his evaluation of markers expressed, stability, and affordability. To conclude the presentation session, Virginia Litwin demonstrated that control cells were found to be important reference material to monitor variation between daily runs, assays, instruments, analysts, and to identify reagent issues.

Discussion

Controls are used to establish a baseline to compare results against introduced variables in cytometry. Control cells can be used as positive and procedural controls for flow cytometric applications such as phenotyping of leukemia and lymphoma, immune monitoring and drug discovery. Scientists have been exploring cell preservation techniques to achieve both consistency and performance for a few decades (68,76,77,83). This workshop covered all current products as well as laboratory-developed approaches.

The following key technical issues were discussed during the panel session:

- Assay values and expected ranges of percentage positive cells for specified markers on all instrument platforms are desired. This is important for the lot-to-lot transfer of the QC tests as well to be able to use different instrument platforms. However, current expected ranges provided by the supplier are often too wide to provide practical details for specific markers on specific instruments regarding a particular lot. This remains a challenge for the field due to the complexity of both markers and instrumentation.
- Large lots of control materials are desired in order to standardize results across instruments and laboratories; an alternative was suggested of comparing the lot in use with the incoming lot when a large lot of control materials is not readily available.

Control cells are important yet remain challenging for the field of flow cytometry. Forming a consortium, for example, Flow Cytometry Quantitation Consortium, to visit the topic annually will enable advancement in the field and establish collaborative efforts to share best practices. The Control Cell Workshop Committee is committed to advancing the field forward to provide better quality of research and clinical results for years to come.

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WS13: Building Measurement Assurance in Flow Cytometry

Lili Wang, Stephen Perfetto, Robert Hoffman, John Elliott, Sheng Lin-Gibson, Steven Bauer, Heba Degheidy, Judith Arcidiacono, Virginia Litwin.

Introduction and Aims

This report summarizes key findings, including the need for high quality reagents, reference standards or materials, and documentary standards.[1] Advances of cell-based therapeutics have increased the need for high quality, robust, and validated measurements for cell characterization. Flow cytometry has emerged as an important platform due to its ability to rapidly characterize heterogeneous cell populations and subpopulations. For example, flow cytometry was critical for establishing identity, purity, and potency for CAR-T cell manufacturing (78); and associated data supported the approval of Biological License Applications (BLA)[2] by the U.S. Food and Drug Administration (FDA) and the approval by the European Medicines Agency (EMA). In addition, multiparameter cytometric measurements are routinely carried out in vaccine and cancer research, clinical diagnosis, and immunotherapies. However, challenges remain with respect to measurement confidence and comparability of results, hindering critical decision-making based on flow cytometry data.

As a part of the joint efforts to develop standards to support innovation and translation of regenerative medicine products, NIST and FDA hosted a workshop titled "Building Measurement Assurance in Flow Cytometry" in October 2017. Approximately 70 experts representing industry, academia, and government agencies attended the workshop. Most participants completed a pre-workshop survey (Supplementary Information WS13_SI1). The survey indicated that the most industrial and academic participants used flow cytometry as a release assay and/or for monitoring of cell manufacturing process (Supplementary Information WS13_SI2). The workshop agenda and presentations are available online (73).

WS13 was held in April 2018 to continue dialogue with respect to broader cytometer users for identifying application challenges and potential solutions. Nearly 100 participants joined the workshop, where the majority use flow cytometry for basic medical research, contract research organizations (CROs) under the category of 'other,' or clinical disease diagnosis (Supplementary Information WS13_SI2).

The different user perspectives from the two workshops enable us to identify common measurement challenges and actionable solutions for obtaining sufficient assurance for the intended flow cytometric measurement.

Methods

For the joint NIST-FDA workshop (73), a pre-workshop survey (Supplementary Information WS13_SI1) was used to gauge participants' experiences and identify common challenges. In addition to introductory content, the one-day

[1] A "documentary standard" is a classification, guide, practice, specification, terminology standard, or test method developed and established by knowledgeable people according to agreed principles of consensus, such as those of ASTM (American Society for Testing and Materials) International.

[2] A biologics license application (BLA) is defined by the U.S. Food and Drug Administration (FDA) as follows: The biologics license application is a request for permission to introduce, or deliver for introduction, a biologic product into interstate commerce (21 CFR 601.2). The BLA is regulated under 21 CFR 600–680.

workshop covered three general topics: 1. standardization strategies, 2. biological and nonbiological reference materials, and 3. best practices and use cases from two CROs and the clinical laboratory at National Institute of Health (NIH). The first two topics were followed by discussions on calibration and reference materials. The last session was followed by guided discussion on strategies for moving forward in the areas of quantitation, gating, and best practices.

For WS13, the content of the NIST-FDA workshop, including the survey and presentations, was provided as the pre-workshop information on the CYTO 2018 webpage. The workshop consisted of four 10-min presentations that covered measurement assurance concepts, instrument standardization and detector operating voltage optimization, cell-based reference controls, and information regarding a CLSI effort to develop a guidance document for flow cytometry validation (Supplementary Information WS13_SI3). The workshop presentations were followed by live polling of selected survey questions and discussions.

Survey responses from both workshops as well as live polling results from the WS13 have been combined to generate a single report identifying common needs and gaps (Supplementary Information WS13_SI2).

Results/Outcome

The most widely reported survey respondents used multiparameter flow cytometry assays (either 6–12 colors or > 12 colors) to investigate blood samples or cell lines (Graph #3 of Supplementary Information WS13_SI2). The most common objectives were to quantify specific cell subsets and characterize the mean fluorescence intensity (MFI) of antigens they express (Fig. 4A). A generalized flow cytometry measurement process for an individual laboratory consists of roughly six major steps: sample collection, cell processing and staining, cytometer QC, calibration and standardization, compensation, quantitative measurement, and data analysis and reporting as shown in the first column of Table 4. Some survey questions were intended to highlight participants' assurance controls for each process step. Most respondents used a combination of beads and cells to perform cytometer compensation as well as manual gating for data analysis (Graph #8 and #9 of Supplementary Information WS13_SI2).

The most widely reported responses indicated that cytometer standardization is of a high priority to their organizations due to the use of different cytometer platforms or at different sites (Graph #12 of Supplementary Information WS13_SI2). In spite of the fact that close to half of the respondents previously participated in interlaboratory studies comparing flow cytometry results, most are not satisfied with or are uncertain about compatibility across instrument platforms or sites (Graph #13 of Supplementary Information WS13_SI2). The three biggest obstacles for obtaining high confidence assay results are 1. a lack of high quality reference materials, 2. lack of confidence in the procedures from standardization/inter-laboratory studies, and 3. uncertainty associated with specimen quality and/or preanalytical processes (Fig. 4B). A key workshop finding is the need for high quality reference standards as shown in Figure 4C. There remain significant needs for common control materials, documentary standards, well-defined measurement procedures, and proficiency training studies as well (Fig. 4C).

Discussion/Conclusion/Perspectives

Measurement assurance requires a systematic approach that informs the confidence in a measurement, and hence the comparability of results. Reference materials, process controls, experimental design, quality by design (QbD), assay validation, and interlaboratory comparisons are examples of measurement assurance strategies needed for achieving traceability and uncertainty qualification of the results (79,80).

The primary goal for utilizing flow cytometry (Fig. 4A) is to measure specific cell subsets and antigen expression; yet quantification via flow cytometry remains a challenge. Significant sources of variability can be introduced at each of the six major steps in the flow cytometry measurement process as well as entitle assay performance/standardization across different locations (Table 4). Measurement assurance strategies, particularly the use of reference methods and materials, could effectively minimize these sources of variability (Supplementary Information WS13_SI4). Workshop experts agreed that appropriate use of these and other strategies can improve their confidence in measurements made by Flow Cytometry (Fig. 4B).

As multiparameter flow cytometry assays are increasingly used to simultaneously characterize and quantitate multiple cell subsets and their antigen expression, a primary requirement is that all detector channels have sufficient sensitivity and resolution to identify and measure the full range of cell surface antigen expression levels, especially the antigens with low abundance. In addition, fluorescence channels should be operated within a linear range (nominally within 2%) to ensure correct spectral compensation. To address these concerns, well established practices and methods are recommended. A set of multi-intensity beads (unstained and stained, including dimly stained fluorescent beads) with defined fluorescent intensity units is deemed adequate to characterize cytometer performance with respect to linearity, dynamic range, electronic noise, sensitivity (Q), and background (B) (81,82). The (CV) for the brightest microsphere population can be used to assess the laser alignment to the sample core stream. The fluorescence intensity unit can be expressed or normalized via the numbers of equivalent reference fluorophores (ERF) assigned by NIST (83). The use of a pulsed LED source can provide consistent evaluations of Q and B without added variability inherent to beads and optical alignment (82). Q and B provide the flow cytometer's ability to resolve dim fluorescent populations from negative populations (84). In this overall scheme, assigning ERF values traceable to NIST SRM 1934 (85) is essential for the standardization of cytometer performance characteristics and enables comparability in multi-instrument and/or multisite longitudinal studies, which should address attendee concerns for adequate assurances (86). Considering that users largely rely

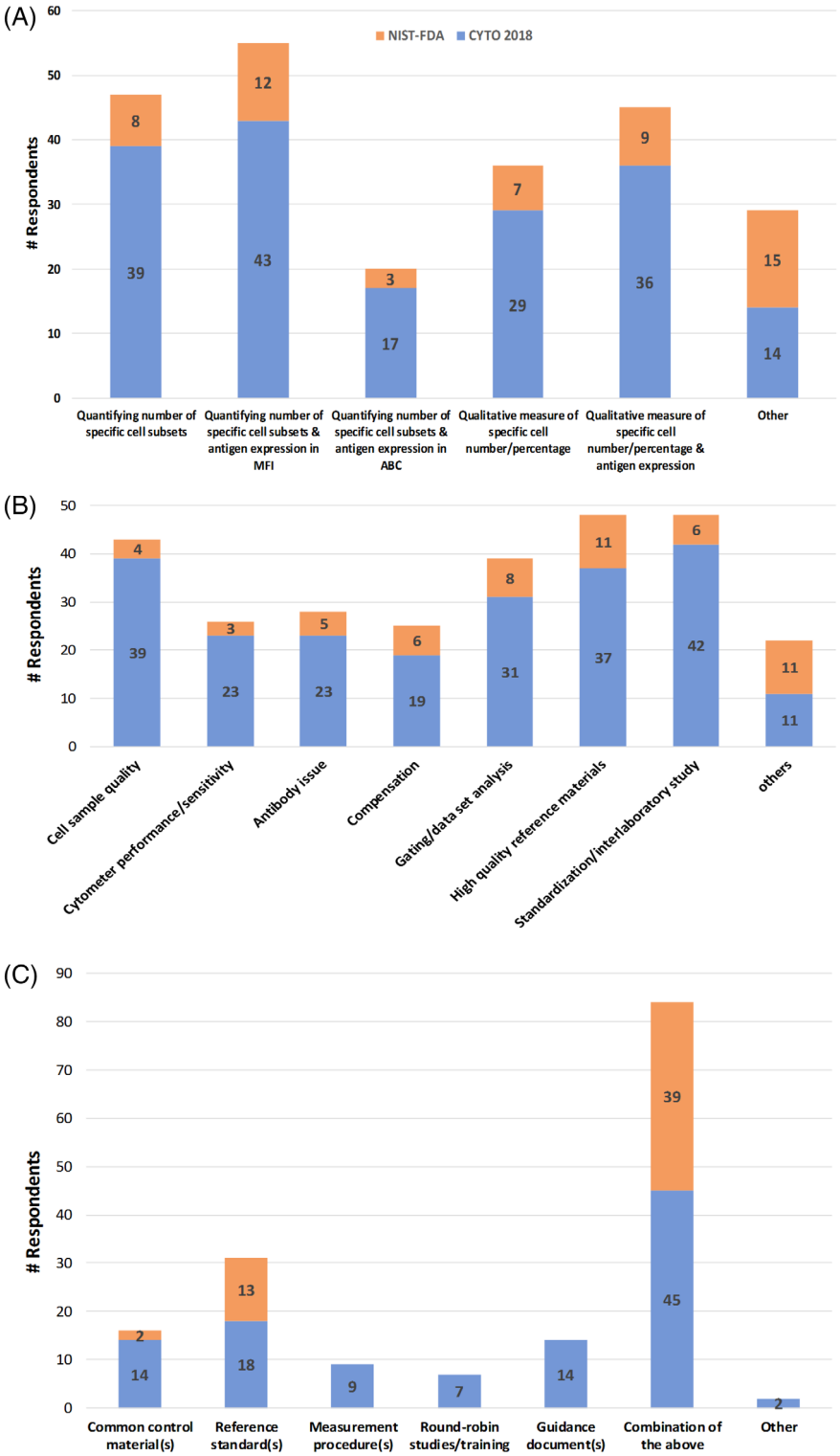

Figure 4. Illustration of the survey results from CYTO 2018 WS13 (Blue) and NIST-FDA Workshop (Orange) on (A) objectives of flow cytometry assays, (B) the biggest obstacle in flow cytometry assays performed, and (C) tools that would help most to achieve measurement assurance.

on built-in software for instrument QC and sensitivity characterization, and there are very few cytometer QC beads with fluorescence intensity values assigned traceable to available NIST standards (Graph #6 and #7 of Supplementary Information WS13_SI2), significant improvements can be made through a joint effort from instrument

Table 4. Flow cytometry measurement process, sources of variability, reference methods, control materials, and procedures

MEASUREMENT PROCESS	SOURCES OF VARIABILITY	REFERENCE METHODS, MATERIALS, AND PROCEDURES
Sample collection	• Fixed versus fresh samples • Anticoagulant • Cell count and viability • Cell debris	• Counting bead reference • Cell (live/dead) reference control material(s)
Cell processing and staining	• Antibody quality: fluorophore labeling quality, binding affinity, and titer • Cell debris	• Method(s) for evaluating antibody quality • Cell reference material(s)
Cytometer QC, calibration and, standardization	• Linearity, sensitivity, and resolution • Instrument threshold and voltage setting • Volumetric cytometers: volume calibration	• Bead reference materials • Beads or beads/LED methods
Compensation	• Linearity range • Choice of labeling fluorophores/panel design	• Compensation beads • Cell reference material(s)
Quantitative measurement	• Tube-to-tube variability of counting beads • Cell reference material(s) with known cell concentration and/or antigen expression • Assay format (single tube or separate tubes)	• Reference counting beads • Cell reference standard(s)
Data analysis and reporting	• Number of events collected • Population gating • Underlying assumptions of automated software	• Reference cell FMO (fluorescence minus one) controls • Cell reference standard(s)
Assay performance/ standardization across locations	• All issues described above • Different cytometer operators • Different assay procedures	• Reference materials and methods described above • Standardized procedure(s) • Round-robin study • Training/certification

manufacturers and users toward cytometer standardization. Furthermore, once more traceable standards are available, meaningful correlations of antigen density can be quantitated by all labs.

Very few cell-based reference materials are currently available. One is *CD34+ Cell Enumeration System Suitability* from the United States Pharmacopeia (USP), which has a certified value for CD34+ cell concentration of 16 to 34 cells/μL via flow cytometry (87). This material is made from fixed and lyophilized peripheral blood cells and provides a stable material for CD45 and CD34 staining and serves as an *in process* control for measurements needed for clinical bone marrow and related stem cell transplantation. The other material is the *FITC-CD4 Positive Control Cells* from the National Institute for Biological Standards and Control (NIBSC) with a certified value for CD4+ T cells of 212.1–438.1 cells/μL by flow cytometry (88). This material contains lyophilized human PBMC prelabeled with a FITC conjugated monoclonal antibody and serves as a positive control for CD4+ T cell enumeration. Furthermore, this material is CE marked for use as an IVD within the EU member states and EEA countries. Because both cell reference materials are made from human blood, they contain clinical analytes of interest and are appropriate controls for detection of cell debris, testing different antibody clones to the same antigen, inter- and intra-laboratory performance monitoring, and training and qualifying new users for their intended use.

Additional cell-based reference materials are needed for counting of other cell types and for quantifying antigen expression in antibodies bound per cell (ABC) (89). The ABC is an instrument independent unit unlike the instrument-dependent MFI. In particular, cell reference standards with well characterized antigen expression (90) are greatly needed for advanced cell manufacturing and cell therapies. NIST and FDA along with industrial partners and user communities are actively collaborating on projects to address these standard needs.

Timely updates on current efforts to develop control materials, documentary standards and methods can be found in WS09 on "Control Cells or Not" reported in the same journal issue, a pending CLSI guideline on "Validation of Assays Performed by Flow Cytometry," and novel computational methodologies for unbiased analysis of complex cytometry data (91). In addition, discussions on antibody quality for

flow cytometry occurred between manufacturers, users, and CYTO meeting management in CYTO 2018. As a result, future actionable steps will be forthcoming. All these activities will ultimately lead to building measurement assurance in flow cytometry (Table 4 and Supplementary Information WS13_SI4).

WS11: Flow Cytometry Application in Multi-Center Global Clinical Studies: The Importance of Standardization and Harmonization

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Introduction and Aims

Flow Cytometry (FCM) is a powerful technique with applications from basic biology and exploratory endpoints to critical safety and efficacy decision-making during drug development. FCM assays should be developed and validated in accordance with recommendations presented in seminal papers (67,92). These support best practices for performing FCM in a regulated environment and are cited in feedback from the experience of cytometrists within the European Bioanalysis Forum (68).

WS11 gave opportunity to present and discuss key aspects considered to be critical to achieving standardization and harmonization of FCM applications in multicenter clinical studies. The attendees were currently involved in, or considering, implementing sophisticated FCM-based assays in multisite clinical testing for global clinical trials in regulated laboratories and among the workshop participants were manufacturers of FCM controls, reagents, and analysis software. Here we report on the survey findings and outcome of the live discussions regarding current challenges and possible solutions. We highlight some of the most advantageous approaches to reduce both the variability and the time required to produce results.

Methods

The online survey and live discussion (Supplementary Information WS11_SI1 for details) were conducted across five categories. The workshop started with a general introduction to the topic (Supplementary Information WS11_SI2), followed by group discussions in each subtopic. Each of the categories, including the live discussions, was handled by one of the WS chairs and the most relevant aspects summarized. Many of the results from the live discussions were in agreement with the online results. In the following sections, we present the main outcomes for each topic and discuss major findings and recommendations.

Results/Outcome

Considerations for specimen type. The most common specimen types involved are peripheral blood and PBMC. Reasons for selection were biological relevance (48.7%), followed by clinical study setup and the ease of sampling/repeat sampling. Stability is also considered as an important factor in specimen selection (10%). To stabilize blood samples, 49% of respondents rely solely on anticoagulants. Blood stabilizers of different types are used by 40% of respondents.

WS participants identified robust sample transport, low stability of rare specimens, and limited availability of samples from diseased individuals for stability testing as major issues; largely driven from the fact that diseased samples often manifest different behavior to healthy samples.

Challenges and opportunities of local versus centralized analysis. There was an equal separation between those shipping samples to multiple testing sites for processing and acquisition, and those sending their samples to a single test lab (40% each). Fifteen percent of respondents used a mixed model approach and 5% used local clinical site facilities; >70% considered the main advantage of local testing was the ability to overcome limitations in specimen stability, or reduced data turnaround (30%). The top ranked advantage of centralized sample testing was the ability to process samples using the same protocol and measuring on the same instruments. Major challenges for centralized sample testing were considered to be sample logistics and stability (30%), instrument alignment between testing sites (30%) and protocol harmonization for sample processing (22%).

When using local, or multiple analytical facilities the discussion clustered around consistency in 1. personnel (training, experience, compliance with local quality systems); 2. instrumentation (calibration, monitoring, and comparability between different analyzers); and 3. concerns relating to data management and reporting. The use of the 'mixed model' approach was considered to be a pragmatic compromise.

Critical steps for assuring process harmonization and instrument standardization. Standard Operating Procedures (SOPs) were agreed to be the main factor in process harmonization assurance followed by periodical laboratory cross testing (75%). Only 5% ensure between-lab process harmonization by involving robotic/automated sample preparation at each testing location. Nine percent of respondents do not monitor inter-instrument standardization. The competency of technical staff is preferably assessed by internal training prior to or throughout the study, followed by internal blind testing/gating assessment. Only 12% indicated participation in an external Quality Assurance (QA) program.

There is a need for guidelines describing standardization and monitoring of instruments alignment. Users indicated that the procedure for instrument standardization is complex and not well described by producers and more could be done in these areas by the instrument vendors. The majority of participants indicated the importance of well written SOPs and continuous staff training in successful process harmonization.

Control of critical reagents and within study controls in multisite studies. For QC, the majority use beads (41%) and/or stabilized blood (38%) followed by fresh blood and frozen cells for each run (59%) or daily (24%). Presence of all