Antigen-specific T cell characterization by simultaneous detection of activation induced markers and intracellular cytokines: a multi-site testing experience

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Abstract

Introduction. During the COVID-19 pandemic, the capacity of several SARS-CoV2 variants to escape the vaccine-induced neutralizing antibody response, together with the observation that T cell mediated immunity was essentially conserved, led to an increased interest in the characterization of antigen-specific T cells by flow cytometry. In this context, we recently published an optimized protocol for simultaneous detection of T cell activation-induced markers and intracellular cytokines, which is based on overnight PBMC stimulation with Spike peptide pool and subsequent flow cytometry characterization using a 12-marker panel (Live-Dead, CD3, CD4, CD8, CD45RA, CCR7, CD137, CD69, CXCR5, IFNγ, TNFα, IL-2)(see references). With the aim of understanding if this protocol could be successfully reproduced in different laboratories, we established a network of five testing sites who characterized a total of 40 SARS-CoV2 reactive subjects accounting for 200 acquired samples.

Methods. Each site worked with a different flow cytometry platform: four analyzers and one cell sorter, three out of five instruments being equipped with a yellow-green laser. Operators were trained and supported during the first experiment execution, a common acquisition template was distributed, instrument settings were harmonized using hard-dyed beads, and data analysis was centralized (Figure 1). Five specific aspects were investigated: 1) the capacity to successfully report data for each analyzed sample; 2) the impact on Stimulation Index (S.I. = ratio between activated T cells in stimulated and unstimulated samples) of intracellular (ic) vs surface (s) CD4+ and CD8+ staining; 3) the impact on S.I. of aCD28 co-stimulation during PBMC activation; 4) the consistency of analysis when different operators evaluated the same raw data files; 5) the estimated limit of detection of the assay.

Results. Two donors out of 40 were discarded for poor sample quality and three were not reported after analysis due to low cell counts; in addition, three samples required a modification in the gating strategy to remove unwanted background. CD4+ and CD8+ intracellular vs surface staining were assessed on nine donors, revealing a lower ic-CD4+ vs s-CD4+ S.I. (ic-CD4+ S.I. = 5.3; s-CD4+ S.I. = 8.7; p-Value = 0.036) (Figure2). No significant differences were detected for the CD8+ subset. Interestingly, samples activated without aCD28 (n=26) revealed a significantly higher CD4+ S.I. (9.4 vs 4.3; p-Value = 0.001) and cytokine-producing CD4+ S.I. (13.6 vs 6.4; p-Value = 0.002) (Figure 3), which was driven by a 54% reduction of background activation in unstimulated samples for the total activated CD4+ subset and by a 43% reduction of background activation for the cytokine-producing CD4+ subset (Figure 4). Corresponding stimulated samples were also characterized by lower activation yet to a milder extent (18% and 16%, respectively) (Not shown). No relevant variations were reported for the CD8+ compartment.

When different operators analyzed the same raw data from three stimulated samples, coefficients of variation for reported subsets were inversely related to the abundance of cells. CD4+ and CD8+ being respectively 65.8% and 22.6% of CD3+ were reported with %CV of 2.6 and 9.4, whereas activated CD4+ and CD8+ being respectively 0.11% and 0.30% of parental T helper and cytotoxic cells were reported with %CV of 18.6 and 30.5.

10^6 PBMCs were stimulated per sample, leading to an average acquisition of 21,611 and 11,720 live CD4+ and CD8+ cells/sample. Considering the requirement of 20 recorded events to detect a given subset, the average lower limit of measurable activation was 0.09% for CD4+ and 0.17% for CD8+. One stimulated sample out of 26 displayed CD4+ activation below the limit and two stimulated samples displayed CD8+ activation below the limit. S.I.≥3 was required to define responder samples: CD4+ response was detected in 20 out of 25 samples and CD8+ response was detected in 8 out of 24 samples.

Conclusions: The method was successfully implemented in all the testing sites, use of s-CD4 and s-CD8 staining as well as avoidance of co-stimulation with aCD28 are recommended. Limit of detection is estimated at 0.09% for CD4+ and 0.17% for CD8+ events.

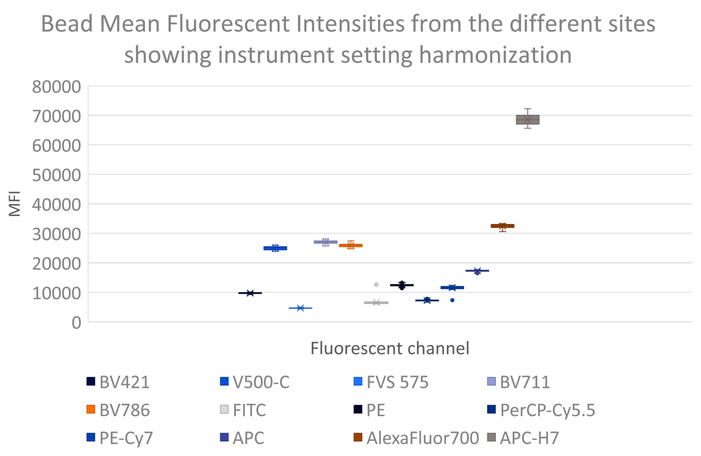
Figure 1 - Characterization of different donors performed by diverse operators on diverse instruments provides similar staining patterns

A

Specificity	Dye
T cell Backbone	
CD3	PerCP-Cy5.5
CD4	APC-H7
Live / Dead	FVS V575 (BV605)
T cell maturation	
CD45RA	BV786
CCR7	PE
Activation Markers	
CD137	APC
CD69	PE-Cy7
Th1 cytokines	
IFNg	FITC
TNFa	AlexaFluor™700
IL-2	BV711

Alexa Fluor is Trademark of Thermo Fisher Scientific
BD Horizon Brilliant Violet is Trademark of Becton, Dickinson & Company

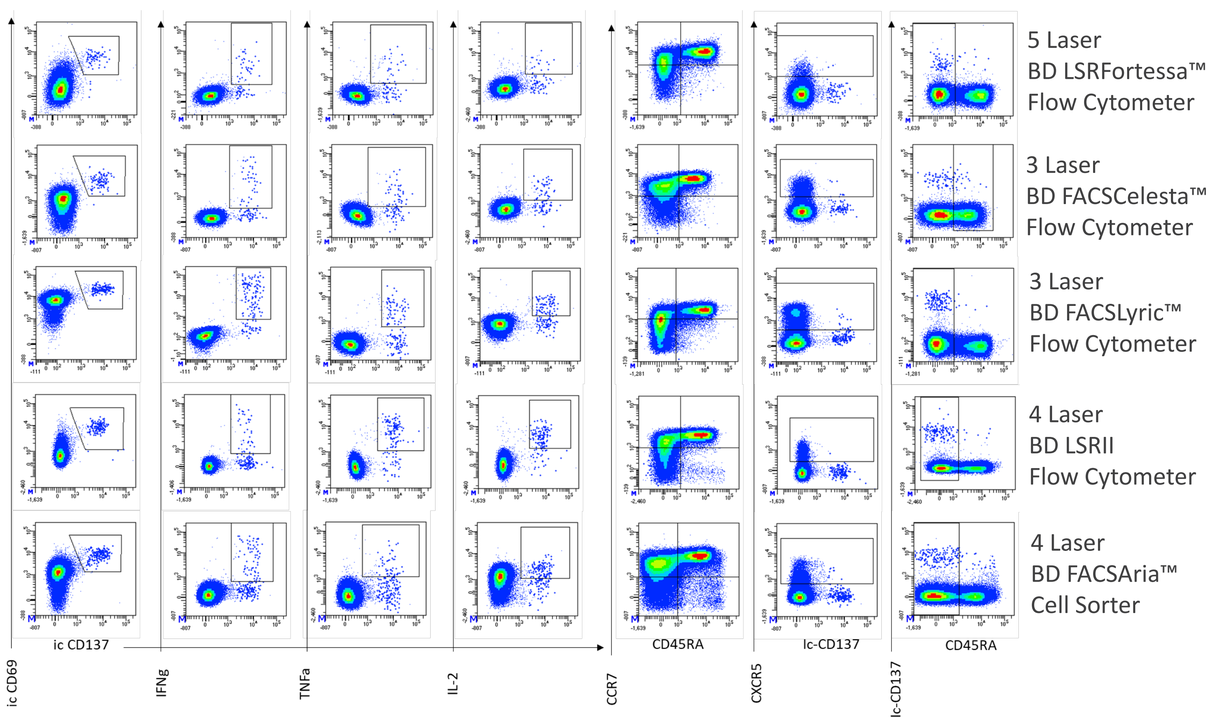
B



Dye	BV421	V500-C	FVS 575V	BV711	BV786	FITC
Average MFI	9743.1	25048.6	4649.3	27031.9	25898.5	6454.5
% CV	2.2	2.3	2.9	2.0	2.5	3.8

Dye	PE	PerCP-Cy5.5	PE-Cy7	APC	AlexaFluor700	APC-H7
Average MFI	12499.4	7246.8	11668.9	17297.5	32440.3	68572.0
% CV	3.1	3.3	3.6	1.9	2.0	2.8

C



A **Flow cytometry panel overview**

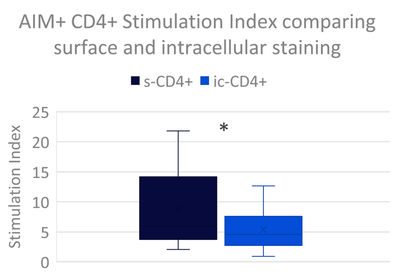
B **Instrument setting harmonization**

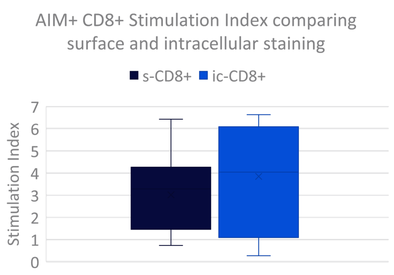
Instrument settings were harmonized by means of BD FACSDiva™ CS&T Research Beads. The same fluorescent target values were set in all instruments for every channel and beads were acquired before every working session.
The panel shows channel specific MFIs and %CVs across instruments and acquisitions.

C **Exemplary CD4+ T cell staining pattern**

The panel shows exemplary CD4+ T cell staining patterns from independent SARS-CoV-2 reactive samples (frozen PBMCs) activated with Spike peptide pools. Cells were processed by different operators working in different sites and acquired with diverse platforms. Each operator was trained on the protocol and the analysis was centralized. Staining patterns always looked similar during the testing phase and could be analyzed using the same gating strategy pending small sample specific gate adjustment.

Figure 2 – CD4 and CD8 markers do not require intracellular (ic) staining





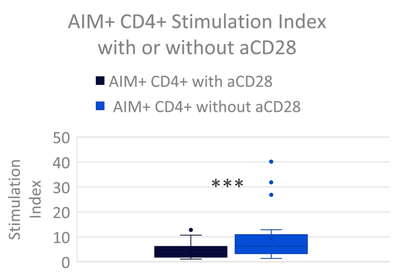
Average ic-CD4+ S.I. = 5.3
Average s-CD4+ S.I. = 8.7
p-Value = 0.036

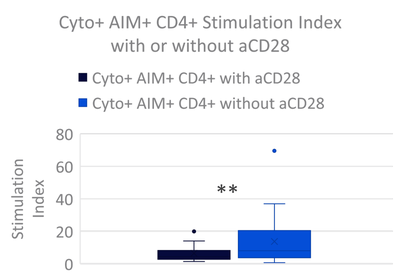
* = p<0.05
** = p<0.005
***=p<0.0005

CD4+ and CD8+ T cell Stimulation Index with surface and intracellular staining

9 samples were stimulated across the different sites with Spike peptide pools and subsequently stained with the 12-color panel using surface or intracellular CD4 and CD8 antibodies. Stimulation Index (S.I.) was calculated as the ratio between Activation Induced Marker positive (AIM+) cells in stimulated and unstimulated samples. The use of intracellular CD4 and CD8 markers did not result in higher Stimulation Index, rather ic-CD4 staining resulted in slightly higher background activation thus lowering the related Stimulation Index.

Figure 3 –CD4+ Stimulation Index is higher without using aCD28 co-stimulation





Average CD4+ S.I. without aCD28 = 9.4
Average CD4+ S.I. with aCD28 = 4.3
p-Value = 0.001

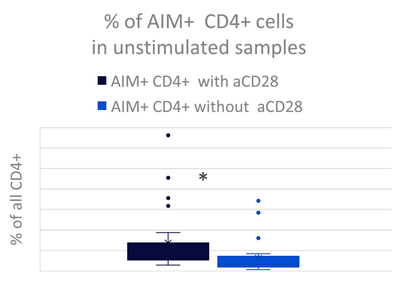
Average Cyto+ CD4+ S.I. without aCD28 = 13.6
Average Cyto+ CD4+ S.I. with aCD28 = 6.4
p-Value = 0.002

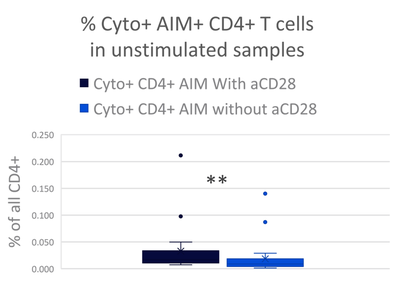
* = p<0.05
** = p<0.005
***=p<0.0005

CD4+ Stimulation index with or without aCD28 co-stimulation

26 samples were stimulated across the different sites with Spike peptide pools in the presence or absence of aCD28 used as co-stimulus. Unstimulated samples were treated accordingly with or without aCD28. Multicolor flow cytometry revealed a significantly higher Stimulation Index when samples were not co-stimulated with aCD28. The pattern was conserved for total activated and for cytokine producing CD4+ T cells.

Figure 4 – Absence of aCD28 results in lower background activation of unstimulated samples





CD4+ background activation
-54% without aCD28
p-Value = 0.0059

Cyto+ CD4+ background activation
- 43% without aCD28
P-Value = 0.0025

* = p<0.05
** = p<0.005
***=p<0.0005

CD4+ background activation

CD4+ T cell background activation was lower in the absence of aCD28 co-stimulation, either for total activated CD4+ T cells (AIM+) and for the cytokine-producing subset (Cyto+ AIM+).

Figure 5 –Lower limit of detection and reproducibility of analysis

Lower limits of detection are 0.09% for AIM+ CD4+ and 0.17% for AIM+ CD8+

10^6 PBMCs were stimulated per sample, leading to an average acquisition of

- CD4+ average acquired events = 21,611
- CD8+ average acquired events = 11,720.

Requirement of 20 recorded events to detect activated cluster was considered according to F. Buccisano et al. Haematologica. 2022 Dec 1; 107(12): 2823–2833. doi: 10.3324/haematol.2021.279777

Different operators provided consistent results when analyzing the same raw FCS data (N=3)

%CV for reported CD4+ = 2.6
%CV of reported CD8+ = 9.4

%CV for reported AIM+ CD4+ = 18.6 being AIM+ CD4+ = 0.11% of total CD4+

%CV for reported AIM+ CD8+ = 30.5 being AIM+ CD8+ = 0.3% of total CD8+

Methods

10^6 cells/sample were seeded per well in a 96-u bottom plate and stimulated with 1 µg/ml of PepTivator® SARS-CoV-2 Prot S, Prot S1, Prot S+ or with 1 µg/ml of PepTivator® of CMV pp65 and CMV IE-1 (Miltenyi). Monoclonal antibodies anti-CD28 at 1 µg/ml were added as co-stimulus. After 4h of incubation at 37°C, 5% CO2, the protein transport inhibitor BD Golgi-Plug™ (Brefeldin A, 1µl/ml) was added for a further incubation of 20 hours.
Detailed methods are available at
https://www.sciencedirect.com/science/article/pii/S002217592300025X?via%3Dihub#s0055

Conclusions

This method was adopted in five different laboratories and the 12-color panel provided meaningful results on 38 samples out of 40 when acquired on different flow cytometry platforms. The use of intracellular staining for CD4 and CD8 markers is not required and the co-stimulation with aCD28 increased T cell background activation thus reducing the final CD4+ Stimulation Index.

REFERENCE: J Immunol Methods. 2023 Apr;515:113443. doi: 10.1016/j.jim.2023.113443. E pub 2023 Feb 24. Tiziana Altosole et al.
An optimized flow cytometry protocol for simultaneous detection of T cell activation induced markers and intracellular cytokines: Application to SARS-CoV-2 immune individuals.