Flow Cytometry in Cancer Immunotherapy: Applications, Quality Assurance, and Future

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25.1 Introduction

Cancer immunotherapy seeks to elicit or augment the antitumor immune response in a patient with detectable tumor or remaining tumor cells in the adjuvant setting in order to enlist the help of the patient's own immune system for tumor control. In this context, active cancer immunotherapy refers to the use of cytokines (e.g., IL-2 in melanoma

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and renal cell carcinoma), immunomodulatory monoclonal antibodies (e.g., antibodies (Abs) against CTLA-4, PD-L1, and PD-1), cell-based products (e.g., sipuleucel-T for metastatic hormone-refractory prostate cancer), or experimental vaccines based on various antigen (Ag) formats. When evaluating immunotherapies, particularly in experimental settings, it is essential to monitor the immune response elicited by the treatment. Immunomonitoring delivers evidence of immunogenicity, guides the choice and dosage of antigens, assesses the effects of immune modulators and therapy combinations, and has the potential to reveal early biomarkers of clinical efficacy. In this respect, immunomonitoring is helpful for rational clinical development and supplements clinical efficacy parameters such as disease-free period or survival, which are often only available at later clinical trial stages.

In view of their role in the anticancer immune response, the quantity and quality of tumor antigen-specific effector $CD4^+$ and $CD8^+$ T cells are of particular interest. In addition, the role of immune regulatory cells, e.g., regulatory T cells (Tregs) or myeloid-derived suppressor cells (MDSCs) that can suppress the effector immune response to a tumor, is increasingly recognized. Informative analysis requires multiple markers for identification of phenotypic and functional properties and the accurate quantification of cell subsets that are typically found at relatively low frequencies in the peripheral blood. These characteristics call for an assay that is multiparametric, robust, and sensitive enough to characterize rare individual cells.

The canonical multiparameter assay for the characterization of single cells in solution is polychromatic flow cytometry, and hence, it is ubiquitously used for immune monitoring in preclinical tumor immunology and in cancer immunotherapy trials. While the first fluorescence-based flow cytometer dates back to 1968, the past several years have brought major advances in cytometer technology, reagents, range of applications, automated analysis techniques, and minimal information standards. Much has also been learnt from large-scale proficiency testing programs about the challenges facing th use of increasingly complex flow cytometry assays, and what needs to be done to harmonize the assays across multiple laboratories. This chapter describes the main flow cytometry methods being applied in cancer immunotherapy, with an emphasis on recent progress in the field, challenges associated with quality control, its promise to reveal biomarkers of clinical efficacy, and further developments that are likely to be rapidly implemented in routine cancer immunology.

25.2 Main Flow Cytometry Assays in Cancer Immunotherapy

Together with immunohistochemistry, immunophenotyping by flow cytometry is probably the most commonly used assay to investigate immune and other cell subsets of interest in cancer immunology. Flow cytometry distinguishes human immune cells via a combination of physical properties and fluorescent markers such as labeled monoclonal antibodies (mAbs) targeted against cell-specific molecules. Physical properties measured by the cytometer are forward scatter light (FSC) which is roughly proportional to the cell size and side-scattered light (SSC) which reflects the granularity of cells. Markers targeted by fluorescent mAb are mostly categorized in Clusters of Differentiation (CD) nomenclature [1]. To date, the Human Cell Differentiation Molecules Association (http://www.hcdm.org) has indexed more than 360 CD markers. Commonly used "basic" CD markers are CD3, CD4, and CD8 for T-cell subsets, CD19 for B cells, CD14 for monocytes, CD11c for subsets of dendritic cells, CD56 for natural killer (NK) cells, and CD15 for granulocytes. In addition to whole blood and PBMC samples, enumeration of the number and frequencies of immune cell types can also be performed on single-cell suspensions obtained from tissues (for instance malignant tumors) [2, 3]. When analyzing tumors, further markers can be added to identify endothelial cells (CD31), fibroblasts (ER-TR7), epithelial cells (EpCAM, i.e., CD326), and particular tumor cells (e.g., CAIX for renal cell carcinoma).

Many cell populations can currently only be identified by the use of multiple mAb

simultaneously; this is the case for natural regulatory T cells (nTregs) [$CD4^+$/CD25high/$Foxp3^+$/CD127low or various subsets of MDSCs [4]. Polychromatic flow cytometry is also necessary to characterize the activation status, maturity, clonality, and differentiation status of T lymphocytes. Commonly used markers for this purpose include CD25, CD27, CD28, CD45RA/RO, CD69, CD137, and CD154, as well as antibodies to different TCR Vβ family members [5–9]. A combination of mAbs against activation markers and chemokine receptor (i.e., CCR7) can be used to identify naïve, effector memory, central memory, terminally differentiated effector memory (TEMRA), and memory T cells with stem cell-like features [10–12]. These differentiation stages are associated with changes in functional and proliferative properties [13], are altered in the elderly [14], and hence are relevant for adoptive transfer therapy and for possibly predicting response to vaccination in aging cancer patients. However, up to now, there is no gold standard for markers that are necessary and sufficient to identify most immune cell subsets; this is not surprising as our appreciation of the complexity and plasticity of human immune cell subsets is constantly evolving.

A major interest in immunotherapy clinical trials is to characterize the specificity of tumor antigen-specific T cells, most notably in settings of active immunotherapy with defined Ags. The most direct characterization of antigen specificity is via the use of HLA-peptide multimers, which bind directly to the peptide-specific T-cell receptors (TCR). First described more than 15 years ago [15], the HLA-class I multimer assay currently serves as a versatile tool for enumerating, characterizing, and following $CD8^+$ T cell immune responses, and staining protocols are broadly available [16–18]. Hence, HLA-multimers are widely used to monitor T-cell responses, especially in the context of peptide-based vaccination approaches [19–22]. They can easily be combined with mAb panels to determine the phenotype and differentiation status of antigen-specific $CD8^+$ T cells [23–25]. Limitations of HLA-multimers are that both the precise T-cell epitope (i.e., the exact amino-acid sequence of the peptide recognized by the TCR) and its HLA-restriction (i.e., the HLA-molecule which binds and presents the peptide to the TCR) must be known in advance. To date, there also remains a lack of general availability of class II multimers for $CD4^+$ T-cell detection [26].

Intracellular cytokine staining (ICS) is another common assay used for antigen-specific T-cell immune monitoring. It is the flow cytometric method of choice when HLA-multimers are not available, if the exact T-cell epitope is unknown, and for routine monitoring of $CD4^+$ T-cell responses. ICS enables monitoring of multiple effector functions of both $CD4^+$ and $CD8^+$ T-cell subsets [27–29], including polyfunctional T cells that have been associated with pathogen protection [30, 31]. A few groups have described polyfunctional T cells after cancer vaccination in patients, but whether these cells are associated with beneficial and long-lasting antitumor T-cell responses remains an open question [32, 33]. Optimized Ab combinations, protocols, and standardization approaches have been published [34–36], and ICS assays are widely used in clinical studies.

Cytotoxicity or proliferation assays, which have traditionally relied on the detection of radioactivity (i.e., ^{51}Cr release or ^{3}H thymidine incorporation) can also be conducted by flow cytometry. For assessment of killing activity, target cells (control and antigen-loaded cells or tumor cells expressing the antigen endogenously) are differentially labeled using fluorescent dyes (e.g., Paul Karl Horan (PKH) or 6-carboxyfluorescein diacetate succinimidyl ester (CFSE)) and incubated with the effector T cells to be tested. Apart from the obvious safety aspects over radioactivity-based assays, advantages of flow cytometry methods are that (1) several targets can be tested in the same tube; (2) as compared to a classical ^{51}Cr release assay, effector-target incubation time can be significantly prolonged (up to 24 h); and (3) the assay has been reported as being sensitive and effective even when low numbers of effectors are available [37, 38, 39]. Another approach to indirectly determine the cytotoxic capacities of T cells is the use of a mAb directed against CD107a (LAMP-1)

which becomes extracellularly detectable after cytotoxic granules have fused with the cellular membrane (degranulation) [40]. For measuring proliferation by flow cytometry, effector cells are first labeled with fluorescent dyes (CFSE or other tracking dyes such as CellTrace™ reagents) and cultured for several days in the presence of relevant stimuli. Since the dyes are diluted from the mother to the daughter cells, the number of cell divisions is visible in the number of fluorescent peaks detected [41]. The frequency of proliferating cells can also be assessed directly *ex vivo* by staining of the proliferation-associated nucleus Ag Ki67, expressed at all phases of the cell cycle except the resting G_0 stage [4, 42]. These measurements of target killing or cell division by fluorescent dyes have rarely been used in large-scale vaccine studies so far [38, 43], probably because they are time-consuming and require careful optimization and technical expertise to achieve reproducible results.

Finally, cell-free cytokine analysis can also be performed by flow cytometry with the use of multiplex beads, a method that has been recently adapted to meet GCLP standards [44–46]. The method uses mixes of beads of different size and fluorescence that are each coated with Abs specific for the different cytokines of interest. The soluble cytokines present in the sample (i.e., culture supernatant, serum, or plasma) bind to these Ab-coated beads, and a second Ab coupled to another fluorescent label is used to visualize the amount of bound cytokine. Simultaneous quantification of several soluble factors in one sample can be done by comparison to standard curves provided by the manufacturer, for example, to evaluate Th1/Th2 profiles [28]. The assay is as sensitive as ELISA, with detection limits in the range of 20 pg/mL for most cytokines, and can be even more sensitive when an enhanced sensitivity system is used.

The examples above clearly show that flow cytometry is a versatile tool for investigations of the phenotype, frequency, and functional properties of immune cell subsets. Furthermore, assays can often be combined for multiparametric probing of cell properties which is beneficial as precious patient samples are spared. However, the need for both robustness and sensitivity to detect tumor antigen-specific T cells and/or rare cell subsets poses specific challenges for the use of this complex tool in clinical research applications. This is addressed in the following sections.

25.3 Panel Development and Quality Assurance

Current state-of-the-art polychromatic flow cytometry in cancer immunotherapy involves multistep, multi-reagent assays followed by sample acquisition on sophisticated instruments that are able to capture up to 20 parameters per cell at a rate of tens of thousands of cells per second. Analysis of these data can be a challenge, as standard tools require multistep gating strategies and preselection of the parameter combinations to be investigated. Obtaining reproducible results from such a complex assay requires well-trained staff, stringent quality management, and detailed standard operating procedures (SOPs) for panel development, cytometer calibration, reagent qualification, sample preparation, use of appropriate technical and biological controls, and careful data analysis.

We start by considering the factors important to understand when developing a mAb staining panel. Target molecules in flow cytometry for cancer immunotherapy can have vastly different expression levels. While lineage markers such as CD45, CD3, or CD8 can be expressed at very high copy numbers per cell, some important markers such as transcription factors (e.g., FOXP3 for CD4 Tregs) or chemokine receptors (e.g., CCR5 on CD4 Th1 cells) are expressed at much lower levels. In addition, the available probes (such as mAb or HLA-peptide multimers) can have variable affinities for their respective targets. Probes are labeled with different chemical classes of fluorescent dyes that must be matched to the instrument, considering factors such as the availability of a high-power laser line with a wavelength close to the maximum absorption of the fluorescent dye and with a detector (photomultiplier plus filters/mirrors) that has a high sensitivity in the spectral emission range of the

given dye. Complicating matters further, cellular autofluorescence (i.e., fluorescence due to cellular molecules such as NADPH even in the absence of all dyes) limits the sensitivity that can be achieved with a given fluorescent probe, laser, and detector. In practical terms, autofluorescence of lymphocytes is usually limited to a distinct range of emission and absorption wavelengths [47, 48]. In general, the degree of autofluorescence determines the limit of detection, which in earlier reports was of 3,000 molecules for a standard flow cytometer [49]. Consideration of all these factors leads to the following recommendations for detecting cellular markers expressed at very low levels: use a high affinity Ab conjugated to a fluorescent dye with high quantum yield with emission spectral range far away from cellular autofluorescence, for which the cytometer has an appropriately matched high-power laser line and detector.

For polychromatic flow cytometry, additional constraints are set by the phenomena of optical spillover and spreading. In flow cytometry, cells are analyzed in a near-physiological aqueous solution to preserve the structural properties of biomolecules. Due to the spectral absorption of water and air, the useful spectral space is limited to the range from Near-UV (ca. 200 nm) to Near-IR (ca. 1,000 nm). Also, in aqueous solutions, both the absorption and emission of fluorochromes show relatively broad spectral lines. Together, this means that the number of fluorochromes that can be analyzed at the same time is ultimately limited: the combination of 15–20 different fluorochromes appears to be the upper feasibility limit [50].

As a further consequence, spectra of fluorescent dyes routinely overlap ("spillover") [51], requiring software deconvolution of true and observed signals ("compensation"). However, compensation cannot correct other errors caused by measurement, binning, and photon noise, and these errors accumulate to give an irreversible effect termed as "spreading error" [52] or "spillover spreading" [53]. Spreading error will cause the presence of one bright fluorochrome to reduce sensitivity for spectrally close fluorochromes present on the same cell. Use of a high-power laser close to the absorption maximum can reduce errors in photon counting, and narrow bandpass filters can reduce spillover; both these measures will reduce spreading error. Finally, probe combinations should be designed so that overlapping fluorochromes are chosen for labeling markers which are expected to be expressed on different cells.

In practice, panel development usually starts with the definition of a "wish list" of cellular targets, followed by the prioritization of these cellular targets, characterization of their expression levels, and checking for the availability of probes and conjugated dyes appropriate for the cytometer to be used. Guidance documents [54] and helpful software (CytoGenie: www.woodsidelogic.com, Fluorish: www.fluorish.com, Chromocyte: www.chromocyte.com) are available. A practical limitation can be the lack of commercially available fluorochrome conjugates for individual antibody clones. Indirect staining with secondary reagents (such as the biotin-streptavidin system) is possible but often not practical for multicolor applications. A better alternative is the use of new methods now available for the self-conjugation of small amounts of Ab to fluorescent dyes [55, 56]. Based on the discussion above, the cornerstones of panel development guidance are the assignment of "bright" probes for "dim" targets and strategies to avoid spreading error and autofluorescence in channels relevant for "dim" targets. It is also possible to change the optical pathway of the flow cytometer to optimize the instrument (e.g., choice of filters) according to the requirements of the panel. As the amount of potential interference between dyes rapidly increases with the number of colors in the panel, and as a large number of critical parameters should be optimized, development of large ($\geq$8 colors) panels and especially those involving separate staining steps for intracellular and extracellular targets can be an expensive iterative process requiring several man-months of dedicated work. Hence, the flow community is encouraged to share rigorously calibrated and optimized polychromatic panels via the "Optimized Multicolor Immunofluorescence Panels" (OMIPs) project [57].

Quality assurance of a flow cytometry assay starts with the flow cytometer itself, consisting of optimization, calibration, and standardization of the machine, and we refer the reader to the technical report by the Roederer group for details [58]. These optimization steps must not be neglected, as they may identify faulty parts that need replacement, such as a photomultiplier tube (PMT) with reduced sensitivity or suboptimal filters, and are important to optimize general instrument parameters. Conveniently, some (but not all) of these steps have been incorporated in vendor software packages, such as the Cytometer Setup and Tracking (CS&T) application within BD FACSDiva 6 that uses a proprietary mixture of calibration beads. For long-term immunomonitoring, it is essential to maintain accurate records of daily monitoring checks to track reproducibility and stability.

For cell staining, reagent quality can be an issue, especially if the assay is performed repeatedly over time. Often, reagents used are classified as "research use only" (RUO) and can show considerable batch-to-batch variation in important properties, such as concentration of antibody-dye conjugate, concentration of free dye, and even in the spectral properties of the dye (as in the case of tandem dyes). In addition, the shelf life designated by vendors is not always based on quantitative specifications. As a result, individual reagent batches have to be pretested and pre-titrated, and tests repeated even during the designated shelf life of a reagent. As batch sizes available from vendors are often limited, this can result in the requirement of reagent bridging (demonstration of the comparability of reagent batches) during the course of a study, leading to complex logistic and tracking processes. Reagent quality assurance may be facilitated by the preparation of mixtures of lyophilized reagents ("lyoplates") [59] that can reduce pipetting error and lead to increased reagent stability.

Appropriate use of technical and biological controls is also vital for assay interpretation. In addition to instrument calibration beads, unstained and single-stained beads are used to determine the spillover matrix for compensation. Isotype and "fluorescence minus one" (FMO) controls can help with setting gate boundaries at the analysis stage by defining the "negative" region. Pretested, aliquoted, cryopreserved samples with prescreened, predictable properties (such as being positive or negative for individual markers in the mAb panel) can serve as valuable biological controls which can be used in each assay run to track the variations in assay performance between operators and over time.

As flow cytometry-based methods become incorporated into clinical trials, the need for a stable and unlimited source of cell specimens that contains defined numbers of functional antigen-specific T cells as batch controls becomes paramount. Moreover, cell samples containing a known number of T cells specific for a defined Ag would allow easy assessment of the quality and accuracy of assays and provide standard controls for comparison of results across laboratories or time. Currently available sources for reference samples are either (i) based on leukapheresis or buffy-coat material from healthy donors – which are restricted to reactivity against immunogenic viral Ags, expensive and available in limited amount, or (ii) dependent on the ability to generate and propagate T-cell lines/clones on a repetitive basis which is a burdensome task. The Cancer Immunotherapy (CIMT) Immunoguiding Program (CIP) group has recently established a process for the generation of reference samples (RS) that can be used in T-cell assays. In a first proof-of-principle study, we showed that retrovirally TCR-transduced T cells spiked at defined numbers in autologous PBMC can be used as standard samples. The T cells could be accurately detected at all dilutions in a linear fashion, down to frequencies of at least 0.02 %, and the feasibility of RS was confirmed in a small-scale proficiency panel [60]. Subsequently, we established, optimized, and standardized the production of RS obtained by transfection of modified and stabilized RNA. Such a platform offers a simple, virus-free, and scalable process for the manufacturing of reference samples. In proof-of-concept studies for HLA-multimer experiments, the feasibility of using such RNA-engineered RS was shown. RS offered favorable properties across a variety of $CD8^+$ and $CD4^-$ T-cell-derived

TCRs against multiple Ags, including clear clustered populations, reproducible results, high stability over time, and the potential for linear dilution. Moreover, the analysis of the RS is similar to that of the tested cell samples in that the same gating strategy (and even the same gates) can be used. This suggests that RS are a useful tool to control T-cell assay performance. The suitability of these RS samples was subsequently tested in a proficiency panel organized recently (manuscript submitted).

A final, critical aspect of quality management is the careful documentation of each procedure performed, as well as provision of detailed standard operating procedures (SOPs) for each stage including data analysis. Technical staff needs to be well trained and perform the analyses on a regular basis to keep up performance. Participating in proficiency panels will also help improve laboratory standards.

25.4 Proficiency Programs Addressing Flow Cytometry Assays

While HLA-multimers and ICS are commonly used for monitoring experimental vaccines or other anticancer immunotherapies such as adoptive transfer of *in vitro* expanded T cells, there are still notable obstacles to the advancement of these T-cell monitoring assays as robust biomarkers for clinical trials [61, 62]. First, there is no gold standard protocol for any of these assays. Second, correlations between *in vitro* immunomonitoring results and patient clinical benefits have rarely been reported [4, 28, 63–67]. The reality is that assays performed at different institutions are not equal; this results in difficulties in comparing the efficacy of the various immunotherapy approaches tested for recruiting a meaningful anticancer T-cell response, in turn hampering progress in the field.

One approach for addressing these problems is by assay validation and standardization and/or centralization of the immunomonitoring at a dedicated core facility. An attractive alternative to these strategies is assay harmonization. The pros and cons of assay harmonization *vs*. standardization have been discussed in detail elsewhere [62, 68].

Assay harmonization is based on the participation of single laboratories in iterative testing exercises called proficiency panels. Pretested PBMC samples, synthetic peptides, and/or HLA-peptide multimers are shipped from a central lab to all panel participants who then use their own reagents, protocols, and analysis strategies for detecting antigen-specific T cells. Participants then report their data, which are centrally analyzed, allowing comparison of individual assay variables and performance to detect T cells. Thus, parameters involved in assay performance may be successively identified, corrected, and confirmed to exert an impact in subsequent panels (i.e., multistep approach). Finally, benchmarks and guidelines are formulated and disseminated to the community. Participating laboratories benefit by being able to measure their own performance in reference to peer laboratories, and regularly taking part in proficiency panels over time can also be seen as a quality control of assay performance for individual labs. Additionally, the working group can guide laboratories to improve performance if needed, while providing an exchange platform for assays and their application.

Proficiency panels can in principle be applied for any T-cell assay, including those based on flow cytometry [69–71]. In 2005, two consortia, the European Cancer Immunotherapy (CIMT) Immunoguiding Program (CIP) and the Cancer Immunotherapy Consortium of the Cancer Research Institute in the USA (CIC/CRI) launched a large program of proficiency panels and synergistically pioneered the concept of assay harmonization [62, 68]. From 2005 to 2012, the CIP (www.CIMT.eu/workgroups/CIP) has organized 15 small- to large-scale proficiency panels, dedicated to the measurement of antigen-specific $CD8^+$ T cells by HLA-multimers, ELISPOT, and intracellular cytokine staining.

Proficiency panels have taught us that there are large variations in the performance of T-cell assays among the flow community. While the majority of labs do detect antigen-specific T cells present at quite high frequencies in PBMC samples

(approx. >0.2 % of $CD8^+$ cells), the detection rate drastically decreases for low-frequency effectors (<0.05 % of $CD8^+$ cells). This is very relevant for cancer immunotherapy, as tumor-specific T cells are expected to be present at low frequencies in the blood, even after patient vaccination. Another lesson is that comparable performance is achievable with different laboratory-specific protocols and reagents, and full interlaboratory standardization is not necessary for good results. Surprisingly, we also found that operator experience in a method does not necessarily predict performance, underlining the utility of regular quality control of established methods. Finally, adoption of simple measures can lead to significant improvements in assay performance. For example, staining and acquiring larger numbers of $CD8^+$ cells increase the ability to detect low-frequency HLA-multimer-positive cells, and inclusion of a cell-resting phase improved sensitivity in the IFN-γELISPOT. In contrast, a high background production of the cytokine (IFN-γ) both in ICS and ELISPOT is clearly associated with decreased performance [72, 73].

Over several proficiency panel iterations, it also became clear that all steps of the assays, starting from cell handling (freezing/thawing/resting), assay conditions (reagents and protocols for mAbs and HLA-multimer staining, conditions of antigenic stimulation in ICS), result acquisition including instrument settings, down to the data analysis, can benefit from harmonization for achieving comparable results between laboratories. In flow cytometry specifically, instrumentation performance may be an issue, as we recently observed in a panel dedicated to the simultaneous detection of four Ag T-cell specificities by HLA-multimers (manuscript in preparation). Both CIC and CIP have also observed in independent panels conducted for ICS [73, 74], as well as for HLA-multimer staining [75, 76], that suboptimal gating strongly influenced the ultimate results – i.e., the detection and deduced frequencies of antigen-specific T cells. We also showed that analysis (gating) performed by a unique user substantially decreased the variation in the frequencies of specific cells as compared to those reported by single labs analyzing their own data (unpublished data). This is not a surprise, since manual gating is subjective and highly dependent on the experience of the experimenter and tradition in the lab. Further work is therefore needed with a focus on both data acquisition and analysis, including the potential for automated analysis strategies to reduce the subjectivity inherent in gating as described in Sect. 25.7.

25.5 Structured Reporting of Immune Assay Experiments

An increasing number of minimal information projects have emerged in the last years to provide guidance for structured reporting of biological assays. The first minimal information project that set the scene was the Minimal Information About Microarray Experiments (MIAME) published in 2001 [77]. It is now an established and mandatory standard for publishing microarray data for a growing list of highly recognized journals (http://www.mged.org/Workgroups/MIAME/journals.html). More than 30 such guidelines have emerged, asking for minimal information on reported results, including minimal information for cellular assays (MIACA) (http://miaca.Sourceforge.net/), specification for in situ hybridization and immunohistochemistry experiments (MISFISHIE) [78], and flow cytometry experiments (MIFloCyt) [79]. Information on the majority of available MI projects can be found in a central portal for minimal information on biological and biomedical investigations (MIBBI) (http://mibbi.Sourceforge.net/). These guidelines aim at achieving two major goals: first, to annotate data to such extent that they give transparent evidence on the quality, reliability, and possible error sources of reported results and, second, to use the reporting standard to systematically feed public databases [80].

More recently, structured reporting guidelines have also been provided for the specific context of immune assay experiments. As outlined before, the continuous conduct of proficiency panels over several years led to the identification of steps in the assay that critically impact the results, namely, (i) the sample, (ii) the assay, (iii) the data

acquisition, (iv) the data analysis, and (v) certain characteristics of the lab environment. In concordance with these findings, a flow chart of decisions that can affect the quality of data produced in clinical trials in which immunological parameters are monitored by flow cytometry was listed in a landmark publication [81]. Although the variables critically affecting the quality of results are – for most of them – well known, only very few scientific publications provide sufficient information on these aspects in their material and method descriptions. This lack of transparency is one of the major reasons preventing meaningful comparison of published results generated across institutions. In contrast, study results reported with transparent information on the essential variables of assay conduct, explicitly indicate awareness of the investigator to control critical variables, thus can be much better interpreted and reproduced.

To reduce the discrepancy between available knowledge on immune assay conduct and lack of critical information in scientific publications, a group of T-cell immunologists from the cancer immunology, infectious diseases, autoimmunity, and transplantation fields initiated the Minimal Information About T-cell Assays (MIATA) project [82]. The group conducted an intensive vetting process with two public consultation periods, two open consensus workshops, and several webinars [83]. The process towards reaching a broadly acceptable guideline on the minimum information that should be provided for T-cell assays [84] can be found at the project's webpage www.miataproject.org. With the MIATA consensus guidelines becoming available, the implementation of more structured reporting for T-cell immune monitoring can begin and should be considered by all investigators, especially for conducting T-cell assays in clinical trials [85]. So far, three peer-reviewed journals endorse the MIATA guidelines and assign the "MIATA label." The label indicates that authors of accepted manuscripts take great care about reporting on and control of variables that matter for T-cell assays. All MIATA compliant manuscripts will be listed on the MIATA homepage leading to greater exposure of the published work, which may increase interest and citations over time. The authors therefore recommend considering structured reporting of results from T-cell assays whenever possible.

25.6 Organization of Immune Monitoring in Multicenter Trials

Clinical trials will often require the recruitment of patients at multiple sites in order to reduce the overall duration and costs of the trial. The laboratory data generated from all patients and at different sites should be comparable, but as the regulatory framework for the conduct of clinical trials (ICH-GCP) is not very detailed with respect to standards of laboratory analyses, further details are specified by the more recent concept of good clinical laboratory practice (GCLP) [86–88].

Two general strategies emerge on how analytical assays can be performed among different sites [89]: in the distributed analysis paradigm, each site analyzes its locally derived samples. In contrast, in the central lab paradigm, all samples are transported to a central lab for analysis. In either case, flow cytometry poses additional challenges due to the fragility of the sample and the complexity of the assay.

For distributed analysis, the assay and instrumentation at different sites must be comparable. This can be achieved via full interlaboratory standardization, as is already routinely performed in clinical flow cytometry with *in vitro* diagnostic (IVD)-certified reagents and instruments [90]. Due to the high development costs, the number of clinical flow cytometry products for IVD on the market is limited and focuses on the clinically most relevant tasks as, e.g., the quantification of $CD4^+$ T cells in blood. In many cases, these applications lack the technical capabilities of modern polychromatic flow cytometry. Full-scale interlaboratory standardization (with demonstrated low interlaboratory variation) of research assays with RUO-grade reagents and customized flow cytometric instrumentation has been demonstrated by some groups but requires great efforts [91]. An alternative to full interlaboratory

standardization discussed in Sect. 25.4 is harmonization which can be achieved via regular participation in proficiency panels.

For highly complex flow cytometric assays within clinical trials, having all samples analyzed by the same central laboratory eliminates the need for full-scale interlaboratory standardization of participating institutes and may be less demanding. However, maintaining sample quality becomes a critical issue with this strategy. The initial sample material for flow cytometry contains living cells (in most cases derived from blood with the addition of anticoagulants). From this sample material, cells have to be isolated before the start of the flow cytometric assay. Cells are usually more fragile compared to biomolecules or small molecules. Several studies have been performed to determine how long blood can be stored or transported before peripheral blood mononuclear cell (PBMC) isolation (mostly using density gradient centrifugation) and how stable isolated cells are before the assay is started [34, 92, 93]. For simple phenotyping (e.g., CD4 counting), a 48 h delay before centralized analysis is acceptable, while the most demanding applications (such as some functional T-cell assays) require isolation of the cells within 8 h of venipuncture, followed by immediate analysis or cryopreservation of the cells [94]. Shipment to a central lab followed by processing of blood samples within 8 h is however not feasible in international multicenter trials. Therefore, a mixed model may be chosen [4], whereby cells are isolated and cryopreserved from peripheral blood at individual labs close to the patient and then shipped in the frozen state to the central lab where they are stored frozen before analysis. All stages of isolation, cryopreservation, and transport conditions should be fully standardized in this model. Standardized labeling of samples that allow the unambiguous assignment of a sample to a trial, site, patient, and visit is also critical. GCP regulation also requires special care to protect the privacy of patients, and this may be achieved by pseudonymization. These procedures have to be clearly defined in the clinical trial protocol and are usually further detailed in the clinical trial laboratory manual.

As an example demonstrating feasibility of this approach, an international, multicentric immunotherapy trial was conducted recently including T-cell immunomonitoring in which more than 40 clinical sites were trained in blood sampling, labeling, and shipping, with labels and collection tubes provided by a central laboratory. Local PBMC isolation laboratories were centrally supplied with pretested kits containing all critical reagents required for isolation and cryopreservation of PBMCs. All laboratory technicians were trained and qualified on central SOPs describing in detail the PBMC isolation and cryoconservation processes. Where required, the fresh blood was transported from the clinical sites to the PBMC isolating labs using temperature controlled shipments. The isolated frozen PBMCs were shipped to the central lab in validated dry ice containers. Patient visits involving a PBMC sampling were carefully coordinated in advance among the clinical sites, the PBMC isolating laboratories, and the logistic service providers to ensure that the blood could be processed within 8 h after venipuncture of a patient. This process led to a successful logistic chain for 361/362 (99.7 %) PBMC samples and an overall evaluability rate of 64/68 (94 %) patients for T-cell immunomonitoring [4].

25.7 Towards Automated Analysis

As discussed in Sects. 25.4 and 25.5, the standard approach for analyzing flow cytometry data is by the visual identification of cell subsets of interest on histograms or two-dimensional scatter plots. With multiparameter data, gating consists of first choosing a gating strategy – a sequence of dot plots that is designed to allow identification of the cells of interest. For example, a possible gating strategy for identifying HLA-multimer-positive $CD8^+$ T cells might be FSC-A/FSC-H (singlets), FSC-A/SSC-A (lymphocytes), CD3/viability dye (viable T lymphocytes), CD4/CD8 (basic T lymphocyte subsets), and CD8/multimer. In each dot plot, cells of interest are included and other events excluded by the use of elliptical or polygonal gates or sometimes by splitting the

dot plot into quadrants. The exact location and shape of these gates may be based on experience or by comparison with negative (e.g., isotype, FMO, or unstimulated control in ICS) and positive (reference sample or T-cell clone or superantigen stimulation) controls. After a gating strategy has been set, it is typically applied in common to all flow cytometry samples in the batch being analyzed. Some researchers will also adjust gates for individual samples to take individual variability into account. In general, there is no consensus or accepted standard gating strategy, and individual laboratories may apply different gating strategies to identify the same target cell subset. Notably, proficiency panels have made it very clear that the subjectivity of gating forms a significant source of assay variability between laboratories in the absence of a harmonization program [72, 95].

To increase the objectivity of flow cytometry analysis, automated methods in which cell subsets are directly quantified by machine algorithms have been proposed [96–98]. In broad terms, these algorithms have to first partition all the events in a data sample into disjoint subsets, based on properties of each individual event and its relationship to other events, and then to assign these subsets to biologically meaningful categories (e.g., HLA-multimer-binding $CD8^+$ lymphocytes). In the context of cancer immunology, a specific challenge for automated approaches is the high sensitivity required, since antigen-specific responses (e.g., HLA-multimer positivity or polyfunctional cells) may be relevant at relative frequencies of 0.01–0.1 %. Data from multiple laboratories significantly increases the challenges for automated analysis, since the algorithms have to also account for the variability across laboratories and issues with harmonization of sample annotation.

A typical automated analysis preprocessing pipeline starts with the extraction of the essential matrix of information stored in a flow cytometer FCS file, where each row represents an event and each column represents a detector channel, either scatter or fluorescent intensity. Preprocessing algorithms may apply compensation or specific transformations to regularize the data distribution (e.g., bi-exponential transformation). Specific channels may be explicitly excluded from analysis at this stage if they are not likely to be informative for the cell subset targets of interest. Often, a quality control filter is also applied at this stage, and data sets with inconsistent annotation, too few events, and anomalous event distributions or signatures may be flagged for manual evaluation [99].

The core of most automated analysis is the unsupervised partitioning of events into cell subsets. There are a variety of approaches that can be taken to partition or cluster events, as summarized in a recent publication [98]. One popular approach is the use of statistical mixture models, either identifying cell subsets with individual mixture components (which are typically multivariate Gaussian, student T, or skewed versions of these distributions) or using features of the estimated density to assign events to cell subsets [100–102]. Such probabilistic approaches provide a declarative framework to model domain knowledge and support formal statistical inferences for structure learning, classification, and prediction. The underlying statistical model for the domain knowledge can also be naturally extended in different contexts – for example, to incorporate specific assay details for combinatorial multimer encoding [103] or to incorporate multilevel effects via hierarchical modeling [104]. The power of probabilistic models comes at a price, in that these models tend to be much more computationally demanding than non-probabilistic approaches [105–108], and the runtime for analysis of high-volume, high-dimensional data sets may be prohibitive. However, recent developments in the use of highly parallel graphical processing units (GPU) [109] have accelerated run-times by orders of magnitude, making the probabilistic approaches a viable approach for many applications in cancer immunology.

The essential step in postprocessing is the alignment of cell subset clusters across multiple data samples, since comparative analysis of equivalent cell subsets is a necessary requirement of flow cytometry analysis in clinical research. Perhaps the most straightforward approach is to align each data sample with respect to either a reference or consensus clustering via an optimization

routine that minimizes some distance between pairs of clusters (e.g., Euclidean distance between cluster centroids). Other possible approaches skirt the problem entirely by enforcing a common clustering across all data samples or partition the clusters from fitting all data samples into "super-clusters" – all clusters in the super-cluster are then assigned to the same cell subset. The final step of assigning meaningful cell subset labels to the aligned clusters is typically done manually, although there have been recent efforts to develop heuristics that can automatically label clusters by establishing a concordance between cluster features and cell phenotype characteristics in the Cell Ontology. Innovations in the visualization of high-dimensional cytometry data have also greatly increased our ability to interpret the results of automated analysis [110–112].

The detection of antigen-specific cells poses a specific challenge for automated algorithms because of the extremely low frequency of these cell subsets in many patient samples – for example, as few as 0.01–0.1 % of the $CD8^+$ T lymphocyte population may be specific for a particular tumor Ag multimer. Two nonexclusive approaches for improving the ability of automated algorithms to improve the limit of detection are biased subsampling to enrich the sample for rare events [111, 113] or to increase the complexity of the statistical model [104]. The development of algorithms that can accurately and robustly identify rare cell populations is a driving motivator for much current research in automated flow analysis, and we expect rapid advances in this area. Illustrative examples comparing manual and automated analysis of antigen-specific cells for HLA-multimer and ICS assays are shown in Fig. 25.1.

Finally, we note that most of these automated analysis tools are developed under open source licenses and so free to use without restriction. Some packages require a modicum of programming ability to use effectively (e.g., R or Python scripting skills) and others are available online, but in general, these algorithms are probably not easily used by the average flow operator in a clinical research laboratory. In the coming years, we expect that these automated analysis tools will become increasingly accessible to the average flow operator with the following developments – developers of these tools will continue to improve their ease of use; the most successful algorithms will be incorporated into commercial software analysis packages; and more workshops will be organized to train people in the use and potential pitfalls of these exciting new technologies.

25.8 New Methods and Technologies

Flow cytometry has played an instrumental role in our comprehension of the immune system and its interplay with human tumors. The technique has recently experienced dramatic advances and the methods and technologies are evolving continuously. Due to space limitations, we focus here on the recent innovations that in our opinion have the potential to transform the field of general cytometry and are directly relevant for cancer immunotherapy.

Since the first description of a tumor Ag targeted by human T cells [114], many tumor-associated proteins and HLA-class I- and class II-restricted epitopes have been identified. However, the antitumor T-cell immune response as a whole, i.e., the repertoire of Ag specificities recognized by T cells of individual patients, has only rarely been dissected [115, 116]. This is indeed a difficult task, due to the inherent complexity of such projects (many Ags and HLA-allele restrictions have to be taken into account), along with the limited amount of patient material generally available, and high requirements in terms of cost and time. Two groups simultaneously described a combinatorial encoding method which is a very elegant way to circumvent most of these hurdles [117, 118]. The technique is based on the combination of many HLA-peptide multimers, whereby a single multimer is coupled to several (two or three) fluorochromes, generating a color code for each tested TCR specificity. Currently, up to 27 HLA-multimers labeled with eight fluorochromes can be combined in routine analysis [117]. Coupled to the production of HLA-monomers by the UV exchange technology, this high-throughput method represents an important technical

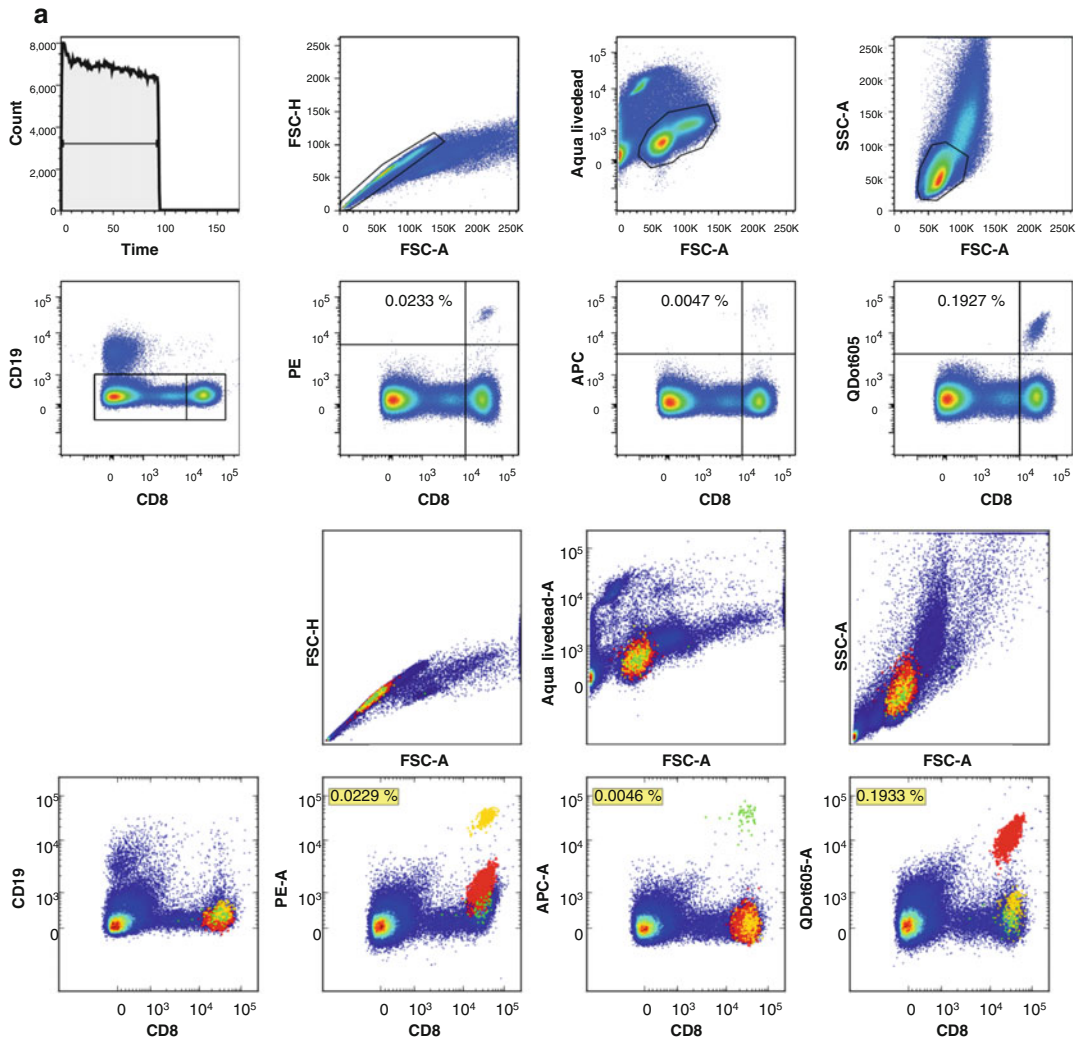

Fig. 25.1 (a) Manual and automated identification of antigen-specific MHC class I multimer-positive CD8⁺ T lymphocytes among PBMC of a HLA-A2⁺ healthy donor. *Top panel* shows a manual gating strategy to identify CD8⁺ T cells specific for three HLA-A*0201-restricted epitopes derived from a EBV, influenza, and CMV viruses. From *left* to *right*, the plots show gates to exclude artifacts due to flow stream bubbles or clumps (count/time), find singlets (FSC-A/FSC-H), exclude nonviable cells (FSC-A/Aqua LiveDead), identify lymphocytes (FSC-A/SSC-A), exclude B lymphocytes (CD8/CD19), and quantify CD8⁺ T cells binding to EBV BRFL1 peptide-MHC multimers (CD8/PE), influenza matrix peptide-MHC multimers (CD8/APC), and CMV pp65 peptide-MHC multimers (QDot605). *Bottom panel* shows the corresponding peptide-MHC binding CD8⁺ T cells identified using an automated analysis approach that fitted a Dirichlet Process Gaussian Mixture Model with 256 components to the data [103]. Essentially identical frequencies of peptide-MHC multimer positive cells are found with manual and automated analyses. (b) Manual and automated analysis of antigen-specific T cells among PBMC of a second HLA-A2⁺ healthy donor tested in an intracellular cytokine staining (*ICS*) assay after incubation with a synthetic peptide corresponding to an HLA-A*0201-restricted epitope of pp65 CMV. Manual analysis finds cells positive for IFN and TNF, and a few events positive for IL-2. Without further gating, it is not possible to tell if the IFN- and TNF-positive events come from two separate or a single bifunctional population. Automated analysis reveals that there is indeed a single-cell population positive for IFN and TNF, with no evidence for an IL-2-positive population. Again, the frequencies of antigen-specific events identified by expert gating and automated analysis are almost equivalent

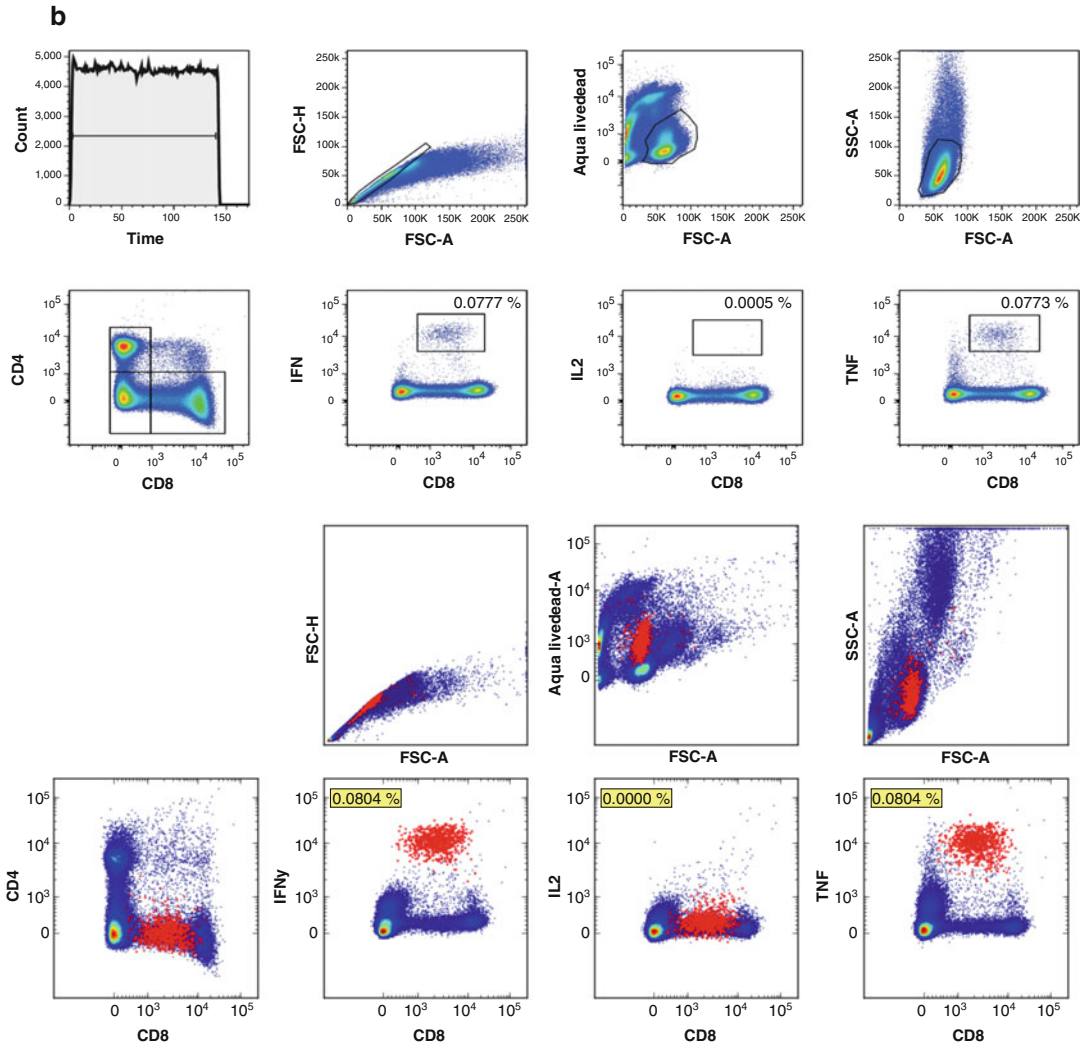

Fig. 25.1 (continued)

achievement for the T-cell immunology field and has started to deliver precious information by dissecting the anti-melanoma TIL repertoire in melanoma patients [119, 120]. Combinatorial staining could easily be implemented for monitoring vaccination trials, for example, when applying cocktails of antigenic peptides for which many specificities need to be tested in a single PBMC sample.

The combination of extracellular phenotyping with determination of intracellular changes in phosphorylation patterns upon stimulation is starting to provide new insights into signaling pathways in healthy and disease conditions [121, 122]. The binding of cytokines to their specific cell surface receptors generally results in the activation (i.e., phosphorylation) of the downstream signal transducers and activators of transcription (STATs), which in turn regulate the expression of many genes involved in cell growth, survival, differentiation, and polarization. Next to cytokines, the effect of unspecific mitogenic stimuli such as phorbol myristate acetate (PMA), phytohemagglutinin (PHA), or MHC-peptide complexes binding to the T-cell receptor (TCR) can be studied by measuring the level of other key signaling molecules such as phosphorylated (p)-Erk, p-S6, and p-NF-κB in T and B cells, whereas Toll-like receptor (TLR)

ligand-induced activation can be followed with p-Akt, p-Erk, and p-NF-κB in B cells and monocytes. The proof of principal for a "single-cell network profiling (SCNP) method" was obtained on healthy donors PBMCs [123]. In this initial study, age as well as race differences were observed, whereas intra-donor variability needs to be established by testing blood samples taken at different time points over time. As T-cell signaling defects have been described in cancer patients [124, 125], insights in the intracellular phosphorylation patterns of T cells, including during immunotherapy, may soon deliver precious information.

A fundamental advance in flow cytometry in recent years is an increase in the number of parameters that can be simultaneously evaluated on single cells. Access to an increasing number of reagents and fluorochromes including tandem conjugates, semiconductor nanocrystals (quantum dots or eFluors), and organic polymers (brilliant violet family) [126–128], together with the wide availability of sophisticated flow cytometers, is making polychromatic analysis mainstream.

However, spectral overlap ultimately limits the number of fluorochromes in a single panel to an upper bound of approximately 20, as described in Sect. 25.3. An exciting new technology that has the potential to greatly increase the number of measurable parameters is mass cytometry (CyTOF), which uses stable heavy metal ions tagged to Abs (or, e.g., MHC multimers) in place of fluorochromes. These isotope labels are detected by time-of-flight mass spectrometry after vaporization of the cell. Although isotope labels generally produce a signal of low intensity, they have a lower background and virtually no spillover, making the measurement of a much larger number of markers feasible.

Mass spectrometry has been reported to be qualitatively and quantitatively equivalent to flow cytometry, with the simultaneous analysis of more than 30 parameters being already possible [129]. However, this promising new technology has the current following limitations as compared to traditional flow cytometry: lower label sensitivity, substantial cell loss, low acquisition rate, and the impossibility to sort living cells. Nevertheless, this method has started to reveal the complexity of healthy hematopoietic cells and of $CD8^+$ T lymphocytes subsets and will certainly mature to become an indispensable technique in cancer immunology and immunotherapy [129, 130].

25.9 Concluding Remarks

Flow cytometry is the prototypical multiparameter single-cell assay, with applications in cancer immunotherapy ranging from epitope screening to immune monitoring of clinical studies. Due to its ability to characterize complex immune phenotypes and flexibility in measuring multiple immune functions such as Ag binding, expression of activation and inhibitory markers, cytokine production, cytotoxicity, and proliferation, flow cytometry is indispensable in cancer immunology research. However, because of the complexity of the assay and the fragility of the sample, it is challenging to apply and maintain robustness, sensitivity, and reproducibility, especially across multiple laboratories. Factors to consider when using flow cytometry in clinical research include understanding the range of flow-based assays available, as well as best practices for instrument, reagent, sample, and data analysis.

In order to harmonize laboratory protocols, practices, and analysis strategies, flow cytometry proficiency testing programs have been organized to learn and raise awareness of best practices. We believe that participation in proficiency testing programs, along with other initiatives delivering protocols, assay guidelines and reporting frames, is critical for raising the standard of flow cytometry analysis and strongly recommend that all clinical research laboratories that perform immune monitoring for clinical trials join such programs.

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