Published in final edited form as:

Immunity. 2020 March 17; 52(3): 419–421. doi:10.1016/j.immuni.2020.02.003.

Worry and FRET: ROS Production Leads to Fluorochrome Tandem Degradation and impairs Interpretation of Flow Cytometric Results

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It is hard to overstate the importance of flow cytometry for immunology research. As the field has advanced, the need for an array of fluorophores to meet different excitation and emission characteristics has increased. A predominant method of extending the range of available fluorophores is through addition of tandem conjugates to existing primary fluorophores (e.g., allophycocin [APC] fluorophore with a Cy7 conjugate yielding APC-Cy7) (Gerstner et al., 2002). This ever-broadening collection of tan-dem fluorophores has allowed further definition of numerous cell populations and are so often utilized that they are now integral to developing suitable flow cytometry antibody panels, particularly with the advent of modern flow spectral cytometers capable of detecting >30 distinct fluorophores (Cytek, 2020).

Tandem conjugates (e.g., Cy7) are activated through Förster resonance energy transfer (FRET) from its primary fluorophore and subsequently alter the emission spectra of the primary fluorophore (Gerstner et al., 2002). However, tandem dyes can degrade and/or lose their FRET capacity, leading to emission in the primary fluorophore's spectrum (Figure S1A; e.g., APC-Cy7 degrades into APC) (Maecker et al., 2004). Tandem degradation is a well-established phenomenon, with several studies exploring how different staining conditions (e.g., light, temperature, cell fixation, presence of serum in staining buffer) contribute to this degradation (Le Roy et al., 2009; Morawski et al., 2019). Establishing the level of degradation and fluorescence overlap is part of the reason instrument compensation prior to using tandem fluorophores is critical. Yet, compensation relies on the assumption of equivalent cell conditions between samples and experimental groups.

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SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.immuni.2020.02.003.

We encountered a substantial problem with this assumption. Specifically, individual sample variability could influence the degree of tandem degradation, thereby variably impacting the signal of the primary fluorochrome on a given cell population. The extent of tandem dye degradation can substantially alter a researcher's interpretation of their data if this "phantom" signal, as a result of tandem degradation, is believed to represent a different protein's expression. When utilizing the cecal ligation and puncture (CLP) model of sepsis induction (Jensen et al., 2018), we consistently observed aberrant results when multiple cell subsets were compared using tandem conjugates in CLP and sham (control) hosts. These oddities were determined to be due to degradation of an APC-efluor780 tandem to elicit a phantom APC signal detectable in the APC channel when no single APC-labeled antibodies are included (Figure S1B). Few $APC^{+}APC\text{-}Cy7^{-}$ cells were observed (data not shown), suggesting that degradation of all tandems on a cell is rare, supporting our model (Figure S1A). If an APC-labeled antibody had been present, this would have impacted the interpretation of the APC antibody target's expression. Using cells only labeled by a tandem fluorophore, this phenomenon was observed across multiple cytometers (data not shown) and occurred regardless of tandem type or antibody target (Figure S1C).

Given that the staining conditions for the two experimental groups were identical we reasoned that some biochemical feature(s) of CLP-induced sepsis must be affecting the cells to more potently degrade fluorophore tandems. Similar to Le Roy et al., we observed that oxidation, through addition of H_2O_2 to the staining buffer, promotes tandem degradation (Figure S1D) (Le Roy et al., 2009). To address whether the sepsis-induced tandem degradation was due to oxidation, 2-mercaptoethanol (BME, a potent reducing agent) was added to the staining buffer (Figure S1E) and was sufficient to reduce tandem degradation, similar to the use vitamin C for the same objective (Le Roy et al., 2009; Morawski et al., 2019). Granulocytes have a greater degree of tandem degradation than lymphocytes (Le Roy et al., 2009), and we observed a trending positive correlation between granulocyte frequency and degree of tandem degradation (data not shown). These observations, along with an increased proportion of granulocytes in CLP samples (data not shown), suggested that reactive oxygen species (ROS) may be the cause of the differential tandem degradation. Indeed, we observed elevated ROS production by cells from CLP samples relative to control (Sham) (Figures S1E and S1G).

To determine whether this occurs in other highly inflammatory infections, mice were infected with non-lethal blood-stage *Plasmodium yoelii* 17XNL (PyXNL) (malaria) or virulent *Listeria monocytogenes*—infections that elicit robust ROS production (Postma et al., 1996; Serbina et al., 2003). Tandem degradation was observed in the context of both murine malaria (Figure S1H) and *L. monocytogenes* (Figure S1I) infections, suggesting that enhanced tandem degradation occurs in multiple experimental model systems and potentially corresponds to the inflammatory status of the individual host or samples. Thus, interpretation of data can be substantially skewed when comparing cells in samples with varying inflammation and/or ROS production.

We noted similar problems with frozen human peripheral blood mononuclear cell (PBMC) samples obtained from septic patients and healthy controls (Figures S1J–S1M). Although the frequency of ROS-producing leukocytes was significantly higher in septic patient

samples compared to healthy controls, the amount of ROS produced, even in healthy controls samples, facilitated tandem degradation. To address whether ROS production and subsequent degradation was a result of sample cryopreservation (Paredes et al., 2018), leukocytes were isolated from blood donation cones then split in two: the first group was left at 4°C and the other frozen in lymphocyte freeze media. After 12 h, the cells in the frozen group were thawed, and both groups were stained. The frozen cells demonstrated enhanced fluorophore degradation and ROS production (Figures S1N and S1O), and this phenomenon appeared to increase with the duration of freezing (data not shown). Therefore, the appearance of the phantom signal in frozen samples could substantially influence accurate detection and/or description of human cell subsets.

Additionally, this phenomenon was exacerbated by prior infection(s) (Figure S1P), potentially due to a trained immune component, and may explain the prevalence of degradation in human samples. A strong linear relationship existed between the degradation of multiple fluorophores for a given sample (Figure S1P), suggesting that if one tandem was degrading, then they were all likely to be degrading, although some tandems showed more pronounced degradation than others (e.g., APC-Cy7 >>> PE-Cy7). Differences in susceptibility of tandems to degradation may be relevant to the likelihood of detecting a problem, such as using PerCP-Cy5.5, which is fairly resistant to degradation. Additionally, using Cy7 alternatives, such as H7, may limit problems; however, it is noteworthy that other “more stable” conjugates like APC-Fire still showed considerable degradation (Figure S1C).

By understanding the factors that underlie non-uniform tandem degradation, we propose some simple adjustments to help detect, limit, or obviate this problem.(1) This problem is most easily detected by including an empty channel for a primary fluorophore that also has a tandem dye included (e.g., if staining with APC-Cy7, leave an empty APC channel). While this reduces the number of available channels, it ensures detection of fluorophore degradation. As there is a strong linear correlation between degradation of different fluorophores (i.e., PE-Cy7 to PE and APC-Cy7 to APC occurred equivalently within an individual sample; Figure S1P), degradation only needs to be monitored by a single fluorophore to determine whether a given sample may have an issue. (2) Incorporation of a reducing agent into the staining buffer. One example was the addition of BME to the staining buffer (Figure S1E). BME was chosen for its common use in complete media formulations and was utilized at a concentration consistent with that formulation. BME addition was able to limit tandem-fluorophore degradation without adversely impacting controls. Alternatively, vitamin C is another option (Le Roy et al., 2009; Morawski et al., 2019). However, addition of a reducing agent does not always fully alleviate the problem of tandem degradation due to the amount and kinetics of ROS being produced within a given sample. (3) Antibodies that recognize highly expressed targets with a clearly defined population can be used for the primary fluorophore. For example, use an APC-conjugated anti-Thy1.1 mAb, an exceptionally bright antibody with a clear positive population, when staining with APC-Cy7. The main caveat to this is that only antibodies with a distinct positive population can be used for the primary fluorophore. Further, relative protein expression (geometric mean fluorescence intensity [GMFI], mean fluorescence intensity [MFI], etc.) cannot be faithfully determined, as degrading fluorophores would non-uniformly influence this value. (4) As ROS production, as well as subsequent tandem

degradation, is dependent on living cells, cells can be fixed prior to staining with tandem fluorophores and potentially further improved through the use of stabilizing fixation buffers. This, however, does pose some issues, as fixation can alter target antigens such that the antibody no longer binds (e.g., the anti-NK1.1 clone PK136). (5) Given the interrelationships between fluorophore degradation, ROS, and granulocytes, removal of granulocytes from samples prior to staining may decrease both ROS and tandem degradation.

These data demonstrate how variability inherent to samples and experimental groups can strongly impact the interpretation of flow cytometry data due to tandem degradation. However, our non-exhaustive list of potential modifications should help alleviate or control for tandem degradation. Importantly, our assessment does not distinguish between the tandem truly breaking free from the fluorophore or merely disrupting the FRET capacity of the tandem. While functionally indistinguishable, future work examining this mechanism may allow for creation of better tandems or linkers for tandems to minimize the problem described here.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

Funding for this study was provided by NIH grants AI147064, GM113961, and GM134880(V.P.B.); AI114543 (J.T.H. and V.P.B.); AI42767, AI85515, and AI100527 (J.T.H.); GM115462 (T.S.G.); and T32AI007511 and T32AI007485 to I.J.J. and Veterans Health Administration I01BX001324 to T.S.G.

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