Challenges of flow cytometry validations

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1 INTRODUCTION

Multi-parametric analysis of thousands of cells per second to adequately identify or functionally characterize complex cell populations of interest can be achieved using flow cytometry platforms. Spanning from basic research, discovery, preclinical to clinical, flow cytometry is a valuable tool, especially with the increasing proportion of biologics in the pipeline. Flow cytometry has proven itself to be an indispensable tool to assess safety, receptor occupancy (RO) or pharmacodynamics (PD).

The development and validation of flow cytometry based methodologies can be challenging, given it involves a cellular aspect, that standardized cellular reference materials are limited and that these assays are often used for multiple different purposes. It is critical to know up front what the flow cytometer assay will be used for in order to conduct the appropriate validation to support GLP studies. No guidelines for the validation of flow cytometry methods are currently available for the preclinical setting. Various working committees have taken initiatives in the writing of guidance documents describing flow cytometry method validation. However, these recommendations have not yet been integrated in an official document released by the regulatory agencies as it has been done for other analytical methodologies.

The validation parameters commonly used for the validation of flow cytometry are presented in this poster as well as three case studies with validation designs adapted to address challenges such as sample stability limitations for shipment , inherent variability of functional endpoints and low frequency populations. For each case study, in addition to the validation parameters presented, all the validation parameters in Table 1 were also included (data not shown).

Table 1: Validation parameters commonly assessed in flow cytometry validations
Each laboratory has slightly different approaches for validation of flow cytometry methods. However, the following parameters are dealt with in a common manner.

Parameter	Assessment	Acceptance criteria
Antibody Titration	≥ 5 dilution/antibody	Optimal dilution: Clear and stable positive staining and minimal background level (negative population). The staining intensity of positive and negative cells are compared by calculating the signal over noise ratio using the MFI values.
Precision - Intra assay - Inter assay/analyst	≥ 5 samples Intra: ≥ 3 replicates/sample, 1 assay Inter: 1 replicate/sample, ≥ 3 assays (*done by different analysts*)	CV ≤ 20% (30% for low frequency populations)
Day-to-day variability	≥ 5 samples Collection over ≥ 3 occasions *Not applicable for terminal sample collections*	CV ≤ 20% (30% for low frequency populations)
Specificity	Isotype matched controls (IC) compared to specific antibodies	ICs should have a low signal and the antibodies should yield a positive signal
Antibody Interaction	Fluorescence minus one (FMO): the panel minus one of the antibodies vs. the full panel.	The FMO is expected to have a similar signal to the full panel when one antibody is removed, while showing low signal in the empty channel
Reference range	≥ 5 samples/sex	None (the mean, range and SD are reported)
Stability (pre/post staining)	≥ 3 samples/sex T=0 (reference): Samples are processed and acquired as soon as feasible T=X: Samples are processed X hours after collection TF= X: Samples are kept for X hours between staining completion and acquisition	Difference to the reference sample ≤ 25-30%

2 Case Study 1

Development of a flow cytometry assay for the measurement of basophil activation in the context of a Phase III clinical study

Assay Design: Human whole blood samples were spiked with the different controls (or compounds in the clinical study) and further stained with an anti-CCR3 and anti-CD63 antibody. The validation stimulation conditions tested included a negative control (PBS) and two positive controls (anti-FcεRI and fMLP). Basophils were identified as CCR3+ and upon activation, CD63 became externalized and present at the surface of the cells. Therefore, activated basophils were quoted as percentages of CD63+ cells from the CCR3+ population. A stimulation index (SI) was calculated for the positive controls (stimulated sample divided by negative control). Samples were considered positive if the % activated basophils was ≥ 5% and the SI was ≥ 2. For this study, commonly used validation parameters were tested (see Table 1), but extending the stability was critical since clinical samples were to be shipped to Canada from various countries around the world including Australia.

Storage condition	Stability Treatment	Acceptance criteria	Results Negative control (% of CD63+)	Results Anti-FcεRI (SI)	Results fMLP (SI)
Fresh blood (RT)	Blood from 10 donors were processed as soon a feasible (used as reference samples)	The percentage difference between the stability sample and the reference sample was to be within ±30% for at least 80% of donors.	24 hrs: ≤ 200%	24 hrs: ≤ 400%	24 hrs: ≤ 250%
Refrigerated blood (4°C)	Blood from the same donor samples were stained at least 24, 48 and 56 hours post collection		48hrs: ≤ 200% 56 hrs: ≤ 400%	48hrs: ≤120% 56 hrs: ≤ 90%	48hrs: ≤ 80% 56 hrs: ≤ 120%

Table 2: Stability Treatment and Results
The results following the stability treatments for most of the donors were not within 30% difference of the reference samples. However, all samples that tested positive with the reference samples (with the positive controls) remained positive after up to 56 hours post-blood collection (% activated basophils was ≥ 5% and the SI was ≥ 2) .

Conclusion:
Stability limitations with whole blood is not unusual for cell functional assays. Given that the assay could be conducted as a qualitative screening assay to determine whether a compound tested was positive or negative, the whole blood sample stability up to 56 hours post-collection was considered acceptable.

3 Case Study 2

Development of a flow cytometry assay for the measurement platelet activation

Assay Design: The assay was designed to either monitor in vivo platelet activation or to assess in vitro platelet activation in the context of anti-platelet drug testing. Non-Human Primate (NHP) blood from naïve animals was activated for exactly 2 minutes with adenine di-phosphate (ADP) as the agonist, and unstimulated blood was used as baseline. Immediately after the stimulation, the platelets were stained for immunophenotyping. Activated platelets were identified as the CD61+ population using side scatter properties. Activation of platelets were assessed by cell surface expression of p-selectin (CD62p) and activated GPIIb/IIIa complex (PAC1). The results were reported as the percentage of platelets that were activated (% of CD62p+ platelets and % of PAC1+ platelets).

Commonly used validation parameters were tested (see Table 1) with the addition of parameters specifically related to the assay design. A) Agonist (ADP) titration and stimulation time course (data not shown). The goal of these evaluations was to determine the optimal assay conditions to have good reproducibility while keeping the non-specific activation to a minimum with the unstimulated control. B) Both the precision of the staining and of the stimulation were assessed. As this method required rapid activation of the platelets followed by staining, assessing the reproducibility of the activation and the reproducibility of the staining separately was required to better understand the assay robustness.

Intra assay	Animal ID	Stimulated platelets CD62+	Stimulated platelets PAC1+	Unstimulated platelets CD62+	Unstimulated platelets PAC1+
Staining Precision	1	3.9	2.9	6.2	25.6
	2	6.6	7.2	47.4	33.5
	3	5.6	11.3	9.5	34.1
	4	2.2	12.0	8.1	18.1
	5	2.7	23.5	18.0	26.3
	6	2.2	5.2	29.4	37.6
Stimulation Precision	1	5.9	12.1	8.1	18.1
	2	6.2	10.9	18.0	26.3
	3	2.2	24.9	29.4	37.6

Table 3: Intra assay precision results (%CV of n = 5 per animal)
- For stimulated samples, the staining and stimulation precisions were within acceptance criteria (%CV ≤ 20). For the animal with a %CV > 20 , the % of PAC1+ platelets was very low for one of the 5 replicates indicating suboptimal activation or staining. This was addressed by processing the samples in duplicate.
- For the unstimulated samples, the staining and stimulation precisions were not within acceptance criteria for both CD62p and PAC1. Since low levels of activation was expected for the unstimulated samples, it was concluded that the platelets were well handled (limited non-specific activation) and that the high %CV was mainly due to the low values obtained for the staining of these cells.

Conclusion:
- The concentration and timing of the agonist should be taken into account when developing functional assays involving stimulation.
- Variability associated with the staining as well as the stimulation should be assessed to better understand the assay and the approach required to address the variability observed with the functional endpoint.
- With functional assessments involving a stimulation step, the acceptance criteria selected for the unstimulated condition should account for the potential absence of signal in the negative or baseline controls.

4 Case Study 3

Development of a flow cytometry assay for the measurement of regulatory T cells in rat whole blood and thymus

Assay Design: Rat blood and thymus samples were stained for regulatory T cells assessment using CD3, CD4, CD25 and FoxP3 as cell markers. Results were reported as percentage of lymphocytes and absolute counts (cells/µL of blood or cells/thymus).

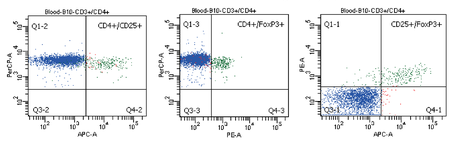

Figure 1: Example of flow cytometry analysis (Blood)
The frequency of regulatory T cells in rat blood was very low (< 1% of lymphocytes). Since this population is rare, the background in the assay could have an impact on the percentage of regulatory T cells reported. Consequently, a limit of detection parameter was included in the validation in addition to the commonly used validation parameters (see Table 1).

Limit of Detection Assessment (LOD): The LOD is used to determine the level of background noise in the overall relative percentage determined for each cell population of interest. Fluorescent minus one (FMO) controls were used to determine the LOD. The frequency of false positive events determines the lower limit of detection. The LOD was calculated as the mean of all animals for each gated region of interest + 3SD. The LOD was assessed on 3 samples, each processed in 5 replicates for each FMO.

Parameters	Blood CD4+/CD25+	Blood CD4+/FoxP3+	Thymus CD4+/FoxP3+
LOD	0.29%	0.07%	0.02%
Reference range (% of Lymphocytes)	1.5 - 3.8%	1.7 - 3.8%	0.2 - 1.8%

Table 3: Blood and thymus LOD Results
LOD were calculated and compared to the reference range.

Conclusion:
For smaller populations, the limit of detection (LOD) was shown to be important in the interpretation of the data especially, when considering the normal range of this population in blood and thymus samples. Therefore, an additional control condition was included , where FMOs were used as daily control for future samples analysis.

5 CONCLUSION

- The parameters included in the validation of an assay depend on the purpose of the assay. Parameters, such as precision and stability, should always be included in validations. However, relevant additional parameters should be well thought of and included on a fit for purpose approach.
- In cases when limitations of the method are identified, then adaptations of the analysis or reporting strategies can be considered based on the purposes of the assay.