier to separate beads from cells. Single beads will be DNA negative and bead ^{140}Ce positive, whereas cells will be DNA positive and ^{140}Ce negative. Double positives are typically bead/cell doublets, and double negatives are regarded as debris.

The next step is to gate for intact cells. This is traditionally done by biaxially plotting the two DNA channels and gating for events expressing both at a balanced level (Figure 5B). If events have too high expression of DNA, they are likely cell doublets, whereas if they express very little, they are potentially debris.

The next step is gating out doublets. This is traditionally done by plotting a histogram of event length (or bi-axially plotting against one of the DNA channels) and gate for events with a length that is within the range of the majority of events, leaving out those that a longer (Figure 5C). Note that if barcoding was used, doublets will likely not pass debarcoding with an n-choose-k scheme (see below).

Lastly, dead cells are gated out using a viability stain such as maleimide-DOTA, cisplatin, or Rh intercalator. It is important to remove dead cells, as they often have anomalous staining (103,104). This is done by staining cells with the viability stain before sample fixation. Dead cells

have compromised cell membranes, and live-dead reagents such as cisplatin stains dead cells by entering these cells and forming covalent bonds with intracellular nucleophile proteins. For live cell assays, live-dead staining is typically done after surface antibody staining. Gating for live cells is traditionally done by plotting a histogram of the live-dead channel or bi-axially plotting live-dead and a DNA channel. Cells with a high amount of live-dead reagent are then gated out (Figure 5D).

The steps above can be automated to a great extent by defining density-based cutoffs. If analyzing a larger set of samples, or if you possess a previously analyzed set prepared in a similar way, these data can be used to train a simple support vector machine (or similar) to automate the pre-gating process for the new samples.

One final type of undesired Events is potential carryover from the previous sample. This arises from insufficient rinsing of the instrument between samples. As would be expected, carryover Events are usually concentrated at the beginning of a sample and decrease in frequency as the sample progresses. However, if some debris is retained in the instrument and then breaks free, carryover can be found in small increments throughout a sample. The only way to ensure lack of carryover is to run wash solution or some other cleaning solution to destroy and remove any residue. However, the time that it takes to run the wash and then run MilliQ water to remove the residual cleaning solution adds to the overall time of acquisition; therefore, many operators simply run MilliQ water between samples. Carryover is not typically a major concern, and is only mentioned here for completeness.

Debarcoding

After running the combined barcoded samples, individual cells can be assigned to their respective samples of origin by debarcoding using the intensity of the dedicated barcoding isotopes. Intuitively, a binary barcode can be generated for each event by manually defining gates to distinguish them as either positive or negative for each of the barcoding isotopes. However, the main drawback of such a Boolean barcoding scheme is that when manually drawing gates to define events as positive or negative for a given isotope, events that fall outside of the gates are discarded. Given that the uptake of barcoding isotopes can differ depending on cell size (larger cells tend to take up more barcode reagents), setting a hard threshold for the intensities of the barcoding isotopes in a mixture of cells comes at the expense of lower yield - and even more troubling, loss of events could be specific to certain cell types or samples.

Instead, the suggested algorithm for deconvolution is the so-called single cell debarcoding (SCD) algorithm, which assigns a barcode to cells independently of each other (85). The algorithm works by first scaling and sorting the expression of the barcoding isotopes for each event. Then, the largest barcode separation between is identified. If the above-mentioned Pd scheme is used, then the largest separation will occur between the 3rd and the 4th isotope. If this separation is higher than a user defined threshold *and* the barcode assigned using this threshold exists in the barcoding scheme, then the cell is preliminarily assigned the barcode, otherwise the event is discarded. Once completed for all events, outliers are removed by applying a user defined threshold of the Mahalanobis distance of each event to the distribution of isotope expression in its respective barcode group. While markedly more robust than the

Boolean debarcoding scheme, like the Boolean scheme, the SCD algorithm requires user decision making during debarcoding and the yield to noise ratio can differ from user to user. The SCD algorithm currently exists as a MATLAB GUI implementation and an R implementation in the CATALYST or Premessa packages.

Parameter harmonization

When co-analyzing data from different center, instruments, or operators, parameter harmonization may be required. Channel naming is entered as free text format in the Fluidigm software, so naming variances such as “HLADR” vs. “HLA-DR” are likely to occur and need to be harmonized before analysis begins, otherwise many software packages will not read them as the same parameter. In rare instances, the name of the mass channel may also vary: for example, one file could have ^{148}Nd while another file could have ^{148}Sm as the mass channel. In both cases, the data for the “148” mass channel will be recorded correctly by the CyTOF®, but these will be seen as different parameters by analysis software (100). The software operating the Helios™ model allows for panel templates to be saved as shareable files, which will hopefully help reduce these issues in the future. Finally, different versions of the CyTOF® software from Fluidigm can slightly vary parameter naming, even for the same instrument model.

Data analysis

Once the data has been preprocessed to a *features x observations* expression matrix similar to those obtained with transcriptomics measurements, the downstream tasks can be addressed using an array of standard and specialized algorithms. An example of a basic analysis workflow consists of: 1) cell subset detection using clustering algorithms, 2) differential cell subset abundance and/or differential feature expression in the different subsets across different conditions using statistical testing, 3) visualization of cell subsets using dimensionality reduction. A number of publications review these tools (105), and it is thus not within the scope of this article to do so here. Instead, we provide an overview in Table 2 (see http://cytof.biosurf.org for a full list), and discuss a few of the more common algorithms and tools in the context of the problems they were designed to answer, and in doing so, enabling bioinformaticians to build upon these efforts and inspire further developments.

Cell subset detection (clustering)

Deconvoluting a cell mixture may be a goal in itself, and certainly a necessity for meaningful comparison of samples or cell populations whether transversal or longitudinal. The traditional approach relies on manual gating on biaxial plots, but as the number of features are increased, manual down gating becomes increasingly laborious. A number of methods are therefore seeking to automate or semi-automate the process - not only in the interest of speed, but unsupervised methods may also reveal cell populations overlooked when following standard gating strategies. Additionally, subset detection by manual gating often lead to unassigned, but potentially interesting events, as well as multiclass membership undesirable in further analyses.

Another feature of haematopoietic cells that can make data-driven clustering difficult, is the fact that since cells in the hematopoietic system develop in a hierarchical structure, the transition between some cells will result in a continuum rather than distinct clusters. The lack of a clearly defined correct answer, makes the true accuracy of clustering algorithms difficult to evaluate, but among the most robustly performing as compared with results from traditional gating are Phenograph (106) and FlowSOM (110) as evaluated by Weber and Robinson (117), where both assignment accuracy and cluster robustness (under different random starts and bootstrap resamples) of the algorithms were evaluated. Another useful metric for cluster quality is the intra-cluster homogeneity of the clusters, as introduced in the SPADEVizR package (118).

Differential abundance

Once subpopulations are clustered in each sample, the next step will often be to elucidate associations between cluster frequencies and/or protein expression and a given condition or time. These exercises closely resemble differential expression analyses used in transcriptomics for decades, and the bioinformatics toolbox contains multiple tools for basic significance tests and linear models. Several specialized tools have also been developed with integrated GUIs, notably Citrus (cluster identification, characterization, and regression) (83), Statistical Scaffold (56) and Cydar (112).

One major shortcoming of these specialized GUI tools is the lack of flexibility when it comes to handling more complex experimental setups, such as longitudinal studies or studies with multiple groups and covariates. Generalized linear models (GLM) for differential expression analysis, popularized for transcriptomic analyses by tools such as limma (119) for microarray data and DEseq2 (120) or EdgeR (121) for RNA sequencing data, accepts model matrices with

co-variates for complex experimental designs, and can be applied to marker expression matrices extracted from FCS files or subpopulation frequency tables calculated from the clustering results.

Lastly, it is important to consider that the immune system consists of multiple functional cell populations, which are coordinated in a complex interplay in response to various conditions. It is therefore sometimes more informative to examine population frequencies as dependent variables by examining correlations or ratios between frequencies (122). Additionally, natural variations in the immune system are likely to mask the difference conferred by time or conditions when looking at a single variable, even if analyzing a large number of samples. Much like transcriptomics analyses, the immune system does not consist of independent variables, and neither the abundance of protein markers on individual cells nor the frequencies of individual cell populations can be assumed independent from the remaining variables.

Dimensionality reduction

The most commonly used algorithm for dimensionality reduction in the biological sciences is principal component analysis (PCA). For mass cytometry data, a PCA will often capture 40-50% of the variance on the first 2-3 PCs, but since the distance between events is linear, data existing on a nonlinear manifold will be misrepresented (105).

The t-Distributed Stochastic Neighbor Embedding (t-SNE) dimensionality reduction algorithm is a more popular tool for dimensionality reduction of cytometry data (116). The distance between data points are represented by a t-distribution, meaning that multidimensional distances are preserved to a greater degree than PCA. However, there are a number of features of the

algorithm that can lead to misinterpretation by the unaware user. For example, t-SNE (intentionally) does not preserve distances nor density, and it only preserves nearest-neighbors if reasonable hyperparameters are selected. This means that the sizes of the clusters are not always indicative of the variability within the cluster, and similarly, the distance between two clusters is not always proportional to how different they really are. In this regard, the algorithm is quite sensitive to the user making an informed choice of the perplexity and epsilon hyperparameters to reasonably represent the underlying structure of the data. Another feature that may be considered a disadvantage to some is that t-SNE depends on a random start and is non-deterministic even on the same data unless the same seed and iteration limit is used for each run. Additionally, removing or adding even a few data points from the input set will affect the layout of the remaining events, and consequently it is not directly possible to add samples to an existing t-SNE plot. Lastly, t-SNE does not allow for identical events, which, in the case of immune profiling where one would expect at least some identical cells in each cluster, means discarding or scrambling data using randomization as discussed above. Dimensionality reduction definitely has a place in mass cytometry analysis pipelines, but for these reasons, it should be limited to exploratory and/or post-analysis visualizations. t-SNE is implemented in a number of languages including R, Python, C++, etc., and as a plug-in to FlowJo X. The MATLAB implementation of t-SNE often used for mass cytometry data in GUI pipelines is referred to as viSNE (123).

Future Directions

A number of areas for future development on the computational side remain. Despite these data being sensitive to noise from sources such as the antibody panel, the isotope labels and spillover (76,77), the instrument cell transmission efficiency, the instrument ion transmission efficiency, the instrument signal capture, instrument signal decay, and sample-related biological sources, no robust error model has been defined. Traditionally, outliers were discarded during manual gating, and variance in the true signal largely ignored. However, with the use of multidimensional analysis tools including all events from a given run, it is necessary to have a well-defined model for the expected noise in order to efficiently filter it out before analysis.

Another issue that remains largely unaddressed is batch effects when comparing data from runs performed at different centers (100), on different instruments or versions, by different operators, or even simply on different dates. In some cases, a well-characterized control sample is stained on the same plate, but primarily as a qualitative quality-control measure (98). This lack of a robust model is somewhat related to the lack of a comprehensive error model, but the need is likely to increase as more and more public data becomes available.

As new technologies develop, analysis must continue to adapt. Recently, development of imaging mass cytometry platforms (Hyperion™ from Fluidigm and MIBI from Ionpath) have been developed (124–127). Rather than requiring tissue dissociation, these allow the intact analysis of tissue sections like fine-needle biopsies or archival paraffin blocks. This preserves additional relational information such as intercellular distances, but also raises new analysis challenges,

such as image segmentation for cell determination that have been explored in light microscopy (128–130).

Lastly, as the data generated using mass cytometry increase both in amount and complexity, the need for data scientists with a deep understanding of the intricacies of these data increases. In this document, we have described the more technical aspects of the data generation, with the intention of providing the insights into the anatomy of CyTOF® data necessary not only to apply existing analysis tools, but also to develop novel algorithms and analysis tools. Notably, the vast majority of the published analysis tools were developed and/or benchmarked on one or more of five reference datasets (84,106,107,131,132), highlighting the need for more systematic data sharing and thorough benchmarking on more diverse datasets. For more in-depth discussions, example data, and code, please visit our data wiki at http://cytof.biosurf.org and follow discussions on bench experiments and data analysis at the online user forums http://cytoforum.stanford.edu.

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Figure legends

Figure 1: Step involved in sample preparation. Steps L, M, and N are specific to mass cytometry, whereas the remaining steps also applies to flow cytometry. Please note that the order of steps may vary slightly between assay types or workflows.

Figure 2: A) At low ion concentrations, the resulting pulses can be counted directly and recorded as ion counts B) At higher concentrations, the intensity distributions will overlap. The signal intensity is calculated by integration of the digital values of the voltage, then converted into ion counts using the dual instrument (Di) coefficient C) The Di coefficient is calculated as the slope of the pulse count vs intensity curve, in the range where the analogue counts correlate with the digital counts.

Figure 3: The central steps and potential error sources (red boxes) in A) reagent preparation and cell staining, B) running the samples through the CyTOF® instrument, and C) instrument data processing.

Figure 4: A) Biaxial plot of the raw counts of CD4 and CD8 on T cells, and B) Biaxial plot of the ArcSinh transformed counts of CD4 and CD8 on T cells. C) t-SNE (perplexity = 60) plot of raw counts of 26 lineage markers on 10,000 PBMC, and D) t-SNE (perplexity = 20) plot of the ArcSinh transformed counts on the same cells.

Figure 5: Manual pre-gating strategies for A) cells vs. beads and debris, B) intact cells vs. debris, C) singlets vs. doublets and debris, and D) live cells vs. dead cells.

Tables

Table 1: Experimental factors affecting cytometry data.

Category	Issue	Example	Effect on Data	Ref
Sample Isolation and Processing	Interference of antibody binding	soluble IgM blocking binding of anti-IgM antibody to cells in complete whole blood	Little or no IgM signal when staining complete whole blood	(4)
		whole-blood CD163 staining OK in EDTA collection tubes but not heparin tubes	CD163 signal in whole blood from EDTA tubes but no signal in heparin tubes	(5)
	Contamination of sample	Iodine during Ficoll separation	Increase in iodine background	(6)
		Lanthanide particles from striker flint in smoker-derived sample	Several lanthanide channels contaminated	(7)
	Distortion of cell distribution	Depletion of monocytes	Decrease in monocyte count in density-separated cell fraction, relative to whole blood	(8)
		Removal of cell types	Ficoll separation removes granulocytes and red blood cells from whole blood, leaving	(9)

			PBMCs	
		Lysis method	Increase in debris, or changes in signal intensity of certain markers	(10)
	Alteration of epitopes during processing	Cleavage of CD4 surface epitopes during enzymatic tissue disaggregation	Usually irreparable loss of epitope; therefore, antigen-specific probe will not bind, resulting in no signal for that marker on that cell	(11)
		Shedding of CD62L during freeze/thaw	Loss of epitope; therefore, antigen-specific probe will not bind, resulting in no signal for that marker on that cell. Sometimes possible to regain during cell rest/culture	(12)
		Increase in expression level due to stimulation	CD69; antibody probe typically added during stimulation to capture transient surface expression of CD107a during degranulation and re-internalization	(13,14)
		Decrease in expression level due to stimulation	CD4 on PMA+I stimulated cells; antigen-specific probe will not bind, resulting in no	(15,16)

			signal for that marker on that cell	
		Fixation causing staining loss	Covalent modification of epitopes; interference with antibody/probe binding; decrease in or complete loss of probe signal; typically affects all cell types	(17,18)
		Permeabilizing agent causing staining loss	Harsh agents such as methanol denature three-dimensional epitopes targeted by specific antibody clones, resulting in loss of signal for those antibodies.	(19)
		Permeabilizing agent causing apparent staining increase	Increase in intracellular staining of eosinophils; can be blocked with heparin	(20)
	Debris and dead cells	General		(21,22)
		Specific, affecting only one high-abundant marker	CD56 signal in disaggregated neural tissue. Debris streaking causes CD56 signal, even for	(M.L., personal

			CD56-negative cells	observation)
		Thawing technique impacting viability		(23)
		Timing of processing (sampling to processing or cryopreservation)	Lower cell recovery, decrease in stimulated T cells when processed 24hr after sampling	(24)
		Cell death due to strong stimulation	ConA, PMA+ionomycin	(25)
	Nonspecific antibody binding	Fc receptor-mediated; cross-reactive to another epitope	Can sometimes be managed with blocking reagents	(26)
Donor Variability--Healthy Donors	Species	human, mouse, bacteria, macaque		(27–29)
	Age	young vs. old		(30,31)
	Gender	male vs. female		(32)
	Ethnicity			(33–35)
	Circadian rhythms	mRNA within cell; cell distributions in tissues		(36–39)

	Differences in cell frequency			(40,41)
	Change in healthy state	Pregnancy		(42)
	Tissue type	whole blood; PBMCs from whole blood; disaggregated tissue from tonsil, pancreas, bone marrow		(43–45)
	Differences in marker expression	CD33 expression on monocytes		(46)
		Genetic loss-of-function		(47)
		Resting cryopreserved samples overnight in media before stimulation	Increase in percentage of cells producing cytokines when stimulated after overnight rest	(48)
		Importance of export inhibitor when measuring intracellular cytokine production	Brefeldin A, Monensin	(49)
	Differences in marker distribution	CD56 on human NK cells vs. macaque monocytes; CD64 in humans vs. macaques		(27,50)
		CD28 in humans vs. macaques		(51)
		CD56 on human NK, muscle,		(52)

		neural cells		
	Ln biological uptake	Bacterial XoxF-family dehydrogenases		(53,54)
Donor Variability--Patients	Cancer	Melanoma		(55)
		Recovery		(56)
	Autoimmune	Sjogren's syndrome		(57)
	Chronic viral infection	Human cytomegalovirus, Varicella zoster virus		(58–60)
	Treatment	Platinum-based chemotherapy agents; Amiodarone treatment; Gd-containing MRI agents	treatment agents usually contain natural- abundance metals, thereby affecting all naturally- occurring stable isotope channels for that element	(61–64)

Table 2: List of most common tools used for CyTOF® data analysis. For a complete list, please visit http://cytof.biosurf.org.

Tool	Purpose	Reference
PhenoGraph	Clustering	(106)
X-Shift	Clustering	(107)
ACCENSE	Clustering	(108)
DensVM	Clustering	(109)
FlowSOM	Clustering	(110)
SPADE	Clustering	(111)
Citrus	Clustering, differential abundance analysis	(83)
Cydar	Clustering, differential abundance analysis	(112)
ACDC	Cell type assignment	(113)
SCAFFoLD	Cell type assignment, cellular trajectory mapping	(36)
Statistical SCAFFoLD	Cell type assignment, cellular trajectory mapping, differential abundance analysis	(56)
Wanderlust	Cellular trajectory detection	(114)
DREMI/DREVI	Cellular trajectory detection	(115)
t-SNE	Dimensionality reduction	(116)

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