

1:41 PM JUL 05, 2023

THE MOMENT YOUR
RESEARCH MOVES FROM
BENCH TO BEDSIDE_



THE DIFFERENCE OF **BREAKTHROUGH RESEARCH** TO ENABLE MORE CURES

WITH BD AS YOUR PARTNER IN IMMUNO-ONCOLOGY FROM BENCH TO BEDSIDE. BD is your partner in immuno-oncology from discovery research to clinical applications, with a range of high-performance solutions designed to give you high-quality, reliable research data for even the most complex experiments. More solutions. More answers. More data you can trust. Discover more with BD at your side. **Discover the new BD.**

Learn how you can advance your research >

PAID ADVERTISEMENT



BD

Advancing the
world of health

Rigor and Reproducibility of Cytometry Practices for Immuno-Oncology: A Multifaceted Challenge

Tamara J. Laskowski,^{1*}  Amy L. Hazen,² Renata S. Collazo,¹ David Haviland³

¹Department of Immunology, MD Anderson Cancer Center, Houston, Texas, 77030

²Shared Research Resources, University of Texas Health Science Center at Houston, Houston, Texas, 77030

³Flow Cytometry, Houston Methodist Research Institute, Houston, Texas, 77030

Received 1 April 2019; Revised 5 June 2019; Accepted 8 August 2019

*Correspondence to: Tamara J. Laskowski, Department of Immunology, 7455 Fannin Street, SCR3.3020, Houston, TX 77030
 Email: tjlaskowski@mdanderson.org

Published online 27 August 2019 in Wiley Online Library (wileyonlinelibrary.com)

DOI: 10.1002/cyto.a.23882

© 2019 International Society for Advancement of Cytometry

• Abstract

The rapid advancement of immunotherapy strategies has created a need for technologies that can reliably and reproducibly identify rare populations, detect subtle changes in modulatory signals, and assess antigenic expression patterns that are time-sensitive. Accomplishing these tasks requires careful planning and the employment of tools that provide greater sensitivity and specificity without demanding extensive time. Flow Cytometry has earned its place as a preferred analysis platform. This technology offers a flexible path to the interrogation of protein expression patterns and detection of functional properties in cell populations of interest. Mass Cytometry is a newcomer technology that has generated significant interest in the field. By incorporating mass spectrometry analysis to the traditional principles of flow cytometry, this innovative tool promises to significantly expand the ability to detect multiple proteins on a single cell. The use of these technologies in a manner that is consistent and reproducible through multiple sample sets demands careful attention to experiment design, reagent selection, and instrumentation. Whether applying flow or mass cytometry, reaching successful, reliable results involves many factors. Sample preparation, antibody titrations, and appropriate controls are major biological considerations that impact cytometric analysis. Additionally, instrument voltages, lasers, and run quality assessments are essential for ensuring comparability and reproducibility between analyses. In this article, we aim to discuss the critical aspects that impact flow cytometry, and will touch on important considerations for mass cytometry as well. Focusing on their relevance to immunotherapy studies, we will address the importance of appropriate sample processing and will discuss how selection of suitable panels, controls, and antibodies must follow a carefully designed plan. We will also comment on how educated use of instrumentation plays a significant role in the reliability and reproducibility of results. Through this work, we hope to contribute to the effort toward establishing higher standards for rigor and reproducibility of cytometry practices by researchers, operators, and general cytometry users employing cytometry-based assays in their work. © 2019 International Society for Advancement of Cytometry

Edward Jenner, in the late 1700s, made the first crucial observations that would lead to the eradication of the plague of their generation, smallpox (1). Regarded as the foundation of immunology, his work paved the way to the understanding that specialized cells in our body, today known as immune cells, have tremendous power to recognize and eliminate threats. Fast-forward a few centuries, the field of immunotherapy is born. Today, the realization that immune cells not only destroy foreign threats, but can be armed and trained to detect and kill cancer cells has fostered new hope that we will indeed win this long-fought battle against cancer. Genetically enhanced T-cells, known as CAR T-cells, have eliminated unrelenting hematological tumors and extended the life of patients once deemed terminal (2,3). Immune checkpoint inhibitors have unleashed the power of killer immune cells against vastly metastatic disease, eradicating all tumors and restoring long-term health (4,5). These unprecedented results boosted new research efforts toward developing strategies that can overcome the complex barriers of more challenging cancers. Flow Cytometry, and more recently also mass cytometry, are used both during the course of research

and development of new therapies, as well as in clinical studies that monitor the progress of patients enrolled in immunotherapy trials. Important questions are answered with the help of these technologies. Therefore, it is critical that analyses are performed accurately and data interpreted with the highest confidence. In this article, we will discuss best practices related to sample preparation and experiment design, proper use of instrumentation, and potential strategies for overcoming the limitations of these technologies.

SAMPLE PREPARATION AND STORAGE: THE CRUCIAL FIRST STEP

When planning protein analysis by Flow or Mass Cytometry, the primary considerations researchers often focus on include the sample type to use, number of markers to assay, the choice of antibodies, and antibody conjugate selection. Sample preprocessing and storage are often disregarded. These, however, are of paramount significance to any cytometry-based assay, and variations in sample preprocessing limit potential downstream applications, affect results, and impact assay reproducibility. Therefore, understanding the different methods used for preservation of sample integrity and how these impact the utility of a sample are crucial determinations that should be carefully considered early on in the study.

Implementation of sample preparation protocols is often based on the type of samples that are obtained for a given study. For immuno-oncology research, sample sources primarily consist of blood, tumor tissue (liquid or solid), or bone marrow. Each requires its own optimized processing procedure. Bone marrow and peripheral blood samples often undergo standardized Ficoll gradient separation through which mononuclear cells are harvested. This well-defined procedure is relatively simple and straightforward, yielding large numbers of cells. Solid tumor biopsies, conversely, are more complex tissues that require additional processing steps. The first hurdle is tissue disaggregation, which is highly dependent on successful disruption of extracellular matrix, a mixture of cohesive factors and structural proteins. This can be achieved by a combination of mechanical and enzymatic methods, but should be carefully performed, as excessive exposure to enzymatic digestion may result in destruction of antibody-binding epitopes, thus decreasing the effectiveness of downstream immunostaining for cytometric analysis (6–8). Furthermore, unlike cell isolation from blood samples, tissue dissociation is a multi-step process that involves serial washes for removal of reagents, clumps, and debris in order to produce a single-cell suspension that can subsequently either be prepared for analysis or frozen for later use. Through these steps, cell loss often occurs leading to decreased final yield. Good dissociation techniques are therefore critical to effective cell isolation from tissues. Guidelines for optimal practices are available, such as the Worthington Tissue Dissociation Guide, and can be very useful when generating standard operating procedures.

Subsequent to cell isolation, a fundamental consideration is sample storage, if immediate use is not feasible. Cryopreservation is often the method of choice, and typically consists of

re-suspension of cell pellet in 10% Dimethyl sulfoxide (DMSO) either in total fetal bovine serum (FBS) or in a medium formulation comprising high FBS concentration. DMSO serves as a cryoprotectant during the freezing process, and upon thawing and removing DMSO, viable cells can be recovered for analysis or subsequent culture (6,7). It is important to consider, however, that cryopreservation may induce functional and phenotypic changes and may significantly affect the relative frequencies of viable peripheral blood mononuclear cell (PBMC) subpopulations (7–13). From our own experience, subsets such as granulocytes or mast cells often experience greater loss of viability, and therefore, studies focusing on these subpopulations must apply alternative protocols to ensure optimal recovery. One feasible strategy to counter the inherent loss of PBMC heterogeneity due to cryopreservation is to employ a Fix-Freeze method through which mononuclear cells are collected, incubated in paraformaldehyde at room temperature for 10 minutes, and subsequently frozen at -80°C . Though cells lose viability in the fixation process, the advantage of this approach is that cell morphology, architecture, and protein structures are preserved and stabilized, while proteolytic enzymes are inactivated, protecting the sample from degradation (14,15).

Determining the appropriate sample preservation approach is therefore a critical first step. It is primarily dependent on the desired downstream applications and the longevity of the study. Decisions such as the type of analysis desired (e.g., Flow Cytometry or Mass Cytometry), and whether additional assays will be performed must be considered before samples are processed for storage. For applications such as flow or mass cytometric analysis of phenotypic markers, both cryopreservation with DMSO or fix-freezing are viable options for sample storage. However, if interrogating cells for functional or activation markers that require prior culture time, DMSO-based cryopreservation is the recommended approach. Furthermore, the subpopulation of interest in a study must be considered. As previously mentioned, specific cell populations may exhibit greater sensitivity to freeze-thawing, and appropriate storage is required to ensure maximum sample preservation.

As stated above, longevity of a study is an important factor that may also affect reproducibility. Specifically for clinical trial samples, which are often collected at defined stages during treatment and may be needed for multiple assays, proper planning should dictate the necessary processing approach. If sample size allows, multiple aliquots should be cryopreserved under both protocols, so as to expand sample usefulness and ensure quality standards are met for each designated application.

Finally, operator introduced variability is a significant contributing factor that affects reproducibility. It is also a challenging problem to address. Establishing strict guidelines for isolation of mononuclear cells and characterization of final product (e.g., viability and cell count assessments, flow cytometric analysis to detect frequency of major cell populations), along with well-defined sample thawing and recovery protocols may help to alleviate this problem. Additionally, meeting optimal sample quality requirements for each downstream application helps to minimize assay

variability. Another potential solution is to integrate automation to the sample processing workflow. Instruments such as liquid handlers offer protocols for automated processing which have been shown to perform as efficiently as the gold-standard Ficoll density gradient method, as measured by overall yield, viability, recovery, white blood cell subpopulation distribution, and gene expression (16–19).

ANTIBODY VALIDATION

Designing appropriate panels with adequate controls and well-titrated antibodies is essential for clear interpretation of biological results. Initial titration of antibodies is a well-established criterion for good experimental design. Most researchers will begin with the manufacturer recommended titration and test multiple doses until an optimal stain index is achieved (20–24). Once an initial titration has been established for a particular antibody within a group, the other members tend to move forward with the same values over the course of the life span of that project. However, lot-to-lot variations would indicate that each new lot should be re-optimized. Importantly, when tandem fluorophores are involved, the intensity and stability of the signal may exhibit greater variability, and hence, even a vial-to-vial titration is advisable.

Rarely do we question the specificity of a commercially purchased antibody. If a particular marker is critical to the outcome of our data, user validation is strongly encouraged. Most reliably, a biological negative control can determine antibody specificity with greater certainty (22–25). In addition, creating antibody master mixes to minimize variability between experiments can be helpful to ensure reproducibility, however the stability of all master mixes must be tested to determine the duration of appropriate use. In our hands, most of our 17-color flow cytometry panel mixes have been tested and shown sustained signal for up to 15 days when stored at 4°C. Antibody staining mixes for Cytometry by time-of-flight (CyTOF), conversely, do not seem have the same stability. Leipold et al. demonstrated that antibody cocktails showed significantly higher background when stored for 2 weeks. This was presumed to be a consequence of antibody aggregation that may likely occur in highly concentrated antibody mixes (26). We have observed that a maximum storage time of 3 days at 4°C for CyTOF staining cocktails is acceptable. It is important to note that efforts continue to be employed toward achieving successful results with premade antibody cocktails in order to minimize variability that is introduced when multiple batch preparations are required. Recently, Schulz et al. demonstrated that 41-antibody mixes were stable for up to 19 weeks when stored at temperatures of –80°C or lower. t-SNE mapping of PBMC samples labeled with cryopreserved cocktails showed impressive consistency in staining patterns when compared with PBMC stained with fresh antibodies (27).

Overall, antibody titration and validation are areas where additional rigor needs to be applied in the field of cytometry. Proper education and training of cytometry users in panel design and assay validation for both flow and mass cytometry is highly recommended as a way to help decrease variability and, thus, improve assay reproducibility.

INSTRUMENT DESIGN

Another area of opportunity is understanding how instrument design can and should influence experimental design. It is common for researchers to attempt to reproduce panels published by other groups to investigate the same cell phenotypes in their specific model or hypothesis. However, major differences in the optical configurations between instruments, such as laser wavelength, power, band pass filters, and detector efficiency, will alter how the data are collected. Educated use of instrumentation plays a key role in reproducibility of results. When examining rare populations or dimly expressed antigens, for instance, it is crucial to optimize the panel design specifically considering the instrument you intend to use for the study. Frequently, published panels can be altered to achieve optimal performance by adjusting one or two fluorophores to better suit the instrument being used.

In addition, proper quality control and cleaning of instruments affect their stability and the overall ability to generate reproducible data over time. For specific longitudinal studies, such as immune monitoring of patients receiving sequential immunotherapy treatments, the use of a dedicated instrument is strongly recommended, as most facilities have not standardized multiple instruments to each other. Consistency with the same instrument is a key factor, as Cytometer Setup and Tracking (CST) protocols may not always standardize across instrumentation. It is important to note that an additional step can be taken to cross standardize cytometers to generate directly comparable results. BDFACS Diva application settings is a well-defined mechanism to establish and set consistent target voltages to help achieve normalization across various instruments as well as normalize for instrument variance over time, which is especially necessary for longitudinal studies. The use of application settings may not be available on all platforms. In such cases, there are alternate mechanisms that can be used to establish standardization (28–31). One example of an alternative method, which in our experience has been successful, is setting target median fluorescence intensities (MFI) values for eight-peak beads. Overall, whether employing traditional CST measurements or alternative methods, some standardization should be implemented to normalize for instrument changes and minimize the impact of instrument variance on assay results. This process may vary for different platforms (32–34). However, there are guidelines for common measures used to monitor instrument performance over time (34–36). It is important to also note that, if utilizing services from a shared resource facility where instruments are communal, and performance and maintenance are monitored by facility staff, it may be helpful to discuss the nature of the study, whether there is need for dedicated instrument, and the quality controls required over the course of all experiments. Utilizing the expertise of shared resource staff can help to mitigate problems with reproducibility.

INSTRUMENT SETUP AND OPTIMAL USE

Proper instrument setup is essential for reliable analysis. Some of the first critical steps include optimizing detector

gains to ensure the positive peak is within the linear range of detection of the photomultiplier tube (PMT) and the negative population is above the noise of the detector.

Inherent to multicolor assays is fluorescence spillover. After proper determination of PMT voltages, a matrix can be generated to determine and remove the effects of spillover. Compensation is gain-dependent and should only be determined after optimal PMT voltages have been established for the experiment (36,37). Compensation must be considered in all multicolor experiments. The use of web resources and antigen density charts is strongly recommended to minimize spectral overlap and reduce compensation. To remove user-induced bias, computer-assisted compensation, whether through the cytometer or thirdparty analysis software, is highly favored. Properly compensated data can be critical when attempting to distinguish small dissimilarities in positive and negative events (34,37).

Regarding compensation control assays, what is the recommended approach? Most groups rely on capture beads for setting their compensation parameters. Capture beads provide a uniform bright signal independent of antibody specificity (35). This is an especially helpful feature because it allows for a clear signal to be produced for dimly expressed antigens, culminating in a more accurate and well-defined compensation matrix.

Biological controls are often regarded as most relevant and in many cases these are indeed the appropriate sources for compensation controls. However, biological controls become inadequate when the expression of certain antigens is too low for cell-based controls to serve as robust compensation controls. This can result from either low antigen expression or antigen expression that is restricted to rare cellular subsets. In either case, the lack of appropriate controls hinders the ability to develop a precise compensation matrix.

PANEL DESIGN

Designing the correct panel to interrogate your samples is a fundamental part of any cytometry experiment. There are several criteria one should consider when developing a flow cytometry panel: fluorochrome brightness, antigen expression patterns and density, antigen coexpression, and spread (36).

When targeting multiple populations, including rare subtypes such as immune cell infiltrates in tumor cells, optimized panel design is a critical step that requires basic knowledge of the biological properties of the sample such as cell type frequencies and antigen expression levels, for instance (38). The recommended strategy is to begin your panel design by assigning the brightest fluorophore to the antigen that exhibits lowest expression level, and giving that antigen priority in the panel. It is important to also consider the number of labeled proteins that will be present on the same given cell, and their overall densities. In these cases, careful planning is necessary to ensure selection of fluorochromes that preferably display little-to-no overlap in their emission spectrum patterns.

Without prior understanding of the markers and populations of interest, as well as their estimated frequencies

within your sample, the optimization of a multiparameter panel becomes a tremendous challenge, affecting even the ability to estimate the number of data points to collect to reach sample size significance. Because the larger the panel, the more complex its design becomes, many in the field have turned to the idea of minimizing panel design to only include markers critically relevant to answer the biological questions in a given study. Under this premise, panels become smaller but less complex, thus resulting in clearer analyses and increased confidence in data interpretation.

FMO CONTROLS

Fluorescence-minus-one (FMO) controls were first introduced by Roederer in 2001 as a strategy for properly defining thresholds for gate boundaries in multiparameter flow cytometry data (35). FMOs provide a method to measure the effects of spillover from individual populations into other channels of interest and are crucial when trying to maximize sensitivity for dim expression (34,36). In addition, FMOs will account for the “spread of the data” in multicolor experiments where single-stained background controls are less than appropriate and will lead to overestimating the actual percentage of a population. To generate appropriate FMO controls, especially in the case of clinical studies, the most comprehensive approach is to build FMO controls for each individual patient to account for sample-specific variability. However, in most cases, the sample size alone is prohibitive, and therefore, sacrificing valuable patient sample for controls is not a viable option. In the instances when cell number is insufficient, one potential strategy is to pool together a small fraction from each sample, creating a mixture that comprises characteristics of all samples included in the analysis set. If running the same analysis several times within the same week (e.g., drug dose–response analysis over the course of a few days) FMO controls can be prepared in advance, fixed, and stored for use over several days. In our experience, FMOs can be used for up to three days with no significant loss of signal.

DATA ACQUISITION PROTOCOL

Generating very detailed standard operating protocols (SOP) is a valuable effort that helps to minimize variability between experiments by improving data reproducibility by multiple operators. As protocol variability is minimized, differences can be more confidently attributed to true biological observations, rather than artifacts due to improper practices. Such SOPs should include the experimental layout with gating established in experimental templates. Using previously discussed application settings or instrument controls ensures that gating adjustments are kept to a minimum, thus reducing subjectivity. Once instrument setup is complete, all data should be acquired under the same instrument settings. For long-term studies, having a control sample that is aliquoted to run internally in each experiment over time is advisable. Applying such control serves as an internal check of both instrument and staining consistency over time. Monitoring the MFI fluctuations of an eight-peak bead set using fixed

PMT voltages over time can provide a good internal control for instrument variations. However, it will not reflect errors in the staining protocol. An additional internal biological control such as a normal leukocyte sample from which small aliquots are frozen and used within each sample batch can serve as a way to monitor interassay variability between multiple experiments. Timing of sample acquisition also plays a critical role. MFIs can decrease over time; therefore, consistency in protocol timing is critical to reduce variability. The impact of cell frequency should be considered in the acquisition protocol. Special attention should be given to rare cell populations, as these are at greater risk of variation between samples (37–39).

DATA ANALYSIS

Despite significant efforts in the field to standardize most aspects of cytometry such as reagents, instruments, and protocols, significant variability is introduced due to subjective gating by independent users. Collectively, the field has focused on moving away from, or minimizing, subjective data analysis. Analysis by iteratively plotting cells as bivariate plots and manually gating introduces subjectivity and variability as the shapes and boundaries of the gates can be modified thus affecting reproducibility over long periods of time. Additionally, subjectivity in the analysis may introduce bias toward the anticipated outcome, obscuring the true meaning of the data.

Manual gating can be a laborious and time-consuming task when dealing with large data sets and may be a source of errors, as the gating strategy and order are critical to resolve the interplay between various markers. Data from studies involving multi-centers, each performing its own supervised analysis, have indicated that manual gating introduces the largest variable in standardized experiments (17–21,40). If no other options are feasible and manual gating is to be performed, it should be centralized to one location of a multisite study to help minimize variability. Furthermore, the use of appropriate controls and templates to standardize gating is encouraged in an effort to ensure analysis has as little user-introduced bias as possible.

As an alternative approach to manual gating, one might consider using computational analysis tools such as Spade and tSNE, which apply mathematical principles to assess all markers simultaneously. These unsupervised algorithms offer far more objective, and therefore reproducible, analysis of data over time (40,41).

MIFLOWCYT

In an effort to bridge the gap of analysis and data reproducibility, journals such as this one have instituted the requirement for publishing according to the Minimum Information about Flow Cytometry Experiments (MIFlowCyt) standard (42,43). This initiative ensures the sharing of sufficient information to ensure experiment reproducibility, such as antibody details (vendors, clones, and concentrations), instrument description (model, optical configurations), data analysis

information (gating strategies, compensation matrices), and so on. As the field increasingly adopts MIFlowCyt, it will enable scientists to more clearly interpret published data and reproduce findings from other groups, thus promoting a better collaborative environment that fosters greater scientific advancements (43,44).

MASS CYTOMETRY: NEW TOOL, SIMILAR REQUIREMENTS

Mass Cytometry, or CyTOF, is quickly becoming an established technology for high-dimensional analysis. Unlike flow cytometry, which requires the labeling of cells with antibodies conjugated to fluorescent proteins, mass cytometry relies on elemental isotope conjugates for detection, a method that permits achievement of greater dimensionality levels (50+ parameters) currently unattainable through flow cytometry (45–48). Just as efforts have been dedicated to the standardization of analysis and interpretation of flow data, similar requirements exist for proper use and reproducibility of mass cytometry analysis. While mass cytometry is not affected by fluorescence spectrum overlap, other sources of background exist, and for markers that do not display clear separation between positive and negative populations (such as CD25 and CD57, for example), it becomes difficult to identify true populations in the absence of controls. One source of background is signal interference due to overlap between mass channels. Though this overlap occurs at low levels, careful planning is recommended when designing mass cytometry panels. Testing panels on a control sample (e.g., healthy donor PBMC) is a good approach for determining whether or not the populations of interest can be clearly resolved. Online tools, such as Fluidigm's Maspar Panel Designer, allow users to optimize their panels by minimizing signal overlap into low signal targets.

Another potential source of background is isotope impurity (49). Commercially available metal-conjugated antibodies are typically enriched and show greater isotopic purity. However, many groups conjugate antibodies in house, generating a product that is dominated by the desired species of isotope, but often contains trace contaminant impurities. Impurities in antibody conjugation are a major contributor to signal overlap. In flow cytometry, employing FMO controls is particularly important for identification of positive and negative populations when marker expression shows minimal separation. Because mass cytometry data are uncompensated, the use of Mass-minus-one (MMO) controls is a plausible strategy to visualize signal interference and guide the assignment of threshold levels. Takahashi et al. demonstrated the importance of MMO controls for resolving true signal from interfering background when assessing the expression of integrin $\beta 7$ in immune cells using a 36-marker panel (49). In their experiment, they observed a discrepancy in integrin $\beta 7$ signal that was most pronounced in a cell population that expressed a marker in an adjacent channel. This discrepancy was confirmed as signal interference by MMO controls.

Similar to flow cytometers, the performance and signal detection accuracy of mass cytometers may also change over

Table 1. A brief guide to cytometry workflow requirements, potential challenges, and best practice recommendations, as discussed in this review

WORKFLOW	CONSIDERATIONS/PITFALLS	RECOMMENDATIONS	REFERENCES
Sample preparation			
Standardization of sample processing protocols per sample type (e.g., solid vs liquid)	<ul style="list-style-type: none"> - Variations in sample processing may affect downstream results - Extensive enzymatic digestion may cause destruction of epitopes—Multi-step liquid processing may result in loss of cells/decreased cellular yield 	<ul style="list-style-type: none"> - Determine sample type and desired downstream applications - Opt for shorter enzymatic digestion followed by mechanical dissociation when processing solid tissues - Optimize sample processing to maximize cellular yield and viability without compromising antigen expression 	6–10,16
Standardization of sample storage protocols such as cryopreservation and fix-freeze methods when longitudinal studies are required	<ul style="list-style-type: none"> - Sample storage methods impact sample utility - Cryopreservation methods may induce phenotypic changes and alter relative antigen frequencies - Cryopreservation allows for reconstitution and live-cell cultures - Fix/Freeze storage: preserve sample characteristics at time of freezing but prevent downstream live-cell applications 	<ul style="list-style-type: none"> - Determine downstream applications early on and then select appropriate sample preservation and storage methods - Consider potential impact of sample storage method on your target proteins prior to sample processing 	6, 8–10, 13
Antibody validation			
Titration of antibodies to determine optimal stain index	<ul style="list-style-type: none"> - Lot-to-lot variations in antibody production - Tandem fluorophores exhibit even greater production variance and instability 	<ul style="list-style-type: none"> - Titrate each lot of antibody or order larger batches from same lot to minimize variability - Tandem fluorophores require vial to vial titration 	22, 23, 25
Validation of antibody specificity	<ul style="list-style-type: none"> - End users must validate antibody specificity as a large percentage of commercially purchased antibodies do not recognize the appropriate antigen 	<ul style="list-style-type: none"> - Use a biological negative to confirm antibody specificity 	20, 21, 26
Instrument design			
Education surrounding instrument configuration such as laser wavelengths, power, band pass filter, and detector efficiency	<ul style="list-style-type: none"> - Researchers should understand instrument differences when reproducing panels or running samples on multiple instruments - Differences in optical configurations, even within the same model instrument, can influence data 	<ul style="list-style-type: none"> - Confirm instrumentation optics are the same when using multiple instruments - When replicating a panel, confirm the fluorophores are optimally excited and emission is optimally collected - Try to collect longitudinal data on a dedicated instrument 	29, 31
			29–32

(Continues)

Table 1. Continued

WORKFLOW	CONSIDERATIONS/PITFALLS	RECOMMENDATIONS	REFERENCES
Proper instrument quality control	- Inadequate instrument cleaning and quality control can impact the stability of the instrument, which can greatly influence the reproducibility of data collected over long periods of time	- Use application settings or MFI target voltages to help normalize instrument variance over time - Use core facility technical support to ensure proper instrument quality control	
Instrument setup and optimal use			
Optimize detectors	- It is critical to ensure signal is detected within the linear range of detection of the PMT	- Optimize detector gains to ensure the positive peak is within the linear range and the negative peak is above noise	33–35
Compensation controls	- Computer-assisted compensation can help reduce user-introduced bias and improve reproducibility	- Capture beads can be helpful in providing bright signal for computer-assisted compensation.	35, 36
Panel design			
Tenants of appropriate multicolor panel design	- Considerations to improve panel design can help elucidate rare populations.	- Fluorochrome brightness, antigen density and coexpression and cell frequency should all be considered.	33, 36–39
FMO controls			
Gating controls	- Fluorescence spillover can result in “spread of the data” making it difficult to visually estimate populations	- FMO controls provide a strategy to define gating boundaries	35, 36
Data acquisition protocols			
SOPs for data acquisition	- Minimizing variability over multiple acquisitions by normalizing with controls. - Protocols should describe timing of sample acquisition as well as file acquisition size to account for cell frequency	- Employ internal biological controls to monitor intra-assay variability - MFI fluctuations can be monitored with eight peak beads and fixed PMT voltages - Ensure consistency in protocol timing	35–37
Data analysis			
Standardization of acquisition protocols	- Manual gating introduces experimenter bias and is considered a large variable in standardized experiments - Manual gating of high-order data is time-consuming	- Use computational analysis tools to increase efficiency and minimize bias - Centralize analysis to one location to minimize variability	35, 36, 39, 40
Mass cytometry			
Standardization of analysis and interpretation	- Background and gradient distributions can make it difficult to identify positive populations - Sources of background: signal interference due to overlap	- Regular instrument calibration and performance checks are required to ensure optimal conditions. CV value is an important metric to assess accuracy of detection.	50–52, 57, 58

(Continues)

Table 1. Continued

WORKFLOW	CONSIDERATIONS/PITFALLS	RECOMMENDATIONS	REFERENCES
	<ul style="list-style-type: none"> between mass channels; Isotope interference - Signal detection accuracy can change over time and create variability across experimental runs 	<ul style="list-style-type: none"> - The use of tuning solutions and calibration beads with known standard elements allow performance tracking over time - Barcoding samples and pooling for batch analysis can help to minimize assay-related inter-sample variability - Use of Mass-minus-one (MMO) controls is a viable strategy to resolve signal from background 	
Panel Design	<ul style="list-style-type: none"> - Panel design must take into account potential overlap between channels 	<ul style="list-style-type: none"> - Computational tools such as Maspar Panel Designer (Fluidigm) help to predict channel overlap and provide a platform for optimizing panel design - Test panel on control sample to ensure target populations can be resolved 	47,48, 50, 52
Antibody cocktail preparation and storage	<ul style="list-style-type: none"> - Conjugating antibodies in house often leads to increased levels of trace contaminants, thus decreasing overall purity - Improper storage of concentrated antibody cocktails leads to antibody aggregation and increased background signal 	<ul style="list-style-type: none"> - Antibody mixes can be stored for 3–5 days at 4°C - Recent studies show that antibody cocktails are stable for an extended time when stored at temperatures ≤ -80°C. 	28

time and with increased use (50,51). This can be a significant source of variability as it causes experimental noise between sample runs, altering staining patterns. This is of special concern when analyzing clinical samples that must be compared together. One strategy that may allow for more precise comparisons is to barcode individual samples which can then be pooled for batched acquisition. Validated methods that employ different permutations of a panel of metals to create unique barcodes that are assigned to individual samples are widely applied in mass cytometry (51–54). Barcoded samples can be stained and run together, and later be deconvoluted prior to analysis. One important consideration is that sample sizes must be relatively similar in order to ensure all pooled samples have the same level of coverage. Furthermore, for low cell-count samples, our recommendation is that these are run individually, and not barcoded. Barcoding protocols often introduce additional washes that are inevitably accompanied by some loss of cells.

Instrument calibration is another critical aspect to consider. Daily calibration is required to ensure optimal running conditions and minimize problems that may interfere with signal

detection and affect instrument accuracy (55). Oxidation due to plasma ionization of isotopes is one such example. Using a tuning solution for calibration helps to minimize this effect (49). It is also critical that regular checks for instrument performance are implemented, as changes can occur even between sample runs within the same day. Calibration beads manufactured by Fluidigm (EQ Four Element Calibration Beads) contain known standards of four elements—cerium, europium, holmium, and lutetium—at natural abundance, and are recommended for daily instrument calibration. EQ bead-derived signal can be used to generate several metrics that allow operators to track instrument performance and to monitor changes between runs. An important metric is the coefficient of variance (CV), which represents the instrument precision of detection of repeated measurements (26,56). User-defined thresholds are typically set for acceptable CV values. We and others use a CV of more than 20 (26). An additional advantage to using EQ calibration beads is that having them as a continuous reference throughout each sample run allows for batch-normalization of raw files. This strategy may be helpful in mitigating variability issues across instruments and even instruments operated at different sites (26).

Analysis of mass cytometry data is also an area for which clear guidelines have not yet been defined. Analysis of high-dimensional data comprising 30+ markers is complex and a standardized analysis method has not yet been established. The use of computational tools (e.g., software packages such as Cytokit, and Cyt, or analysis platforms such as Cytobank) for supervised or unsupervised clustering of data is common among mass cytometry users. Many of these tools employ tSNE, a dimensionality reduction algorithm that allows for visualization of data in two dimensions, while conserving the high-dimension structure of the data (57–59). Other popular clustering algorithms are Phenograph, SPADE, and X-Shift, which stratify all events into subpopulations with distinct gene expression signatures (58,59).

CLOSING REMARKS

This is an exciting time for immuno-oncology research. The field is prolific with high-quality work that has led to remarkable discoveries. The 2018 Nobel Prize in Physiology and Medicine, awarded to Drs. James Allison and Tasuku Honjo for their ground-breaking work in immunotherapy, is further evidence that this is a game-changing approach in the treatment of cancer. Technology is, likewise, experiencing tremendous advancement in order to support the growing needs of the field. As scientists entrusted to apply these new tools to translational research and clinical studies, it is incumbent upon us to maintain the highest standards of quality and rigor to ensure the reproducibility of our data. As we described in this manuscript, we must consider a long list of variables and how they affect the reliability and reproducibility of our work (Table 1). Each must be carefully optimized, planned, and executed in order to confidently interpret experiment outcome. Although no standardized protocol exists within the field, concerted efforts toward cytometry standardization are well underway, as demonstrated in the initiatives led by the International Society for Advancement of Cytometry and extensive work by EuroFlow in the last decade (60,61).

LITERATURE CITED

1. Riedel S. Edward Jenner and the history of smallpox and vaccination. *Proc (Bayl Univ Med Cent)* 2005a;18(1):21–25.
2. Boyiadzis MM, Dhodapkar MV, Brentjens RJ, Kochenderfer JN, Neelapu SS, Maus MV, Porter DL, Maloney DG, Grupp SA, Mackall CL, et al. Chimeric antigen receptor (CAR) T therapies for the treatment of hematologic malignancies: Clinical perspective and significance. *J Immunother Cancer* 2018b;6(1):137.
3. Bhoj VG, Arhontoulis D, Wertheim G, Capobianchi J, Callahan CA, Ellebrecht CT, Obstfeld AE, Lacey SF, Melenhorst JJ, Nazimuddin F, et al. Persistence of long-lived plasma cells and humoral immunity in individuals responding to CD19-directed CAR T-cell therapy. *Blood* 2016b;128(3):360–370.
4. Wolchok JD, Hodi FS, Weber JS, Allison JP, Urba WJ, Robert C, O’Day SJ, Hoos A, Humphrey R, Berman DM, et al. Development of ipilimumab: A novel immunotherapeutic approach for the treatment of advanced melanoma. *Ann N Y Acad Sci* 2013a;1291:1–13.
5. Yuan J, Ginsberg B, Page D, Li Y, Rasalan T, Gallardo HF, Xu Y, Adams S, Bhardwaj N, Busam K, et al. CTLA-4 blockade increases antigen-specific CD8(+) T cells in prevaccinated patients with melanoma: Three cases. *Cancer Immunol Immunother* 2011a;60(8):1137–1146.
6. Riedhammer C, Halbritter D, Weissert R. Peripheral blood mononuclear cells: Isolation, freezing, thawing, and culture. *Methods Mol Biol* 2016;1304:53–61.
7. Tsuji K, Ojima M, Otabe K, Horie M, Koga H, Sekiya I, Muneta T. Effects of different cell-detaching methods on the viability and cell surface antigen expression of synovial mesenchymal stem cells. *Cell Transplant* 2017;26(6):1089–1102.
8. Angel S, von Briesen H, Oh YJ, Baller MK, Zimmermann H, Germann A. Toward optimal cryopreservation and storage for achievement of high cell recovery and maintenance of cell viability and T cell functionality. *Biopreserv Biobank* 2016;14(6):539–547.

9. Zhang W, Nilles TL, Johnson JR, Margolick JB. The effect of cellular isolation and cryopreservation on the expression of markers identifying subsets of regulatory T cells. *J Immunol Methods* 2016;431:31–37.
10. Lemieux J, Jobin C, Simard C, Néron S. A global look into human T cell subsets before and after cryopreservation using multiparametric flow cytometry and two-dimensional visualization analysis. *J Immunol Methods* 2016;434:73–82.
11. Weinberg A, Song LY, Wilkening C, Sevin A, Blais B, Louzao R, Stein D, Defechereux P, Durand D, Riedel E, et al. Optimization and limitations of use of cryopreserved peripheral blood mononuclear cells for functional and phenotypic T-cell characterization. *Clin Vaccine Immunol* 2009;16(8):1176–1186.
12. Perdomo-Celis F, Salgado DM, Castañeda DM, Narváez CF. Viability and functionality of cryopreserved peripheral blood mononuclear cells in Pediatric dengue. *Clin Vaccine Immunol* 2016;23(5):417–426.
13. Hønge BL, Petersen MS, Olesen R, Møller BK, Erikstrup C. Optimizing recovery of frozen human peripheral blood mononuclear cells for flow cytometry. *PLoS One* 2017;12(11):e0187440.
14. Caldwell CW. Preservation of B-cell-associated surface antigens by chemical fixation. *Cytometry* 1994a;16(3):243–249.
15. Zwang NA, Ganesh BB, Cardenas KT, Chong AS, Finn PW, Perkins DL. An optimized protocol to quantify signaling in human transitional B cells by phospho flow cytometry. *J Immunol Methods* 2018a;463:112–121.
16. Hamot G, Ammerlaan W, Mathay C, Kofanova O, Betsou F. Method validation for automated isolation of viable peripheral blood mononuclear cells. *Biopreserv Biobank* 2015;13(3):152–163.
17. Maecker HT, McCoy JP, Nussenblatt R. Standardizing immunophenotyping for the human immunology project. *Nat Rev Immunol* 2012;12(3):191–200.
18. Barcelo H, Faul J, Crimmins E, Thyagarajan B. A practical cryopreservation and staining protocol for Immunophenotyping in population studies. *Curr Protoc Cytom* 2018;84(1):e35.
19. Reichard A, Asosingh K. Best practices for preparing a single cell suspension from solid tissues for flow cytometry. *Cytometry A* 2019;95(2):219–226.
20. Cossarizza A, Chang HD, Radbruch A, Akdis M, Andr i I, Annunziato F, Bacher P, Barnaba V, Battistini L, Bauer WM, et al. Guidelines for the use of flow cytometry and cell sorting in immunological studies. *Eur J Immunol* 2017;47(10):1584–1797.
21. Maciorowski Z, Chattopadhyay PK, Jain P. Basic multicolor flow cytometry. *Curr Protoc Immunol* 2017;117:5.4.1–5.4.38.
22. Taussig MJ, Fonseca C, Trimmer JS. Antibody validation: A view from the mountains. *N Biotechnol* 2018;45:1–8.
23. Bordeaux J, Welsh A, Agarwal S, Killiam E, Baquero M, Hanna J, et al. Antibody validation. *Biotechniques* 2010;48(3):197–209.
24. Bradbury A, Pl uckthun A. Reproducibility: Standardize antibodies used in research. *Nature* 2015;518(7537):27–29.
25. Berglund L, Bj orling E, Oksvold P, Fagerberg L, Asplund A, Szgyarto CA, et al. A genecentric human protein atlas for expression profiles based on antibodies. *Mol Cell Proteomics* 2008;7(10):2019–2027.
26. Leipold MD, Obermoser G, Fenwick C, Kleinstuber K, Rashidi N, McNevin JP, Nau AN, Wagar LE, Rozot V, Davis MM, et al. Comparison of CyTOF assays across sites: Results of a six-center pilot study. *J Immunol Methods* 2018;453:37–43.
27. Schulz AR, Baumgart S, Schulze J, Urbicht M, Gr utzkau A, Mei HE. Stabilizing antibody cocktails for mass cytometry. *Cytometry A* 2019;95:910–916.
28. Kalina T, Flores-Montero J, van der Velden VH, Martin-Ayuso M, B ottcher S, Ritgen M, et al. EuroFlow standardization of flow cytometer instrument settings and immunophenotyping protocols. *Leukemia* 2012;26(9):1986–2010.
29. Parks DR, Moore WA, Brinkman RR, Chen Y, Condello D, El Khettabi F, et al. Methodology for evaluating and comparing flow cytometers: A multisite study of 23 instruments. *Cytometry A* 2018;93(11):1087–1091.
30. Perfetto SP, Ambrozak D, Nguyen R, Chattopadhyay PK, Roederer M. Quality assurance for polychromatic flow cytometry using a suite of calibration beads. *Nat Protoc* 2012;7(12):2067–2079.
31. Holmes KL, Fontes B, Hogarth P, Konz R, Monard S, Pletcher CH, et al. International Society for the Advancement of Cytometry cell sorter biosafety standards. *Cytometry A* 2014;85(5):434–453.
32. Perfetto SP, Chattopadhyay PK, Wood J, Nguyen R, Ambrozak D, Hill JP, Roederer M. Q and B values are critical measurements required for inter-instrument standardization and development of multicolor flow cytometry staining panels. *Cytometry A* 2014;85(12):1037–1048.
33. Wang L, Hoffman RA. Standardization, calibration, and control in flow Cytometry. *Curr Protoc Cytom* 2017;79:1.3.1–1.3.27.
34. Maecker HT, Trotter J. Flow cytometry controls, instrument setup, and the determination of positivity. *Cytometry A* 2006;69(9):1037–1042.
35. Roederer M. Spectral compensation for flow cytometry: Visualization artifacts, limitations, and caveats. *Cytometry* 2001;45(3):194–205.
36. Nguyen R, Perfetto S, Mahnke YD, Chattopadhyay P, Roederer M. Quantifying spillover spreading for comparing instrument performance and aiding in multicolor panel design. *Cytometry A* 2013;83(3):306–315.
37. Ratei R, Karawajew L, Lacombe F, Jagoda K, Del Poeta G, Kraan J, et al. Normal lymphocytes from leukemic samples as an internal quality control for fluorescence intensity in immunophenotyping of acute leukemias. *Cytometry B Clin Cytom* 2006;70(1):1–9.
38. Rundberg Nilsson A, Bryder D, Pronk CJ. Frequency determination of rare populations by flow cytometry: A hematopoietic stem cell perspective. *Cytometry A* 2013;83(8):721–727.
39. Gutensohn K, Jessen M, Ketels A, Gramatzki M, Humpe A. Flow cytometric analyses of CD34+ cells with inclusion of internal positive controls. *Transfusion* 2012; 52(2):284–290.

40. Mair F, Hartmann FJ, Mrdjen D, Tosevski V, Krieg C, Becher B. The end of gating? An introduction to automated analysis of high dimensional cytometry data. *Eur J Immunol* 2016;46(1):34–43.
41. Mazza EMC, Brummelman J, Alvisi G, Roberto A, De Paoli F, Zanon V, et al. Background fluorescence and spreading error are major contributors of variability in high-dimensional flow cytometry data visualization by t-distributed stochastic neighboring embedding. *Cytometry A* 2018;93(8):785–792.
42. Lee JA, Spidlen J, Boyce K, Cai J, Crossbie N, Dalphin M, et al. MIFlowCyt: The minimum information about a flow Cytometry experiment. *Cytometry A* 2008a;73(10):926–930.
43. Minimum information about a flow cytometry experiment (MIFlowCyt) checklist (numbered in accordance with MIFlowCyt 1.0 document). *Cytometry A* 2010a;77(9):813.
44. Spidlen J, Breuer K, Brinkman R. Preparing a Minimum Information about a Flow Cytometry Experiment (MIFlowCyt) compliant manuscript using the International Society for Advancement of Cytometry (ISAC) FCS file repository (FlowRepository.org). *Curr Protoc Cytometry* 61:10.18.1–10.18.26
45. Nair N, Mei HE, Chen SY, Hale M, Nolan GP, Maecker HT, Genovese M, Fathman CG, Whiting CC. Mass cytometry as a platform for the discovery of cellular biomarkers to guide effective rheumatic disease therapy. *Arthritis Res Ther* 2015b;17:127.
46. Maecker HT, Lindstrom TM, Robinson WH, Utz PJ, Hale M, Boyd SD, Shen-Orr SS, Fathman CG. New tools for classification and monitoring of autoimmune diseases. *Nat Rev Rheumatol* 2012;8(6):317–328.
47. Newell EW, Sigal N, Bendall SC, Nolan GP, Davis MM. Cytometry by time-of-flight shows combinatorial cytokine expression and virus-specific cell niches within a continuum of CD8+ T cell phenotypes. *Immunity* 2012a;36(1):142–152.
48. Hines KM, Ford GC, Klaus KA, Irving BA, Ford BL, Johnson KL, Lanza IR, Nair KS. Application of high-resolution mass spectrometry to measure low abundance isotope enrichment in individual muscle proteins. *Anal Bioanal Chem* 2015; 407(14):4045–4052.
49. Takahashi C, Au-Yeung A, Fuh F, Ramirez-Montagut T, Bolen C, Mathews W, O'Gorman WE. Mass cytometry panel optimization through the designed distribution of signal interference. *Cytometry A* 2017a;91(1):39–47.
50. Leipold MD, Maecker HT. Mass cytometry: Protocol for daily tuning and running cell samples on a CyTOF mass cytometer. *J Vis Exp* 2012;69:e4398.
51. Spitzer MH, Nolan GP. Mass Cytometry: Single cells, many features. *Cell* 2016;165(4):780–791.
52. Behbehani GK, Thom C, Zunder ER, Finck R, Gaudilliere B, Fragiadakis GK, Fantl WJ, Nolan GP. Transient partial permeabilization with saponin enables cellular barcoding prior to surface marker staining. *Cytometry A* 2014;85(12):1011–1019.
53. Bodenmiller B, Zunder ER, Finck R, Chen TJ, Savig ES, Bruggner RV, Simonds EF, Bendall SC, Sachs K, Krutzik PO, et al. Multiplexed mass cytometry profiling of cellular states perturbed by small-molecule regulators. *Nat Biotechnol* 2012;30(9): 858–867.
54. Mei HE, Leipold MD, Maecker HT. Platinum-conjugated antibodies for application in mass cytometry. *Cytometry A* 2016;89(3):292–300.
55. Kimball AK, Oko LM, Bullock BL, Nemenoff RA, van Dyk LF, Clambey ET. A Beginner's guide to analyzing and visualizing mass cytometry data. *J Immunol* 2018b;200(1):3–22.
56. Krutzik PO, Clutter MR, Trejo A, Nolan GP. Fluorescent cell barcoding for multiplex flow cytometry. *Curr Protoc Cytom* 2011;55:6.31.1–6.31.15.
57. Finck R, Simonds EF, Jager A, Krishnaswamy S, Sachs K, Fantl W, et al. Normalization of mass cytometry data with bead standards. *Cytometry A* 2013;83(5):483–494.
58. Amir E-A, Davis KL, Tadmor MD, Simonds EF, Levine JH, Bendall SC, et al. viSNE enables visualization of high dimensional single-cell data and reveals phenotypic heterogeneity of leukemia. *Nat Biotechnol* 2013a;31(6):545–552.
59. DiGiuseppe JA, Cardinali JL, Rezuze WN, Pe'er D. PhenoGraph and viSNE facilitate the identification of abnormal T-cell populations in routine clinical flow cytometric data. *Cytometry B Clin Cytom* 2018a;94(5):588–601.
60. Kalina T, Brdickova N, Glier H, Fernandez P, Bitter M, Flores-Montero J, van Dongen JJM, Orfao A. Frequent issues and lessons learned from EuroFlow QA. *J Immunol Methods* 2018.
61. Nováková M, Glier H, Brdicková N, Vlková M, Santos AH, Lima M, Roussel M, Flores-Montero J, Szczepanski T, Böttcher S, et al. How to make usage of the standardized EuroFlow 8-color protocols possible for instruments of different manufacturers. *J Immunol Methods* 2017.