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BRIEF REPORT



FRET causing misleading signal from fluorescein excited by the violet laser in flow cytometry

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Abstract

Multiple immunolabeling introduces high risks of interferences between fluorescences. As an example, in analyzing T cell clonality, we recently reported a fluorescence resonance energy transfer (FRET) effect providing an unexpected signal on B770 (PE-Cy7) detector, on the V β -PE positive CD3 APC-Alexa750+ T cell subsets. Here, we report another FRET effect produced by the violet laser in V β -FITC positive CD3-Pacific Blue (PB) T cells providing signal on V550 (Krome Orange; KrO) detector. The study was performed on fresh whole blood, labeled with anti-CD3-PB, CD8-KrO, Vbeta FITC, Vbeta PE, CD4 AA750 then fixed, treated for erythrolysis, and washed before analysis on DxFlex cytometer from Beckman Coulter. Data were analyzed using Kaluza software. Using this panel, we repeatedly observed an added CD8dim-KrO (V550) cell population on all V β FITC positive T cells. The unexpected green signal excited by the violet laser was still observed after removing anti-CD8-KrO (FMO) but disappeared where either anti-CD3-PB or anti-V β -FITC was removed. The effect was also observed with an anti-TCR gamma delta-FITC labeling, but not with another FITC labeled antibody targeting a protein out of the CD3-TCR complex. The analysis of fluorochrome spectra confirms that PB emission and FITC excitation spectra partly overlap. This observation clearly reminds users that FRET can give misleading results in case of labeling of very close markers with complementary fluorochromes. This risk has to be considered in panel design. These observations clearly highlight the potential for FRET to give misleading results in cases where very close markers are labeled with complementary fluorochromes. This risk must be considered when designing panels. To our knowledge, this is the first description of a FRET between PB and FITC as acceptor thus excited by the violet laser.

KEYWORDS

FITC, flow cytometry, FRET, Pacific Blue, phycoerythrin, violet laser

1 | INTRODUCTION

Simultaneous detection of multiple fluorochrome labeled immunolabelings is an exceptional feature of flow cytometers. In most modern

cytometers, Blue (B, 488 nm), Violet (V, 405 nm), Red (R, 630 nm) lasers aim to excite successively fluorochromes to produce new light detected on different optical benches. However, some fluorochromes (acceptors) can also be excited by a near electric fields of the

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oscillating electric dipoles produced by another fluorochrome (as donor) named fluorescence resonance energy transfer (FRET). The probability that fluorescence emission of a dye could directly excite another dye at molecular proximity, leading to secondary fluorescence, is practically zero unless provided the two fluorochromes are in a distance comparable to the critical distance (below 10 nm) as described by Förster's formula [1] and if donor emission spectrum overlap with acceptor excitability spectrum. FRET is commonly used as tandem conjugation, making possible to detect multiple colors with one single light source [2]. Few fluorochromes are well known being good “donor” of signal for FRET like phycoerythrin (PE), peridinin-chlorophyll-protein (PerCP) or allophycocyanin (APC), while other fluorochromes like Texas Red (625 nm), Cyanine 5 (660–675 nm), Alexa Fluor 700 (700–710 nm), Cyanine 7, Alexa Fluor 750 or H7 (780–790 nm) are the most used “acceptor” in conventional tandems [3, 4]. In a recent publication, we have shown an incident FRET effect on the blue laser (488 nm) optical bench in double labeling of two components of the T cell receptor complex [5]. In this example, unexpected B755 signal (on blue laser bench) was detected due to excitation of Alexa Fluor 750 from APC-Alexa Fluor 750 tandem expected to be excited by the red laser (R755). Alexa Fluor 750 was then excited by FRET from PE conjugate that labeled a very close component of the complex. In order to avoid this phenomenon, we have tempted a new fluorochrome combinations to characterize T cell clonotypes and faced a new, unknown, FRET effect generated by the violet laser (Supporting Information).

The new panel included T cell receptor (TCR) labelings using an anti-CD3 conjugated with Pacific Blue (PB) fluorochrome (excitation V405, emission V440–510 nm approximately) with an anti-CD8-Krome Orange (KrO, emission V500–580 nm) and different specific antibodies for the TCR anti-Vbeta ($V\beta$) chain, conjugated with fluorescein (FITC) together with an anti-CD4 APC-Alexa750 (clone13B8.2) in order to analyze the clonality of the T cell receptor. With this combination, we observed that a small fraction of CD4+ T cells expressed diminished level of anti-CD8 labeling and all these apparently “CD4+CD8dim” T cells expressed the Vbeta FITC clonotype.

Here, we explored the causes of this misleading detection that appeared to be due to incidental FRET between double labeling of components of the TCR molecular complex.

2 | METHODS

T cell clonality was analyzed using monoclonal antibodies directed to $V\beta$ clonotypes of the T cell receptor (TCR), conjugated with either FITC or PE (example shown with $V\beta$ 17 FITC—clone E17.5F3.15.13— or $V\beta$ 7.1 FITC—clone ZOE—or an anti-TCR gamma delta; $\gamma\delta$ TCR, clone IMMU510) conjugated with FITC. T cells were selected using anti-CD3 ϵ -PB (cloneUCHT1). T cell subsets were identified using anti-CD8-Krome Orange (KrO, clone B9.11) and anti-CD4APC-Alexa750 (AA750, clone13B8.2). All monoclonal antibodies were provided by Beckman-Coulter (Fullerton, CA).

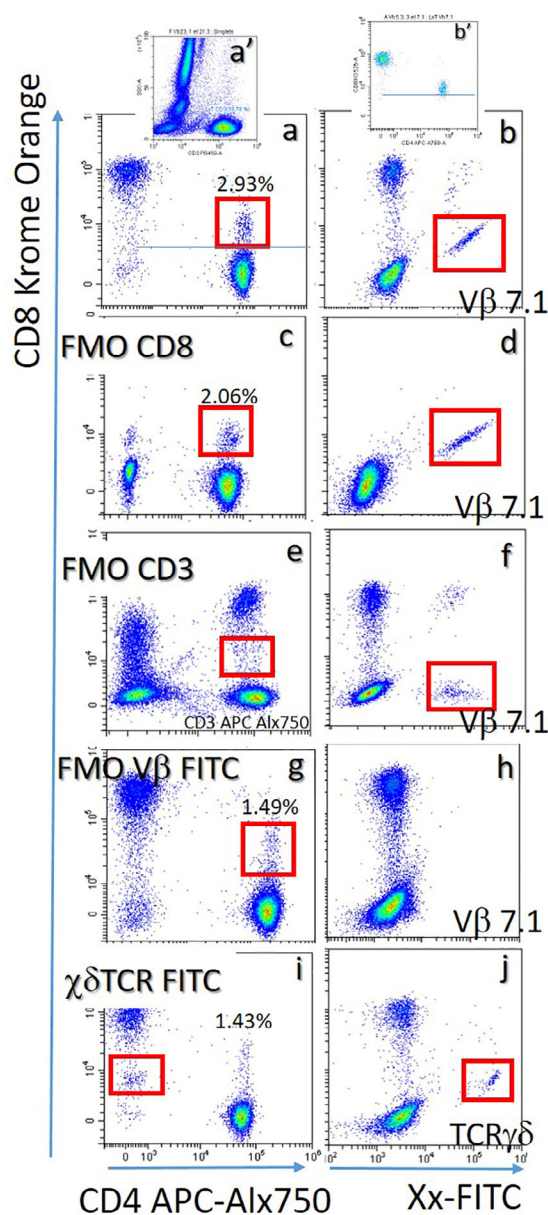


FIGURE 1 Representative graphic of unexpected CD4+CD8dim (panel A) detection of V525 signal on CD4+ T cells (gated on CD3+ T Cells, panel A') as well as CD8+ T cells. Panel B shows correlation between detection of Vbeta 7.1 FITC and CD8 Krome Orange. Accordingly, all Vbeta FITC CD4+ T cells produce signal on V525 detector (panel B'). Similar picture is observed with other Vbeta clonotype, depending on the representation of the T cell subset. The aberrant detection (panel C) and coincidence (panel D) are still observed after removing anti-CD8 KO labeling (FMO) but not after removing anti-CD3 Pac Blue labeling (panels E and F) or removing anti-V β chain FITC labeling (panels G and H) but is observed again when using anti- $\gamma\delta$ TCR-FITC labeling (panels I and J). [Color figure can be viewed at wileyonlinelibrary.com]

The labelings were performed on whole blood anti-coagulated by EDTA. Fixation and erythrolysis was performed with Immunoprep™ using the TQ.prep™ Workstation (Beckman-Coulter). Cells were washed with 4 mL phosphate buffer saline (PBS, Eurobio, France),

centrifuged at 300g and the pellet resuspended in PBS supplemented with bovine serum albumin 10 g/L (BSA, Eurobio). Samples were analyzed within 4 h on DxFlex™ using CytExpert™ software (Beckman-Coulter). Instrument settings and calculation of compensation were performed using single labeling on capture beads Versa-comp™ beads (Beckman-Coulter). Instrument settings were checked every working day using Daily QC™ beads (Beckman-Coulter) according to the manufacturer instruction and to our Iso 15189 procedures. Data were analyzed on Kaluza™ software (Beckman-Coulter).

3 | RESULTS

Results showed an unexpected presence of a CD4+CD8dim population that constantly expressed the single Vβ-FITC chain and not Vβ-PE chain irrespective of the clonotype analyzed (Figure 1A). Furthermore, Vβ-FITC signal was correlated with the CD8-KrO signal, suggesting a compensation problem (Figure 1B).

When removing the anti-CD8-KrO (FMO), the CD4+CD8dim population was still present along with the correlation between the Vβ-FITC signal and the KrO signal, thus confirming the presence of a cytometric artifact (Figure 1C,D).

However, when replacing the anti-CD3-PB with an anti-CD3-AA750 or removing the anti-Vβ-FITC, the CD4+CD8dim population disappeared along with the correlated signal between FITC and KrO (Figure 1E-H).

Interestingly when staining the γδ chains of the TCR with FITC-conjugated antibodies, the correlated signal and the CD8dim population were present (Figure 1I,J). However, they were not present when we stained molecules located outside the TCR complex such as CD5 or CD31 (data not shown).

Fluorescence intensity of CD3-PB was slightly reduced by the adsorption of FITC with a Median Fluorescence Intensity MdFI = 46,559 on Vβ-FITC positive as compared to 51,695 on Vβ-FITC negative CD4+Tcells; one representative example; Figure 1A. Interestingly, CD3-PB MdFI was also reduced on Vβ-PE positive at 44619.

Furthermore, PE excitation spectrum also overlap with PB emission and another FRET effect was also observed for PE, detected on V610/20 filter (MdFI = 1945 on Vβ-PE positive as compared to MdFI = 474 on Vβ-PE negative not shown).

4 | DISCUSSION

Our study brought to light a cytometric artifact when studying multiple chain of the TCR complex. The simultaneous use of anti-CD3-PB and anti-Vβ-FITC led to the appearance of an aberrant CD8dim signal. FMO experiments clearly show the role of PB and FITC but not KrO. FITC excitation spectrum ranging from 475 to 510 nm strongly overlap with PB emission spectrum (from 440 to 520 nm) and FITC emission spectrum ranges from 510 to 575 nm is detected by the V525/40 detector on violet laser. FRET could be further confirmed measuring high depolarization of acceptor emission which we could

not check. The explanation is that after being excited by the violet laser, the PB molecules located on CD3 could excite via FRET the FITC molecules located on the Vβ chain of the same TCR complex. Once excited, the FITC molecules would emit light detected by the V525 detector designed for KrO fluorescence detection. Accordingly, PB MdFI is slightly reduced. Thus, the simultaneous use of anti-CD3-PB and anti-Vβ-FITC would lead to the detection of unexpected KrO signals due to FRET. As FRET only happens when fluorochromes are at very close distance (inferior to 10 nm) we confirmed the PB energy transfer to FITC was still present by labeling another molecule of the TCR complex (γδ TCR) but was absent when FITC labeled molecules out of the TCR complex (CD5 and CD31).

However, PB only partly overlaps the FITC excitation spectrum. This is why the signal produced by FRET to FITC is strongly reduced as compared to KrO CD8 full labeling. The particular unexpected labeling we observed, was misleading because CD4+CD8dim T cell phenotype existed in a small fraction of T cells [6, 7]. It could be noticed because the CD4+CD8dim sub population was varying a lot according to the relative expression of different Vβ-FITC clonotypes. CD8dim T cell phenotype was also observed on CD8+ T cells but the role of FRET was more difficult to observe since a proportion of CD8+ T cells have the CD8dim T cell phenotype.

This FRET effect is probably applicable to Alexa Fluor 488 that has similar excitation/emission spectra. FRET could also be observed in PE and tandems. This was not relevant to our panel but must be considered in case the panel includes conjugates detected on V610, V660, and V780 detectors.

Another FRET effect could be detected. Indeed, PE has a large excitation spectrum ranging from 460 to 580 nm also overlapping with PB emission spectrum (from 440 to 520 nm) and we could observe some signal V610/20 detector on violet laser and a lower CD3 MdFI on Vβ-PE positive T cells. This was not really apparent in our panel as we were not using this detector.

To our knowledge, this is the first time when FITC and PE are described as possible acceptors in a FRET experiment where PB serves as a donor even if its fluorescence is not too strong. The weak fluorescence of the PB could be the reason why PB has not been used too often as a donor molecule in FRET experiments.

Similar effect is also expected but has not been tested for PE tandems like PE-Cy55 on V660/10 filter or PE-Cy7 on V780/60 filter that could be excited by Krome Orange or BV480, BV510 and similar fluorochromes as donor.

In conclusion, multicolor labeling can be exposed to several risks of artifacts that can mislead the results due to phenomena like spectral overlapping, cross laser excitation, fluorescence spreading, fluorescence leaking in tandems, tandem dissociation, or bleaching. FRET is another risk that should be considered in panel designing, even on violet laser, especially when the panel targets components of molecular complexes or molecules that are in close contact. The use of donor/acceptor fluorochromes should be avoided for markers in close contact. [Correction added after first online publication on 15 September 2023. The final three paragraphs of the Discussion section has been updated to increase readability.]

AUTHOR CONTRIBUTIONS

Louis Waeckel, Hana Khenine, were in charge of investigation and formal analysis; Anne-Emmanuelle Berger was in charge of resources, Claude Lambert were in charge of conceptualization, methodology, project administration and supervision.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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