# Beyond Spectral Overlap: FRET-Driven Signal in TCR/CD3 Receptor Complex Staining Profile

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## Introduction

Accurate immunophenotyping of γδ T-cell subsets relies on optimized, high-parameter flow cytometry panels that require meticulous fluorochrome selection to avoid unexpected molecular interactions. Because these panels frequently target co-localized proteins within the same macromolecular TCR/CD3 complex, the close spatial proximity of fluorochromes significantly increases the risk of fluorescence artifacts. During the development of a γδ T-cell panel (comprising CD3, TCR γδ, TCR Vδ2, TCR Vγ9, and CD56), we detected an anomalous signal in the RB780 channel when FITC conjugated TCR Vδ2 antibody was present. Although the artifact resembled typical under-compensation, spectral analysis confirmed no conventional emission overlap between FITC and RB780, ruling out standard fluorescence spillover. To isolate the source of this signal, troubleshooting experiments including single-stain controls, fluorescence-minus-one (FMO) controls, and modified panel configurations were performed. The objective was to characterize the abnormal signal and determine if it was driven by a proximity dependent interaction. Excluding CD3 APC-Cy7 from the panel eliminated the RB780 associated signal. This finding strongly supports the hypothesis of macromolecular Förster Resonance Energy Transfer (FRET) occurring natively within the TCR/CD3 complex. In this mechanism, TCR Vδ2 FITC acts as the donor fluorochrome, non-radiatively transferring energy across the close molecular distance to the CD3 APC-Cy7 acceptor, which then emits a false-positive signal near 780 nm (RB780 channel).

Ultimately, these data underscore the necessity of evaluating non-spectral fluorochrome interactions during high-parameter panel design, particularly when targeting tightly clustered receptor complexes. It also reinforces the critical role of FMO controls in distinguishing true biological phenotypes from physical fluorochrome interaction artifacts.

## FRET Hypothesis

During panel validation on the BD FACSLyric™ instrument, an unexpected compensation artifact was observed between CD56-RB780 and TCR Vδ2-FITC (Fig.1). FITC-associated signal was reproducibly detected in the RB780 channel despite minimal predicted spectral overlap and could not be resolved through standard compensation.

Review of the instrument filter configuration (Fig.2) confirmed that FITC emission (~500-550nm, blue laser) does not directly overlap with the RB780 detector (~750-800 nm). However, the RB780 detection window coincides with the emission range of APC-Cy7 in the red laser path. These observations support a non-conventional mechanism in which excitation of FITC results in indirect energy transfer to APC-Cy7, leading to emission within the RB780 detection window.

**Fig.1: TCR Vδ2/CD56 compensation artifact**

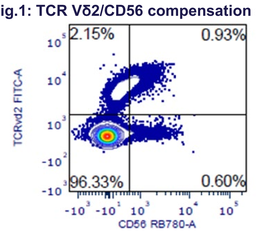



**Fig.2: FITC, RB780, and APC-Cy7 Excitation/emission**

*NOTE: filters for Violet laser is shown for information purpose only*

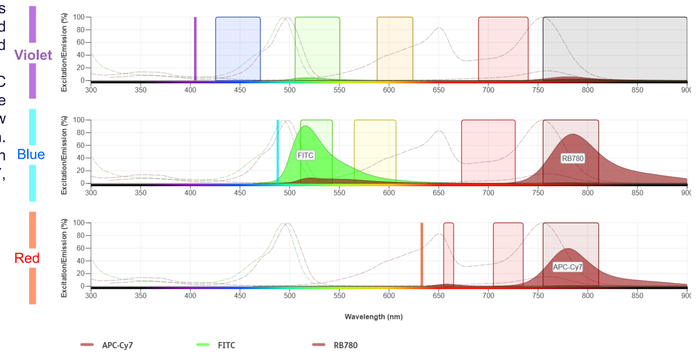



*Image acquired through BD® Spectrum Viewer*

Detection of the CD3/TCR complex in this panel included CD3-APC-Cy7, TCR Vγ9-APC, TCR Vδ2-FITC, and TCRγδ-BV711, raising the possibility of FRET between fluorochromes located in close molecular proximity within the same receptor complex. Because CD3 and TCR molecules are physically associated on the cell surface, fluorochromes conjugated to antibodies targeting these markers can be positioned within nanometer-scale distance of one another. In this configuration, FITC conjugated to TCR Vδ2 can function as a donor fluorochrome, while APC-Cy7 conjugated to CD3 can function as an acceptor fluorochrome.

As illustrated in the figure below, excitation of FITC by the Blue laser (488 nm) promotes FITC into an excited energy state. Rather than releasing this energy solely through its normal FITC emission pathway, FITC can transfer energy directly to the nearby APC-Cy7 fluorochrome through non-radiative dipole–dipole coupling. This energy transfer occurs only when donor and acceptor fluorochromes are within approximately 1–10 nm of each other and when their spectral properties are compatible for FRET interaction. Following energy transfer, APC-Cy7 emits fluorescence near 780 nm, which overlaps with the RB780 detection channel and results in an apparent signal in the RB780 parameter despite the absence of direct FITC-to-RB780 spectral spillover.

**Fig.3: Proposed mechanism of apparent RB780 signal from FITC to APC-Cy7 Energy Transfer.**

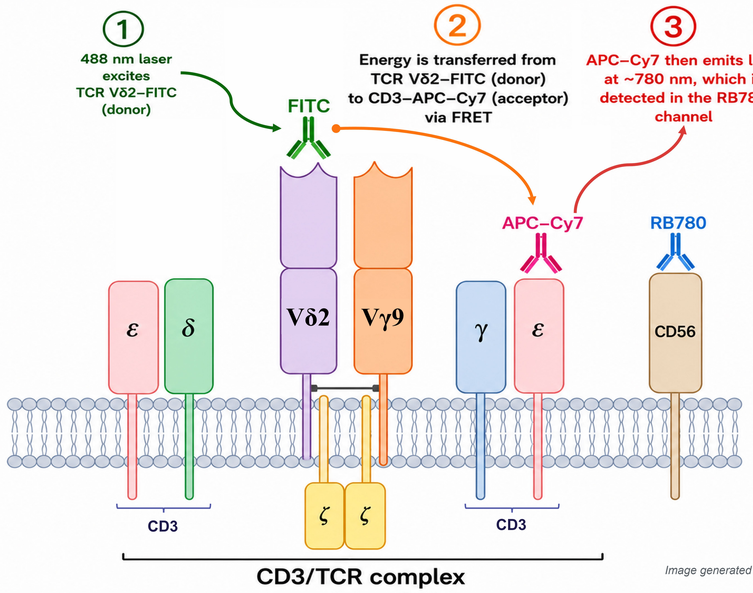



*Image generated by Google Gemini.*

## Method

To evaluate the FRET hypothesis, a series of experiments were performed using T cells stained according to the panel configurations shown in the table below.

Modified panel configurations, fluorescence-minus-one (FMO) controls, and single-stain controls were used to assess whether the abnormal RB780 signal was caused by a FRET-mediated fluorochrome interaction.

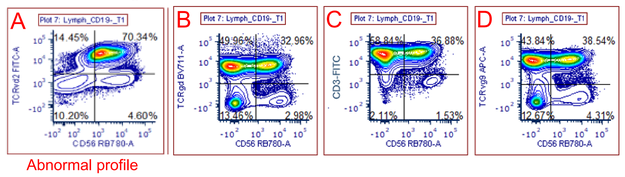
**Experiment setup**

| Detector | FL1 | FL2 | FL3 | FL4 | FL5 | FL6 | FL7 | FL8 | FL9 | FL10 | FL11 | FL12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| (laser) | 405 | 405 | 405 | 405 | 405 | 488 | 488 | 488 | 488 | 640 | 640 | 640 |
| (Filter) | (448/45) | (528/45) | (606/36) | (715/50) | (755LP) | (527/32) | (586/42) | (700/54) | (783/56) | (660/10) | | (783/56) |
| Fluorochrome | | | | BV711 | | FITC | | | RB780 | APC | | APC-Cy7 |
| Full stain-1 | | | | TCRγδ | | CD3 | | | CD56 | TCR Vγ9 | | |
| Full stain-2 | | | | TCRγδ | | TCR Vδ2 | | | CD56 | TCR Vγ9 | | CD3 |
| TCRγδ-BV711-FMO | | | | | | TCR Vδ2 | | | CD56 | TCR Vγ9 | | CD3 |
| TCRδ2-FITC-FMO | | | | TCRγδ | | | | | CD56 | TCR Vγ9 | | CD3 |
| TCRγ9-APC-FMO | | | | TCRγδ | | TCR Vδ2 | | | CD56 | | | CD3 |
| CD56-RB780-FMO | | | | TCRγδ | | TCR Vδ2 | | | | TCR Vγ9 | | CD3 |
| CD3-APC-Cy7-FMO | | | | TCRγδ | | TCR Vδ2 | | | CD56 | TCR Vγ9 | | |
| Single stain TCRδ2-FITC | | | | | | TCR Vδ2 | | | | | | |
| Single stain CD56-RB780 | | | | | | | | | CD56 | | | |

## Results

Fig 4A (Full stain-2) shows abnormal signal profile in CD56 RB780 channel (TCR Vδ2-FITC/CD56 RB780). Similar profile was observed when plotted with TCR γδ BV711/CD56 RB780 and TCRVγ9/CD56 RB780 (data not shown here). To investigate the apparent FITC signal detected in the RB780 channel, TCR Vδ2-FITC was replaced with CD3-FITC while maintaining CD56-RB780 staining (no TCR Vδ2 antibody, Full stain-1). Replacement of TCR Vδ2-FITC with CD3-FITC eliminated the abnormal signal previously observed in the CD56-RB780 versus TCR Vδ2-FITC plot (compare Fig 4A: TCRVd2/CD56 and Fig 4C: CD3/CD56 plots), and also in TCR γδ BV711/CD56 RB780 and TCRVγ9/CD56 RB780 (Fig 4B and Fig 4D respectively). These findings suggest that the apparent RB780-associated signal may result from the combined presence of CD3 APC-Cy7 and TCR Vδ2-FITC within the CD3/TCR complex, potentially due to a FRET-associated interaction between these fluorochromes.

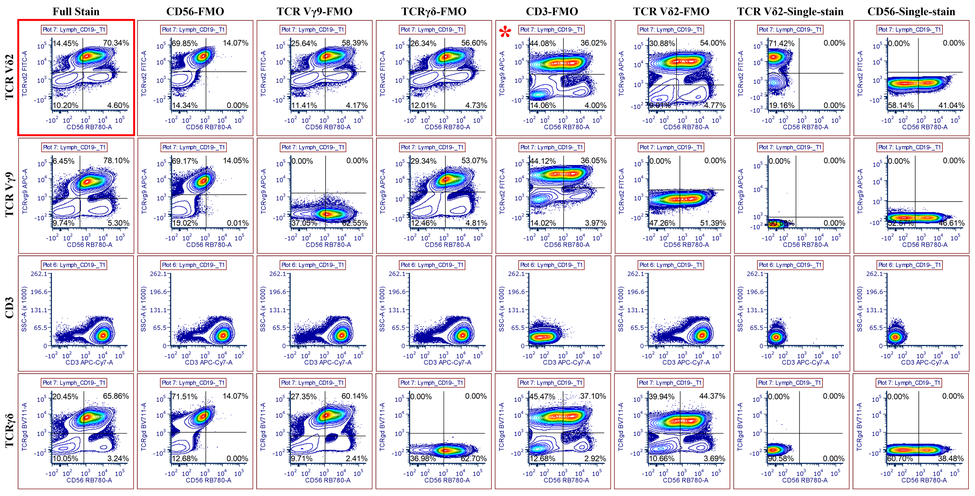
**Fig.4: TCR Vδ2 FITC was replaced with CD3 FITC**




Abnormal profile

For each experimental condition, full-stain, FMO, and single-stain controls were analyzed by plotting TCR Vδ2, TCR Vγ9, and TCRγδ, against CD56-RB780. As shown in the figure below, the full-stain condition demonstrated an apparent abnormal signal in the CD56-RB780 channel (Full stain-2). Similarly, various FMO tubes (as in the Experiment setup table) continued to show abnormal signal in the RB780 channel, except when CD3 APC-Cy7 was not present (CD3-APC-Cy7 FMO tube, shown with the *). As expected based on FRET hypothesis, single-stain control for TCR Vδ2 FITC did not show abnormal profile in the RB780 channel. CD56 RB780 single stain shows normal CD56 positive profile (last column on top). These findings suggest that the apparent RB780-associated abnormal signal resulted from the FRET when CD3 APC-Cy7 and TCR Vδ2-FITC within the CD3/TCR complex were present.

**CD56-RB780 and TCR Vδ2-FITC Interaction Across Experimental Conditions**




## Conclusion

Our findings demonstrate that the abnormal RB780-associated signal is not due to spectral spillover or compensation error, but instead arises from a specific fluorochrome interaction within the CD3/TCR complex, consistent with FRET. This interpretation is directly supported by control experiments in which substituting TCR Vδ2-FITC with CD3-FITC, as well as performing FMOs for either TCR Vδ2-FITC or CD3 APC-Cy7, completely abolished the aberrant signal. Furthermore, the RB780 signal was consistently observed across multiple staining conditions, including both full-stain and FMO controls, confirming that this effect is reproducible.

Together, these results highlight an important technical consideration in multiparametric flow cytometry, particularly when targeting multiple components of a single receptor complex. To ensure data integrity, careful panel design and strategic use of FMO controls are essential for accurately distinguishing true biological signals from fluorescence artifacts.

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