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## Implementation of highly sophisticated flow cytometry assays in multicenter clinical studies: considerations and guidance

Flow cytometry is increasingly becoming an important technology for biomarkers used in drug discovery and development. Within clinical development flow cytometry is used for the determination of PD biomarkers, disease or efficacy biomarkers or patient stratification biomarkers. Significant differences exist between flow cytometry methodology and other widely used technologies measuring soluble biomarkers including ligand binding and mass spectrometry. These differences include the very heavy reliance on aspects of sample processing techniques as well as sample stabilization to ensure viable samples. These differences also require exploration of new approaches and wider discussion regarding method validation requirements. This paper provides a review of the current challenges, solutions, regulatory environment and recommendations for the application of flow cytometry to measure biomarkers in clinical development.

### Technical overview & applications of flow cytometry

Flow cytometry is a powerful tool which uses fluorescence to assess individual cell markers and populations within a given sample. Staining with fluorescently conjugated antibodies is most frequently used for the identification of cell populations and subpopulations based on surface markers. The increased knowledge of surface marker expression on cells and the wide availability of specific antibodies enable advanced immunophenotyping and the detection of specific cell subsets and rare cell population. Fluorescently tagged monoclonal antibodies can also be used to identify intracellular signaling molecules thus allowing for analysis of signal-transduction pathways in specific cell populations. Cell activation can also be assessed by detection of specific surface markers or by looking at cytokine production, which can be measured intracellularly by blocking cytokine secretion prior to staining. Additionally, fluorescent molecules can be used to detect cellular proliferation and apoptosis. Flow cytometry therefore provides an extensive variety of applications which

can be applied to collect multiparameter data from heterogeneous whole blood populations down to single cell analyses.

### Use of flow cytometry for biomarker analysis in clinical studies

Flow cytometry has become increasingly important in the biomarker arena. The various flow cytometry applications provide solutions for different biomarker questions and biomarker use (Box 1).

PD markers are important biomarkers which provide information about target engagement and the effect of the drug on its target. In early human clinical studies, PD assessments are useful to define the PK/PD relationship and to create a PK/PD model which supports proper dose selection for further studies. Flow cytometry provides several ways and methods to measure PD biomarkers: Phosphospecific flow cytometry (Phosphoflow) is a flow cytometry method to detect the phosphorylation of intracellular signaling molecules. This method can be used to determine PD effects of small molecular inhibitors targeting signaling kinases. Perl *et al.* described that

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**Box 1. Flow cytometry applications provide solutions for different biomarker questions and biomarker use.**

- PD markers which provide information about target engagement and the effect of the drug on its target can be assessed by the following flow cytometry applications:
  - Activation/inhibition of cells analyzed by cytokine production, activation markers, signaling pathways and receptor occupancy assays.
- Disease and efficacy biomarkers used to monitor disease severity, progression or improvement can be assessed by the following flow cytometry applications:
  - Disease-specific cells or subpopulations and disease relevant expression markers.
- Biomarkers for patient stratification to enrich or separate patients into subgroups can be assessed by the following flow cytometry applications:
  - Detection of pathway activation, detailed leukocyte subset analysis or measurement of rare cell populations.
- Safety biomarkers used to monitor clinical safety:
  - The monitoring of the balance of cell subsets upon drug treatment can provide important safety information.

monitoring of phosphorylation of S6 serves as a PD biomarker to measure the effect of the mTOR inhibitor (Sirolimus) in acute myelogenous leukemia [1]. Another study demonstrated that inhibition of the JAK/STAT pathway by immunosuppressive drugs in kidney transplant patients could be used to monitor PD effects of a drug [2]. Here the authors also used Phosphoflow to analyze the intracellular signaling pathway. A reliable method to detect the intended PD effect of a biological drug (therapeutic antibody) by flow cytometry is the measurement of cell depletion as the result for the depleting activity of the therapeutic antibody. For example, reduced numbers of B cells can be monitored to prove the direct effect of B cell depleting antibodies

(e.g., Rituximab) on the target cell [3,4]. The depleting effect of S1P receptor modulators (e.g., BAF312) on leukocyte subsets can also be demonstrated by flow cytometry [5]. Another way to determine PD effects by flow cytometry are receptor occupancy assays which detect receptor engagement of a therapeutic antibody and thus provide information of the direct PD effect of the biological drug. Competing and noncompeting fluorescently tagged antibodies are used to detect levels of total receptor expression, and levels of free receptor whose signal can be blocked by the presence of a given drug. This method was employed by Ma *et al.* in order to characterize the PK and PD of a humanized anti-CD40 antibody [6]. Also surface marker staining such as the detection of FcεRI and IgE expression on basophils by flow cytometry demonstrated the PD effect of a novel anti-IGE antibody in clinical trials in atopic patients [7].

Disease and efficacy biomarkers are used to monitor disease severity, progression or improvement. Flow cytometry assays are widely used to measure different kinds of disease markers. For example, flow cytometry is routinely used for the detection and monitoring of leukemia and myelomas, as neoplastic cells can easily be distinguished from normal cells by staining of surface markers. In addition to hematologic malignancies, a study reported the use of flow cytometry in identifying free tumor cells that had metastasized to the peritoneal cavity of patients with abdominal malignancies [8]. Leukocytes were also identified in the staining panel and the results were used to demonstrate a predictive tumor cell to leukocyte ratio that may be used as a biomarker for the severity of peritoneal metastasis [8]. Autoimmune diseases, such as the chronic inflammatory condition ankylosing spondylitis, are also excellent candidates for disease biomarker assessment by flow cytometry. A study published in 2013 described that CD4+ T cells expressing IL-21

**Key terms**

**Flow cytometry:** Laser-based technology that uses emissions of excited fluorochromes to measure the levels of a given marker on or in a cell. Measurement of the scatter pattern of the lasers is also used to discern size and granularity of samples passed through the flow cytometer. Samples are passed in a single cell fluidic stream by an electronic detection apparatus. Flow cytometry can simultaneously analyze multiple parameters across myriad of sample types and can gather data down to a single cell analysis.

**Biomarker:** An indicator that is used to objectively measure normal or pathogenic biological processes as well as pharmacological responses to a therapeutic intervention. Biomarkers have significant utility in clinical studies, for example, PD biomarkers, disease and efficacy biomarkers, patient stratification biomarkers and safety biomarkers.

**PD biomarker:** Biomarker for which a change from baseline (pre-treatment) to post-treatment indicates that the treatment has interacted with its target(s).

**Biomarker method validation:** Process of proving, through scientific testing, that an assay meets predetermined standards and performs reliably and in agreement with its intended purpose.

and the chemokine receptor CXCR5 exhibited a positive correlation with the development of ankylosing spondylitis. Treatment with meloxicam, thalidomide and etanercept for 1 month resulted in a significantly lower percentage of these IL-21 positive follicular helper T cells, also correlating with diminished disease activity. Thus, CD4+IL-21+CXCR5+ cells have been proposed as a disease as well as efficacy biomarkers for ankylosing spondylitis [9].

The monitoring of a change in the balance of cell subsets upon drug treatment can provide important safety information, thus cellular biomarkers can serve as safety biomarkers as well.

Clinical drug development is more and more focused on personalized medicine and the identification of biomarkers for patient stratification is important for this strategy. Flow cytometry can provide helpful information such as detection of constitutive phosphorylation events in patients having mutations in signaling pathways (e.g., RasRafMAPK pathway in Rasopathy patients) or in patients where specific signaling cascades are hyper activated in an autoimmune disorder setting. Suárez-Fueyo *et al.* found for example enhanced PI3K pathway activity in systemic lupus erythematosus (SLE) patients [10]. This activated pathway can be detected by increased pAkt using the Phosphoflow method. Furthermore, depletion of B cells in SLE patients using the anti-CD20 antibody Rituximab has been shown to resolve B cell abnormalities in the peripheral blood, tracked by using flow cytometry to discern B cell phenotypes and depletion efficacy [11]. Multicolor flow cytometry staining has also been used to identify subsets of dendritic cells and has identified CD52 as a marker on peripheral myeloid dendritic cells which are highly stimulatory. Depletion of these cells using alemtuzumab is therefore useful for patients with lymphoproliferative disorders or who will be receiving hematopoietic stem cell transplants [12]. Biomarker discoveries such as this using flow cytometry have led testing the use of alemtuzumab in multiple sclerosis [13] and B cell chronic lymphocytic leukemia [14–16]. Advanced immunophenotyping and the detection of cell subsets or rare populations is clearly an important tool to define subgroups of patients with an impaired balance of cell subsets. For example, more extensive phenotyping of regulatory T cells has led to the identification of CD127 as a potential biomarker for human regulatory T cells. When used in conjunction with CD4 and CD25, cells that showed downregulation of CD127 were as suppressive as the classically identified CD4+CD25<sup>hi</sup> population. This lends much needed specificity to the identification of human regulatory T cells [17].

The application of flow cytometry in clinical drug development is clearly far-reaching. Sophisti-

cated flow cytometry assays like detailed leukocyte subset analysis, measurement of rare cell populations, disease relevant expression markers, receptor occupancy, activation of cells analyzed by cytokine production, activation markers and signaling pathways provide important information on PD, disease progression, treatment effect, safety and patient stratification. Whereas the measurement of soluble biomarkers in serum, plasma and other body fluids is performed by validated assays following standard operational procedures, the implementation of flow cytometry methods in clinical studies is challenging and so far not routinely applied due to sample stability issues, complexity of the assays and the requirement of in-depth flow cytometry expertise. This review is focused on the challenges and approaches of highly sophisticated whole blood flow cytometry assays implemented in human clinical studies. First, key aspects of flow cytometry method validation are summarized and specific advice for the validation of PD assays to be used for PK/PD modeling is provided. Second, we detail aspects which are important for multicenter clinical studies such as on-site sample processing, stabilization of samples and clinical implementation.

## Development & validation of flow cytometry assays for clinical use

### Summary of current guidelines & key challenges

The current draft guidance from US FDA (2013) recommends the fit-for-purpose approach for **biomarker method validations**, which is defined as a method validation performed specifically to support the intended use of the data [18]. This means that the validation process is continually evolving and must be assessed regularly [19]. The FDA also suggests biomarker method validation criteria should follow those of PK method validations: accuracy, precision, selectivity/specificity, assay range, reproducibility and stability. However, the FDA recognizes there will be some variances between PK and biomarker method validations [18].

Flow cytometry method validations present unique challenges. Although no official guidelines for the validation of flow cytometry methods currently exist, several guidance documents have been published in the recent years [20,21]. Also the guidance documents by Cunliffe *et al.* published in 2009, the recommendations from the AAPS (American Association of Pharmaceutical Scientists) flow cytometry steering committee as well as the guideline from Wood *et al.* provide good insight into the goals and challenges of flow cytometry method validations [22–24]. In this

section, we summarize key aspects for the validation of flow cytometry methods.

Accuracy is generally a necessary component of GLP validations [18], but this assessment is not possible for flow cytometry since the needed reference material or quality controls (QCs) are not available [24]. In some instances, it is possible to use commercially available control material such as CD-Chex by Streck Laboratories or receptor density beads [22]. The comparison of results obtained by flow cytometry to results obtained by other methods is suggested by Wood *et al.* [24].

Although traditional quantitative QCs are difficult to implement, there are several uses of qualitative or relative QCs, such as those used by Ramalingam *et al.* in which each day's test for CD4<sup>+</sup> T cell counts included the low and high samples from the previous day as an internal control and the percent variation over the 2-day period was analyzed [25]. While providing some proof of consistency throughout the assay, this method of internal QCs would not detect overarching issues from the method itself or potential mistakes in the initial settings of the flow cytometer provided these settings were used throughout the study. Furthermore, while providing some relative quantitative measure in the form of mean fluorescence intensity (MFI), many QC values are still judged relative to one another, making the measurements semiquantitative at best.

Several methods exist in which flow cytometry samples can be normalized across runs. One method is the use of Molecules of Equivalent Soluble Fluorochromes (MESF) beads [26]. These beads have a known level of fluorescence that allows the user to generate a standard curve independent of the instrument settings and software. Problems in a run on any given day could be confirmed by the bead fluorescence falling outside of its normal range. To best track assay performance over time, a running average of bead values should be maintained for reference.

In the case that the markers being investigated are standard surface markers, stabilized blood with known levels of surface markers can also be used as a QC for each run. Limitations of this method would be cost and potential variations in cell surface markers from lot to lot. Also, fluorescence levels would not be controlled in this method, therefore traditional QCs with high-, medium- and low-expression levels would not be readily available. While the use of standardizing beads and other procedural controls can help control for variations and increase the quality of flow cytometry outputs, true quantitative QCs to accompany each run are not consistently commercially available [21]. It is currently advised that in-house QCs may need to be generated for varying assays, as QCs should reflect

the tissue type and expression patterns of the clinical samples of interest [21].

During assay development the specificity of the used antibodies must be verified: manufacturers claims may be acceptable but there are different methods to confirm antibody specificity in the lab [23]. The detection of a marker on a specific population and the lack of detection of an irrelevant population is one example. Competition assays can be implemented utilizing two antibodies directed against the same antigen, one labeled with a fluorescent tag and the other left unlabeled.

Another potential challenge is the measurement of interassay precision since whole blood samples might not be stable 24 h after blood collection [21]. It is recommended by O'Hara *et al.* that interassay precision testing should contain 3–6 specimens with 3–6 replicates for each specimen, run over the course of 3–6 days [23]. In most cases, the use of a normal paraformaldehyde fixation method will yield enough stability to meet the 3-day requirement when held at 4°C, however, if needed two or more analysts should contribute to the interassay runs to complete the necessary analysis within the required time frame [21]. Clearly, the stability of fresh and fixed whole blood sample needs to be known before interassay precision tests are performed. However, variability between donors and day-to-day variability might be a drawback of this kind of interassay approach. In order to avoid this biological variability between samples and to avoid variations due to whole blood instability, we recommend to perform interassay assessments in parallel on the same batch of blood, but in different plates if applicable and differently prepared reagent-mixes (antibody mixes, lysis buffer etc) should be used to mimic different assay runs.

The biological variability of cellular markers in whole blood provides important information for the analysis of cell based biomarkers in clinical studies. The day-to-day variability in one subject as well as the intersubject variability needs to be evaluated. The sampling of at least 10 individuals for the intersubject analysis may be used to set a reference range for the parameters of interest.

Without quantitative standards available, the establishment of the assay range and limit of quantification is also difficult for flow cytometry assays. Defining a lower limit of quantitation is still not standard practice for flow cytometry validations, although more recent guidelines are beginning to incorporate this idea. The AAPS flow cytometry steering committee recommends that a lower limit of quantitation and limit of detection be assessed for the validation of phenotypic biomarker assays, but does not suggest either of these practices for validations of functional biomarker assays [23]. Wood *et al.* recommend establishing the



optimal cell number or sampling volume required for proper staining [24]. Also it is important to define a minimum number of events needed to get reliable data indicated by replicate precision. For this, the number of acquired events in a parent or grandparent population needs to be defined in order to reach the needed number of events in the population of interest. The user may define the limits of the assay by predefining the gates during the assay development using negative controls such as isotype and Fluorescence Minus One (FMO) controls [27]. In assays where the signal of interest can be inhibited by the drug, for example the inhibition of a signaling pathway and the resulting inhibition of a phosphosignal, the limit of quantification can be assessed by the lowest signal which gives reliable and reproducible results (measured by replicate precision, detailed in the section below). Horton *et al.* addressed some short falls of flow cytometry validations [28]. This eight-color validation addressed assay linearity by diluting stimulated cells with unstimulated cells and performing intracellular staining for cytokines present only in stimulated cells. This allowed the group to not only show that the assay was linear, but also to define the lower limit of quantitation by defining the parameter as ‘the lowest frequency of antigen-specific T cell responses for which the CV was  $\leq 30\%$ .’ The assay showed an impressive lower limit of quantitation, with a median value of 0.01%, showing the highly sensitive nature of flow cytometry [28].

### Validation challenges across multiple instruments & sites

Instrumentation also plays a critical role in the effort to standardize flow cytometry read outs between sites. Standardizing applications across multiple flow cytometers at various sites is perhaps one of the biggest challenges. In order to reduce the interlab variability, outsourcing of laboratory testing and a centralized sample acquisition and analysis becomes more and more popular [29], common procedures for instrument set-up, calibration, maintenance and sample analysis becomes a requisite. Calibrated fluorescent beads can be used to establish a targeted fluorescence level in each channel, for example, BD CS&T beads have bright bead target values that can be used to construct optimal settings for a given application. These target values can then be used to mimic the settings on other flow cytometers [30]. Norman Purvis has elucidated means of standardizing results across multiple instruments using a range of bead types. This method includes using calibration beads for initial instrument set up (CaliBRITE from Becton Dickinson, QC3 Microbead Standards and Full Spectrum Beads from Flow Cytometry Standards Corporation), followed by the use of Quantum Molecules of

Equivalent Soluble Fluorochromes beads and Quantum Simply Cellular Calibration Standards to calibrate and standardize data read outs between flow cytometers. This multistep approach shows remarkable correlation of data sets between instruments [31]. Additionally, the EuroFlow Consortium demonstrated high levels of reproducibility of immunophenotyping assays at multiple sites using Sphero™ Rainbow beads to standardize the instrument settings and strictly following their standard operating procedures and antibody panels [32]. The use of Sphero™ Rainbow beads has also been published by Perfetto *et al.* as a quality tool for establishing PMT linearity and dynamic range as well as tracking performance over time. Sphero™ beads are stable nano particles which contain various fluorophores, each with multiple intensities. PMT linearity is determined by identifying a low (M1) and high (M2) MFI peak within a sample and using the calculation  $(M2-M1)/M1$  across a range of voltages. Signal to background ratios can be determined when used in conjunction with Compensation beads (BD CompBeads), which are made to nonspecifically bind to the kappa chains of antibodies derived from a particular species. Unlike Rainbow beads, Compensation beads are used to adjust for experiment-specific fluorochromes and contain both a positive and negative bead population. Signal-to-noise ratios can be calculated by dividing the M1 value (described above) by the MFI of the negative Compensation beads. In this way, the use of Rainbow beads and other similar calibration particles, can be used to optimize settings and validate the performance of the detectors on a flow cytometer and a cytometer performance can be tracked over time [33]. In an effort to standardize not only the instrumentation for validation, but the reporting itself to ensure appropriate reproducibility and interpretation, validation experiments for flow cytometry should follow the guidelines for *MIFlowCyt: The Minimum Information about a Flow Cytometry Experiment* published by Lee *et al.* in 2008 [34].

### Considerations for the validation of flow cytometry assays used for PK/PD assessment

In this section, we add new considerations for the validation of PD assays to be used to assess PK/PD relationship in early clinical trials to the existing comprehensive guidelines for flow cytometry assay validation.

As described above, PD assays are mainly used in early human clinical studies to monitor the drug effect on its target. These can be assays detecting intracellular signaling events, such as the phosphorylation of downstream targets (Phosphoflow assay). Also, the detection of intracellular cytokines or the expression of activation markers can provide PD information, although these biological events are further downstream of the target.

**Key term**

**PK/PD model:** Combination of PK and PD which provides an integrated set of mathematical expressions in order to assess the time course, concentration effect and biochemical and physiological response to dosing.

In healthy subjects, signaling pathways might need to be stimulated in order to get a measurable PD signal.

During validation of these assays it is recommended to go beyond showing only the reproducibility of stimulation and staining procedures without proving the drug's intended PD effect on the marker. Thus in addition, *in vitro* dose–response curves showing the effect of the drug on its target should be included in the development and validation procedure by *in vitro* incubation of different drug concentrations with the whole blood sample. Drugs should be spiked at clinically expected and relevant PK concentration range. Higher doses lead to strong inhibition of the signaling pathway and thus to a low signal of the PD biomarker. This assessment provides information about the sensitivity and the biological variability of the drug's effect ( $IC_{50}$ ) and limits of quantification (LOQ) of the assay. **Figure 1** shows an example of the effect of a small molecular weight inhibitor on a signaling pathway determined by pErk as PD biomarker. The pathway was activated by 2-O-Tetradecanoylphorbol-13-acetate (TPA) and pErk was detected by Phosphoflow in lymphocytes. In this example the interassay variability was assessed by performing four dose–response curves in separate experiments. In this experiment, it is shown that the interassay variability of the assay is low demonstrated by similar  $IC_{50}$  for each curve. The LOQ of a PD assay is given by the lowest signal at high drug doses which gives reliable and reproducible results. The obtained assay performance criteria can then be applied for use of the assay in clinical trials. Furthermore, *in vitro* dose–response curves will provide information about the sensitivity of the drug's effect on the PD biomarkers in different subjects (interdonor variability). It is also recommended that the day-to-day variability within one donor be approached similarly. The measured biological variability needs to be taken into consideration during the analysis of clinical study samples.

Stability assessments are important measures for the implementation of whole blood flow cytometry assays in clinical studies. In the next section, this topic will be addressed in more detail. Stability testing for PD assays is similar to other flow cytometry assays. The stability of blood samples should be assessed after blood collection, before any further sample processing occurs. Also, storage and stabilization conditions following processing and fixation of the samples should

be tested. However, for these assessments it is recommended to use the PD assays in its intended clinical use without incubating the samples with the drug, because the drugs are usually dissolved in DMSO and stability assessments might be affected by DMSO. Also, the incubation step at 37°C for at least 30 min is a step which is not performed during the clinical study. For these reasons, we suggest a two-step approach for the development and validation of PD assay: first, to test the PK/PD relationship using the *in vitro* dose–response curve and second, to perform stability assessments with the assay in its intended clinical use.

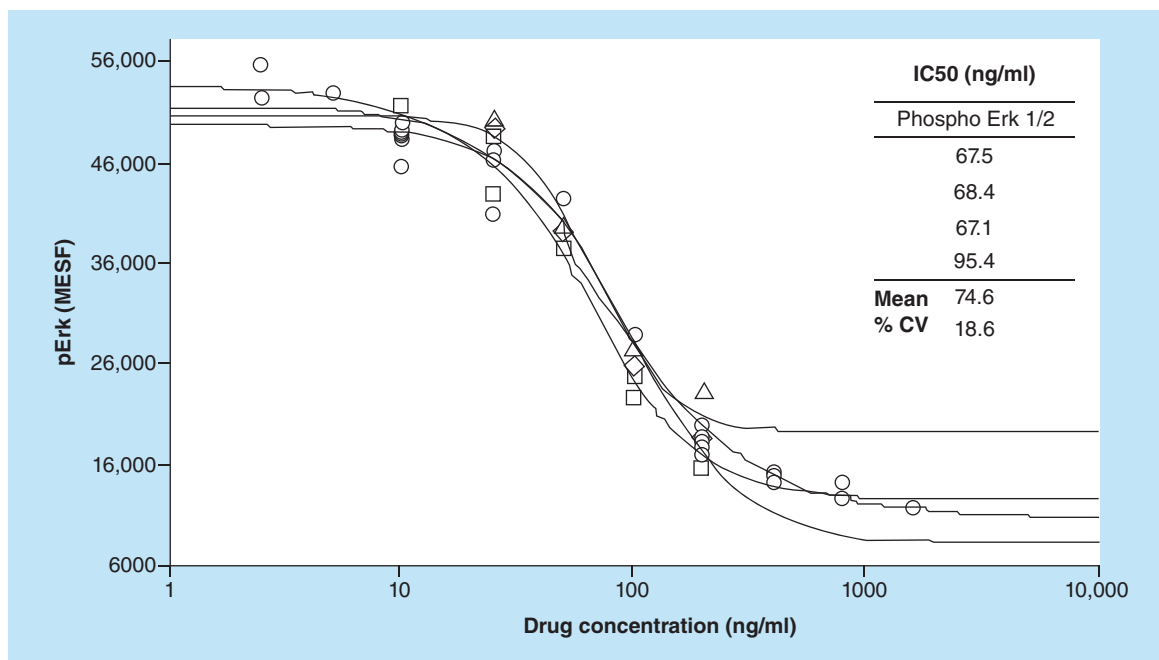
### Whole blood flow cytometry assays in multicenter clinical studies

The application of flow cytometry assays on fresh whole blood samples is restricted to well-equipped (flow cytometer available on-site) and experienced clinical sites. If more sites are involved, lab-to-lab and instrument-to-instrument variability may occur in addition to the challenge of finding these specialized clinical sites. To avoid these issues, isolation of PBMC's or specific cell populations is often conducted, because these can be frozen and send to a central laboratory for flow cytometry analysis. However, the isolation of cells from whole blood samples requires expertise in handling the cells at the clinical site and may affect the results by either washing out the drug or by manipulating the cells. In order to implement reliable flow cytometry assays, it is recommended to use cellular biomarker assays in whole blood. In this section, we summarize key considerations for the use of whole blood flow cytometry assays in multicenter clinical studies (**Box 2**).

### On-site processing of whole blood samples

The use of whole blood flow cytometry assays in multicenter studies is challenging when certain processing steps need to be performed at the clinical site. For immunophenotyping assays, the critical steps are the proper staining of the samples in two or three replicates with the provided antibody mixes followed by the complete lysis of the erythrocytes (when the staining needs to be done before fixation of the samples is discussed in the following section). The lysis buffer needs to be diluted freshly before usage on-site. All steps can be done at room temperature which reduces the needed equipment to a minimum.

Whole blood Phosphoflow assays require even more sample processing at the site, especially when these assays are applied to first in human studies to define a **PK/PD model** and to support dose selection for further clinical studies. In this scenario, the sampling time points for single and multiascending dose cohorts are quite frequent. In the single ascending dose cohorts



**Figure 1. In vitro dose-response curve: phosphoflow assay showing inhibition of pErk.** Phosphoflow assay showing inhibition of pErk after *ex vivo* stimulation of whole blood with 2-O-Tetradecanoylphorbol-13-acetate. Each curve corresponds to an independent experiment (Novartis in house data, blood was received from Novartis internal blood donor bank, healthy human donors).

collection points should be done a couple of times (e.g., every 2 h) together with the PK sampling at the day of dosing in order to determine a proper PK/PD relationship. The on-site sample processing steps for these assays include the proper aliquoting of blood samples into a plate or tubes. An *ex vivo* stimulation might need to be done in order to activate the signaling pathways which is time critical due to the transient nature of signal-transduction events. After stimulation, which is often short (15–30 min), the activated pathway needs

to be stopped by fixation of the cells. Also this step is crucial to get reliable and reproducible results. As for the immunophenotyping assay, the lysis of the erythrocytes needs to be performed properly in order to guarantee good sample quality and the buffer needs to be prepared freshly before usage. Accordingly, the needed equipment at the clinical sites is much more complex than for immunophenotyping assays as sites need incubators and/or a water bath at 37°C. Despite these requirements and on-site procedures we were able to apply various PD

#### Box 2. Key aspects and considerations for the use of whole blood assays in multicenter clinical studies.

- Whole blood is the preferred matrix to get reliable cellular biomarker data.
- On-site processing such as *ex vivo* stimulation, lysis and fixation of the samples requires detailed training (best on-site training) and documentation (video and slide decks).
- Whole blood samples for immunophenotyping as well as Phosphoflow can be stabilized by direct freezing at -80°C in commercially available buffers without the need of centrifugation at the sites.
  - The staining of some markers are altered by the fixation method, thus the staining needs to be done before fixation and freezing at the clinical site
  - Shipment of frozen Phosphoflow samples is critical and when the shipment requirements are not followed samples get clotted and not-analyzable, frozen samples for Immunophenotyping are not affected by shipment conditions.
- Simplification of processing steps at the sites: the 'kit approach' avoids pipetting of a fixed volume of blood or buffer but uses plastic Pasteur pipettes to transfer blood, dilute lysing solution and add antibody mixes to the blood. The use of defined number of drops of blood using the plastic Pasteur pipettes instead of pipetting a specific amount of blood was validated. This kit approach simplifies sample processing and reduces the needed equipment such as pipettes at clinical sites. This also enables clinical sites without laboratory experience to perform these assays.

Phosphoflow assays in human clinical trials to measure intracellular phosphorylation events.

### Stabilization of samples: stabilizing tubes or freezing of whole blood samples

A key challenge for the implementation of whole blood flow cytometry assays in multicenter studies is a procedure to stabilize the samples to avoid the need of flow cytometry acquisition and analysis at the clinical site. The aim is to ship samples to an experienced flow cytometry facility to avoid lab-to-lab variability and to minimize the clinical site requirements. In the meantime several ways are described how to stabilize whole blood samples:

Commercially available blood collection tubes such as Cyto-Chex and TransFix tubes may stabilize blood for up to 7–10 days [35]. This is a very convenient approach for immunophenotyping assays since blood can be drawn directly in these tubes and then shipped to the central analysis lab. However, because the blood is fixed in these stabilizing tubes, these samples cannot be used for *ex vivo* stimulation assays which might be used for PK/PD determination.

Freezing of whole blood samples is another approach to stabilize samples to be shipped to a central laboratory. Perl *et al.* describe a Phosphoflow assay to detect pS6 in whole blood samples [1]. The cells are fixed first before another buffer is added to permeabilize the cells and to lyse the erythrocytes. Afterward cells are centrifuged, washed and the resuspended pellets are frozen at  $-20^{\circ}\text{C}$ . This approach contains several steps which might be technically challenging for clinical sites. We use a more simple method to freeze whole blood samples for Immunophenotyping assays as well as for Phosphoflow assays: the samples are fixed and lysed in commercial buffers (BD FACSTM - BD FACS Lysing Solution, BD PhosflowTM - Lyse/Fix Buffer) and frozen afterwards at  $-80^{\circ}\text{C}$  in the same buffer, without the need of centrifugation [5,36,37]. It is important to mention that both Immunophenotyping and Phosphoflow assays have their own requirements and limits during this freezing approach:

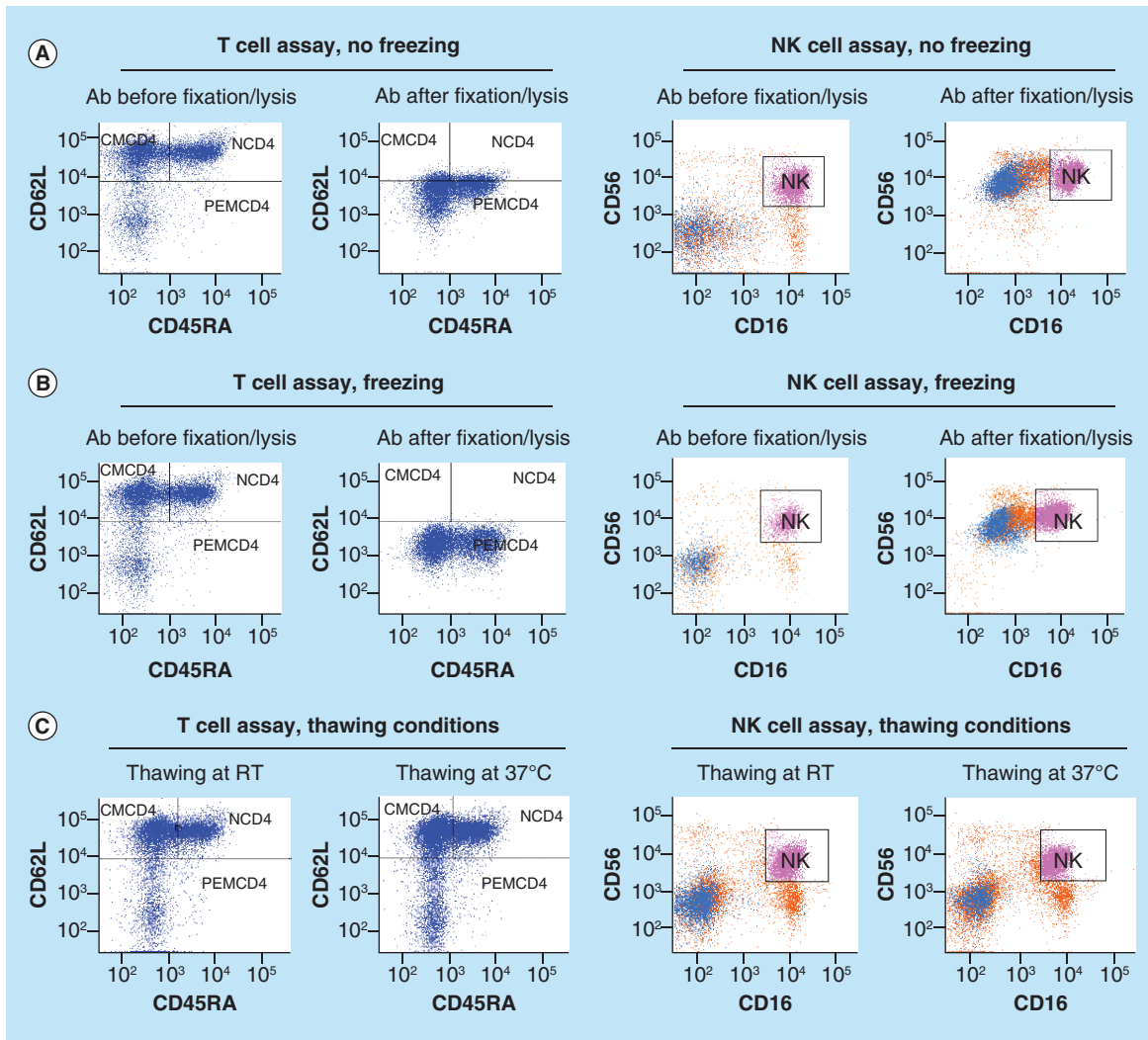
Different staining procedures of Immunophenotyping assays such as staining before fixation and freezing at  $-80^{\circ}\text{C}$  versus staining afterwards might influence staining patterns and intensities. We tested the impact of fixation/lysis, freezing and thawing on a T cell assay detecting CD4 naïve and memory T cell subsets using CD45RA and CD62L staining and on a NK cell assay detecting NK cells using CD16 and CD56 markers. Figure 2A shows the effect of fixation and lysis on the antibody staining. It is clearly visible that the staining before fixation and lysis of the sample gives an appropriate signal whereas the staining after fixation

and lysis affects the results. Interestingly, freezing and thawing of the samples does not influence the staining pattern (Figure 2B&C).

This finding is in line with the publication of Pinto *et al.* in 2005. This group describes a similar approach to freeze whole blood for immunophenotyping assays and they could also show differences in fluorescence intensity for several markers when different fix and stain procedures were used [38]. Therefore, it is recommended to define during assay development which procedure fits best for the given antibody panels used for the staining. If possible, staining before fixation and freezing should be avoided because this would be an additional step for the clinical sites to perform. In cases where no alternative antibodies can be used which would work after fixation, the final antibody mixes need to be provided to the sites and the clinical site personnel should be trained on the staining procedure. Importantly, when stored at  $-80^{\circ}\text{C}$  the samples are usually stable until 4–6 weeks without loss of sample quality or signal intensity, which enables batch analysis of the samples. Using this freezing procedure it is possible to measure complex immunophenotyping panels including panels for B cell subsets, T cell subsets, comprehensive leukocyte subsets and rare cell population in multicenter studies. A similar approach is also described by Hensley *et al.* [39]. This group compared the stability of samples at  $4^{\circ}\text{C}$  versus  $-80^{\circ}\text{C}$  and demonstrated that freezing provides a better stability than  $4^{\circ}\text{C}$ . They also show that samples kept at  $-80^{\circ}\text{C}$  maintain their integrity for up to 120 days for surface markers but that activation markers showed deterioration after only 13 days [39].

The freezing approach of Phosphoflow samples is similar to immunophenotyping assays; however another buffer is needed in order to fix the phosphorylation signal appropriately (we use the BD PhosflowTM - Lyse/Fix Buffer). When PhosphoFlow samples are frozen at  $-80^{\circ}\text{C}$  and measured in house these samples are stable for at least 2 weeks and do not lose quality or show clots. During our first clinical study where this approach has been applied and frozen samples were shipped on dry ice to the analysis lab, we have observed that some of the Phosphoflow samples have been clotty and could not be analyzed. Thus, the combination of freezing and shipment on dry ice might have influenced the sample integrity. In order to avoid these issues and to optimize the freezing and shipment condition for clinical samples, we have investigated different conditions and found out that several steps might influence the sample quality of Phosphoflow assays: one key point is the proper lysis of erythrocytes, which should be done at  $37^{\circ}\text{C}$  for at least 30 min for Phosphoflow assays before





**Figure 2.** Influence of fixation/lysis, freezing and thawing on staining of blood samples. **(A)** Blood samples were either stained before or after lysis and fixation using a commercial lysing and fixation buffer. Cells were acquired without freezing. **(B)** Same blood samples as above have been frozen over night at  $-80^{\circ}\text{C}$  in the commercial lysing and fixation buffer. **(C)** Frozen samples have been thawed either at RT or  $37^{\circ}\text{C}$  water bath. Left: T cell assay: T cell subsets were detected using CD4, CD62L and CD45RA staining. Right: NK cells were detected using CD56 and CD16 staining. (Novartis in house data, blood was received from Novartis internal blood donor bank and healthy human donors). Ab: Antibody; RT: Room temperature.

freezing. Another critical aspect is the time of sample storage which influences sample quality: this includes the storage time before shipment as well as the time the samples can be stored at  $-80^{\circ}\text{C}$  after shipment. We have experienced that samples should be stored maximal 3–5 days at  $-80^{\circ}\text{C}$  before shipment and that samples should be further processed and measured within one week after arrival at the analysis lab. Duration of dry ice shipment should also be as short as possible and not longer than 2 days. When these steps are not performed accurately the samples will be clotty and cannot be analyzed.

Importantly, we have not observed that freezing and shipment condition alters the expression of mark-

ers. This is in line with the data of Hensley *et al.* who show that shipment at  $-80^{\circ}\text{C}$  does not influence the signals [39]. This comparison of fresh, frozen and shipping condition is an important test that we run during assay development and validation of PhosphoFlow assays. In contrast to the PhosphoFlow samples, immunophenotyping assays are not affected by the shipment condition.

In order to optimize sample quality after fixation and freezing we are currently also testing new buffers and conditions. Also the type of blood collection tubes might improve sample quality under freezing and shipment conditions. So far we use mainly Na-heparin monovette collection tubes or EDTA vacutainer. It is

recommended to add the assessment of fresh versus freezing condition and the impact of shipment conditions to the usual procedure during assay development and validation.

### Clinical study implementation

Because of the complexity of flow cytometry assays, read outs and instrument parameters, standardization of methods across sites is an ongoing challenge for multicenter studies. There are several variables that can contribute to intersite distinctions. Maecker *et al.* reported that activation at a centralized site reduced variability as compared with activation of whole blood at the individual sites. However, for some clinical settings it is inevitable that some steps such as antibody staining, *ex vivo* stimulation or lysis and fixation of blood sample needs to be done at the clinical sites [40]. Therefore, the success of the implementation of flow cytometry assays in multicenter studies is heavily based on the possibility to simplify complex assays, on a comprehensive training of the site and provision of clear documentations.

We have developed a unique kit approach which avoids pipetting of a fixed volume of blood or buffer but uses plastic Pasteur pipettes to transfer blood, dilute lysing solution and add antibody mixes to the blood. In our lab we have validated the use of defined number of drops of blood using the plastic Pasteur pipettes instead of pipetting a specific amount of blood. This kit approach simplifies sample processing and reduces the needed equipment such as pipettes at clinical sites. This also enables clinical sites without lab experience to perform these assays.

Another new area is to supply clinical sites with plates containing lyophilized reagents already distributed to the appropriate wells [40] or even to provide clinical sites with blood collection tubes containing stimulation reagents [41]. Introducing these methods greatly reduce variability across sites and simplify processing steps at the clinical sites.

In case assays cannot be further simplified and certain steps need to be done at the clinical site, our strategy is that sites need to be trained and certified for the assay procedure, especially for *ex vivo* stimulation assays. Optimally, on-site training where the sites are visited in person and each steps and the hurdles of the assay are demonstrated to the lab personnel. When this is not possible due to logistics, costs, etc. a video demonstration helps to show the assay procedures and the kit items to be used. The advantage of the video demonstration is that the sites can replay this at any time to refresh their memory. In all cases, the sites need to be qualified by the experts on the assay performance by performing test runs of

the assay independently, and shipping the samples to the flow cytometry analysis lab. The assay procedure, test run set up and acceptance criteria of the test runs are detailed in an analytical study plan which is provided to the clinical sites which enables the similar sample processing procedure at different sites. According to the performance and the acceptance criteria which are set upfront the site is qualified or is requested to repeat the test. Standardization of reporting should follow the MIFlowCyt guidelines to provide further continuity across multiple studies and site locations [34].

### Conclusion & future perspective

In this article, we have reported a summary of the potential uses and challenges for implementing highly sophisticated flow cytometry assays in multicenter settings. Flow cytometry offers a vast array of analytical uses and read-outs which can aid in addressing numerous clinical needs, from identifying circulating leukemia cells to accurately detecting CD4+ T cell counts for HIV patients. Strides are being made to standardize flow cytometry methods and address core issues in harnessing this powerful technology. To this end, we have discussed here recent advances and challenges in validating flow cytometry assays including the implementation of QCs and the general lack of a true accuracy measurement. The inclusion of *in vitro* dose–response curves in the validation process of PD assays is addressed as well in order to mimic PK/PD relationship, to assess variability between donors and day-to-day variability of one donor and to get information about the LOQ of the assay. Furthermore, the implementation of flow cytometry assays across sites is an important factor in making this technology both practical and useful. It has been reported that centralized data analysis and provisions for premade lyophilized reagents can greatly decrease variability across sites. Also the use of a kit approach simplifies and minimizes sample handling and reduces needed equipment at the different sites. Of paramount importance is sample stability and shipment. We report here that fixation with commercially available buffers followed directly by freezing at  $-80^{\circ}\text{C}$  can stabilize blood for both immunophenotyping and PhosphoFlow. This allows the initiation of a myriad of different assays at a clinical site, followed by shipping to an experienced flow cytometry lab for sample acquisition and detailed analysis at one central site, thus, eliminating variability of sample analysis on multiple flow cytometers. This simplified fixing and freezing of whole blood directly, without the need for PBMC isolation or extra centrifugation steps, allows for

greater consistency of sample integrity from various clinical sites. Taken together, it is apparent that the use of flow cytometry in the clinical biomarker setting is rapidly evolving and will continue to play a vital role in the enhancement of human health in the years to come. Continued work on standardization of assay validation and clinical sample analysis, simplification of assay procedures at the sites, the conduct of on-site trainings or the provision of other detailed training documents, etc. will be fundamental for the successful implementation of flow cytometry assays

in multicenter clinical studies and to generate reliable data to support the drug development process.

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## Executive summary

### Technical overview and applications of flow cytometry

- Flow cytometry is a powerful tool which uses fluorescence to assess individual cell markers and populations within a given sample. Staining with fluorescently conjugated antibodies is most frequently used for the identification of cell populations and subpopulations based on surface markers. Flow cytometry provides an extensive variety of applications which can be applied to collect multiparameter data from heterogeneous whole blood populations down to single cell analyses.

### Use of flow cytometry for biomarker analysis in clinical studies

- Flow cytometry assays like detailed leukocyte subset analysis, measurement of rare cell populations, disease relevant expression markers, receptor occupancy, activation of cells analyzed by cytokine production, activation markers and signaling pathways provide important information on PDs, disease progression, treatment effect and patient stratification.

### Development and validation of flow cytometry for clinical use

#### Summary of current guidelines and key challenges

- Accuracy assessment is challenging for flow cytometry assays since the needed reference material or quality controls are not available.
- Antibody specificity to be verified, for example, by lack of detection in an irrelevant population, competition assays, by selective inhibition of the pathway and hereby inhibition of the signal. Limited blood stability is a challenge to perform interassay precision tests: it is recommended to perform interassay variability tests on the same day but using different set of reagent mixes in order to mimic different assay runs and to ensure that blood sample quality is comparable.
- Biological variability is recommended to assess during assay validation: interdonor as well as intradonor (day-to-day variability within one donor) variability.
- Defining the limit of quantification is still not a standard practice for flow cytometry validations.
- Centralizing flow cytometry acquisition and analysis helps to minimize lab-to-lab variations.

### Considerations for the validation of flow cytometry assays used for PK/PD assessment

- The inclusion of *in vitro* dose–response curves in the validation process of PD assays mimics the PK/PD relationship, assesses the sensitivity and the variability between donors and the day-to-day variability within one donor and provides information about the limits of quantification of the assay.

### Whole blood flow cytometry assays in multicenter clinical studies

#### On site processing, stabilization of samples and clinical study implementation

- Whole blood is the preferred matrix for cellular biomarker assays in clinical studies.
- The use of whole blood samples may require complex sample processing steps at the clinical sites which can range from proper staining of samples in replicates for immunophenotyping assays to *ex vivo* stimulation needed for pathway activation.
- Fixation and lysis of the whole blood samples is also an important step to be done at the clinical site.
- The use of commercially available buffers allows fixation, lysis and freezing of whole blood samples at  $-80^{\circ}\text{C}$ , which can stabilize whole blood samples for both immunophenotyping and Phosphoflow assays. This allows the initiation of different assays at a clinical site, followed by shipping to an experienced flow cytometry lab for sample acquisition and detailed analysis at one central site. This simplified fixing and freezing of whole blood directly, without the need for PBMC isolation or extra centrifugation steps, allows for greater consistency of sample integrity from various clinical sites.
- Simplification of assays is recommended for multicenter trials: the use of a kit approach simplifies and minimizes sample handling and reduces needed equipment at the different sites.

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