Rapid light-dependent degradation of fluorescent dyes in chemically-defined media

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Running headline: Fluorescence loss in chemically defined media

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Abstract

Chemically defined media is increasingly used as a tool to help standardize cell biological experiments by eliminating the variability contributed by pooled animal serum. Using chemically defined media during acquisition or collection when performing FACS can contribute to high cell viability and robust experimental design. Careful handling of fluorescently labeled samples for flow cytometric analysis is critical for reliable and repeatable results, but a thorough analysis of how chemically defined media impacts the stability of fluorochrome-antibody conjugates has not been performed. In this study, we expose fluorescent antibody-labeled cells or beads to white light in the presence of various cell culture media and provide evidence that chemically defined media permits rapid light-induced fluorescent dye degradation in a cell-independent manner. This signal loss occurs both to tandem dyes and a photostable AlexaFluor dye, and results in substantial signal degradation and fluorescence spillover into secondary detectors. Finally, we find that photostability of fluorescent dyes in chemically defined media can be restored in the presence of either serum or vitamin C, demonstrating that fluorochrome degradation in this system is mediated by the activity of reactive oxygen species. Thus, our data indicate a need to standardize chemically defined media additives to reduce the impact of oxidative stress before use in cytometric experiments.

Key Terms

Flow cytometry; fluorochrome stability; serum; vitamin C; oxidative stress

Introduction

Biological studies that require sustained growth of cells and tissues increasingly use chemically-defined media (CDM) in place of supplementation with animal-derived sera (1,2). Chemically-defined, serum-free media are now commonly available with varieties manufactured for many cell types, each including a unique but consistent lot-to-lot combination of amino acids, recombinant proteins, growth factors, and inorganics. While the development and use of defined media helps eliminate an element of technical variability from experiments, the impact of such media on antibody-conjugated fluorescent dye stability has not been well studied.

To limit variability in cytometric experiments, great lengths have been taken to standardize sample preparation (3), and the efficient conjugation and proper handling of fluorescently-labeled antibodies (4). While the properties of a dye are largely unchanged following antibody conjugation (5), tandem dyes, which rely on fluorescence resonance energy transfer (FRET) between donor and acceptor fluorophores are susceptible to uncoupling (6). For example, indotricarbocyanine (Cy7) conjugated PE and APC fluorophores (7) may uncouple in response to light, oxygen, or tissue fixation chemicals (6,8,9), and introduce loss of signal intensity and unwanted fluorescence spillover into the detector of the donor fluorochrome. A quantitative assessment of PE tandem dyes revealed that increasing white light exposure results in proportional fluorochrome degradation, with the largest impact on Cy7 conjugates (6). This study also found a small but appreciable tandem dye signal loss during long-term temperature- and light-controlled storage, though overall, light-protected storage of tandem dyes improved their long-term stability (8). Photon-induced tandem dye degradation is thus a well-

documented issue, however a comprehensive analysis of CDM used to maintain the viability of stained cells during or after FACS has not been performed.

In the current study, we evaluated how photon-induced tandem-dye uncoupling was impacted by various chemically defined or serum-supplemented culture media. Our data demonstrate that CDM contain components that promote photon-induced oxidative-uncoupling of tandem fluorochromes on both stained cells and antibody capture beads, and that the addition of serum or vitamin C to these media is sufficient to block this degradation. Thus, careful selection of the media and supplements used for sorting and collection of fluorescently-labeled samples will improve the reliability and reproducibility of flow cytometry experiments.

Materials and Methods

Human tissue and cell isolation

All human samples were obtained upon written informed consent at the Virginia Mason Medical Center (Seattle, WA). All studies were approved by the Institutional Review Board of the Benaroya Research Institute (Seattle, WA). PBMC were isolated using Ficoll-Hypaque (GE-Healthcare; Chicago, IL) gradient separation. $CD4^+$ T cells were enriched using CD4 microbeads (Miltenyi Biotec; Bergisch Gladbach, Germany).

Media and reagents

The following CDM were used in this study: X-Vivo™15 Hematopoietic Cell Medium (Lonza; Bend, OR); Macrophage SFM (Thermo Fisher; Waltham, MA); AIM V™ Serum-free medium (Thermo Fisher; Waltham, MA); Immunocult™-XF T cell expansion medium (STEM CELL Technologies; Vancouver, Canada). Control comparisons to chemically defined media were performed using serum free RPMI 1640 medium with L-glutamine and phenol red (Thermo Fisher; Waltham, MA), 1X calcium- magnesium-free PBS (Sigma Aldrich; St. Louis, MO), or Sphero™ rainbow calibration particles, 8-peak (Spherotech Inc.; Lake Forest, IL).

Inducing and preventing fluorescent signal loss

Signal loss was induced on stained human cells or antibody capture beads where indicated either by direct exposure to white light from the bulb contained within the sample injection chamber (4°C, 100 r.p.m.) of the flow cytometer, or by exposure to

ambient light on the benchtop (20-25°C). Signal loss was prevented by addition of indicated amounts of Hyclone FBS, or vitamin C reagent grade L-ascorbic acid (Sigma Aldrich; St. Louis, MO).

Antibodies and labeling

Human leukocytes were stained with fluorescently-labeled antibodies diluted in staining buffer made up of 1X PBS + 3% FBS for 20 minutes at 37°C in the dark. Antibody capture beads were stained according to manufacturer instructions; in 1X PBS for 5-10 minutes at room temperature in the dark. The following fluorescently conjugated antibody clones were used in this study: CD4-BV421, RPA-4; CCR7-BV605, G043H7; CD103-BV605, BerACT8; CCR6-BV650, G034E3; CD4-BV650, OKT4; CD4-BV711, OKT4; CD4-BV785, OKT4; CD4-AF488, RPA-T4; CD4-FITC, SK3; CCR4-PerCP/Cy5.5, L291H4; CD3-PE, HIT3a; CD4-PE/Dazzle594, OKT4; CD25-PE/Cy5, BC96; CCR6-PE/Cy7, G034E3; CD8α-AF647, SK1; anti-mouse CD45.2-AF700, 104; CD45RA-AF700, HI100; CD4-AF700, OKT4; CD4-APC/Cy7, OKT4; CD45RA-APC/Cy7, HI100; (BioLegend; San Diego, CA). CD45-BUV395, H130; CD28-BUV737, CD28.2; CXCR3-BV421, 1C6; CD3-v450, UCHT1; CD4-BV510, SK3; CD8α-v500, RPA-T8; CD127-BV786, HIL7RM21; CD4-PE/CF594, RPA-T4; CD127-PE/Cy7, HIL7RM21; CD45-APC/H7, 2D1 (BD Biosciences; San Jose, CA). CD4-PerCP/ef710, SK3; CD103-PE/Cy7, B-Ly7; CD45RA-APC/ef780, HI100 (Thermo Fisher; Waltham, MA). CCR10-PE, 314305; (R&D Systems; Minneapolis, MN).

Data acquisition and analysis

Samples were collected on either a FACSAria™ Fusion or AriaII (BD Biosciences; San Jose, CA) each equipped with five lasers: ultra-violet (355 nm), violet (405 nm), blue (488 nm), yellow-green (561 nm) and red (637 nm). For fully-stained human samples, single color controls were generated using a combination of UltraComp eBeads™ (Thermo Fisher; Waltham, MA) and PBMC labeled with eBioscience Fixable Viability dye eFluor780 (Thermo Fisher; Waltham, MA). Where indicated, fluorescence spillover was measured in secondary detectors in which no concomitant primary staining was performed. Compensation was calculated using FACS DIVA™ Software (BD Biosciences; San Jose, CA) acquisition defined matrices. Data were analyzed with FlowJo 10 (TreeStar, Inc.; Ashland, OR).

Results

Fluorochrome degradation in chemically defined media can occur rapidly in response to light and does not require the presence of cells

The use of CDM is increasing, but a thorough characterization of the interaction between antibody-fluorophore conjugates and CDM has not been performed. We therefore wanted to test under what conditions these media might impact the storage or collection of fluorescent-antibody labeled cells, comparing multiple conjugates directed against a variety of lymphocyte surface antigens.

We first labeled freshly isolated human $CD4^+$ T cells with a panel including tandem and non-tandem fluorophores, designed to discriminate both naïve and memory cells, as well as T_{helper} subsets. Labeled $CD4^+$ T cells were resuspended in CDM without serum and run on a cell sorting flow cytometer. Data were recorded immediately and again after 20 minutes of intermittent white light exposure during sorting (Fig. 1A). The source of white light was the internal user-activated bulb in the sample injection chamber. Substantial light-induced signal loss was evident at 20 minutes for CD45RA-AlexaFluor700 (Fig. 1A), which is part of a highly photo-stable family of dyes (10), while the polymer tandem dyes BV605 and BV650 remained intact during this time. We also observed PE/Cy7 tandem dye uncoupling, as loss of FRET to the acceptor fluorophore resulted in an approximate 10-fold loss of $CD127^+$ staining intensity and reciprocal spillover into the PE detector. We next asked whether CDM from different manufacturers, formulated for use with different hematopoietic cell populations, could elicit similar effects. For this, we stained thawed PBMC and resuspended them in

staining buffer composed of PBS with 3% FBS, one of four different serum-free CDM, or serum-free RPMI. Stained cells in various buffers were left uncovered on the benchtop for 1 hour and both PE/Cy7 signal and spillover into the PE detector was assessed (Fig. 1B). When cells were stored in staining buffer signal intensity remained constant for both CCR6-PE/Cy7 and CD4-AF488 after light exposure, with no PE detector spillover. By comparison, varying degrees of tandem-dye uncoupling was evident following light exposure in CDM and serum-free RPMI. Interestingly, in some of the CDM preparations (e.g., AIM V^{TM}) substantial spillover into the PE detector was extensive even in the absence of major loss of the CCR6-PE/Cy7^{+} signal (Fig. 1B). These data suggested that fluorochrome-antibody conjugates are particularly vulnerable to degradation in media lacking serum.

Fluorescence signal loss is a well-known phenomenon. Various conditions impacting dye degradation including light, fixative, or temperature of the sample can cause up to 50% signal loss within an hour (9). We therefore asked to what degree different fluorochromes were susceptible to light-induced degradation in CDM. We generated antibody-stained CD4^{+} T cells and resuspended them in either CDM, standard serum-supplemented media, or staining buffer. Signal stability was measured during sample acquisition over time, first in the dark for three minutes, followed by three to seven minutes of light exposure. We measured drastic signal loss of PE/Cy7- and AF700-, within 30 seconds of light exposure (Fig. 1C). By comparison, we identified no signal loss using a BV421-conjugate, and all dyes remained light-stable within serum-containing RPMI and PBS. CD103-PE/Cy7 and CD45.2-AF700 single-stained antibody capture beads resuspended in CDM were also highly susceptible to signal loss (Fig.

1D). Although tandem dye degradation has been described as a cell-dependent process (9) these data suggest that rapid light-induced fluorochrome degradation does not require cell metabolic processes to occur.

Extensive fluorescence spillover can occur together with tandem dye uncoupling and without substantial signal loss in the primary detector

Having identified light-induced loss-of-signal of several fluorochromes in CDM we next wanted to more broadly assess this kind of degradation across the full visible spectrum. We thus generated single-stained antibody capture beads using 25 unique commercially available antibody-dye conjugates. Stained beads were resuspended in CDM and data were acquired in the dark for 30 seconds, followed by 2 minutes of light exposure (Fig. 2). The starting and ending MFI of the positively-stained antibody capture beads were calculated to assess signal loss over time. We found the largest impact using PE/Cy7- and AF700-stained beads, while several tandem dyes including APC/Cy7, BV785, and BV786 also showed appreciable signal loss over time (Fig. 2A-B).

In addition to assessing fluorescence stability of single-stained beads in the primary detector associated with all tested fluorochromes, we also measured fluorescence spillover following dye degradation by recording data in all other detectors. As expected, during PE/Cy7 FRET loss we found a concomitant increases in all PE and PE-tandem detectors (Fig. 2C). Interestingly, while APC/Cy7 signal loss was minimal (Fig. 2B), substantial spillover into secondary detectors across multiple lasers was detected following APC/Cy7 uncoupling (Fig. 2D). Although comparable signal loss was detected

for BV711, BV785, and BV786 tandem dye antibody-conjugates, no appreciable spillover could be detected with these dyes (data not shown). These data demonstrate that the rapid fluorescence degradation measured in CDM can have a broad impact on the primary and spillover detectors for a given fluorochrome.

Serum and vitamin C are sufficient to protect against light-induced fluorochrome degradation in chemically-defined media

Based on our results, we wanted to identify factors that were sufficient to eliminate light-induced fluorochrome degradation and concomitant fluorescence spillover in samples stored in CDM. Our data to this point suggested that the presence of serum in traditional media such as RPMI limited fluorochrome degradation during light exposure (Figs. 1-2). In addition, a previously published study demonstrated that vitamin C could prevent APC tandem dye signal loss if added to stained cells (9). We therefore tested the capacity of serum or vitamin C to limit signal loss. To do this, we generated antibody capture beads singly-stained with PE/Cy7, APC/Cy7, or AF700 antibody conjugates. Stained beads were placed in CDM alone or in the presence of either 1mM vitamin C or 3% FBS and left uncovered on the benchtop for 1 hour. Consistent with our previous results we found measurable signal loss and secondary detector spillover for all antibody conjugates exposed to light in CDM alone (Fig. 3A). By contrast, dye stability was maintained if CDM was supplemented with either vitamin C or FBS, similar to what we observed following storage in staining buffer during light exposure.

As a final control we tested the impact of CDM and light on 8-peak rainbow calibration beads, which use highly stable fluorescent particles contained within the

beads themselves. As expected, these calibration particles are entirely immune to the effects of light-induced fluorochrome degradation, irrespective of buffer used (Fig. 3B).

Discussion

Using CDM can help circumvent the undefined and variable lot-to-lot characteristics of pooled human- or animal-serum, thus further standardizing cell-based experiments and removing the need to perform large-scale tests to evaluate each new batch of serum. Many cytometry cores and technical manuals on cell sorting suggest using HEPES-buffered, serum-supplemented media to help maintain cell viability during the sorting and collection of live cells (11). To this end, using optimized CDM during cell sorting should serve to further increase cell viability and reduce experimental variability. Our study thus aimed to provide a thorough characterization of antibody-fluorophore conjugate stability in CDM.

Care must be taken with the handling of fluorescently-labeled cells and particles, which are susceptible to signal loss following exposure to light or reactive oxygen species, especially for tandem dye antibody conjugates (6,9). Consistent with these findings, we found that even brief exposure to white light was sufficient to induce rapid tandem-dye degradation when labeled cells were stored in chemically defined or serum-free medium, an effect that was absent in PBS alone (Fig. 1). Furthermore, we found that different manufacturer formulations of CDM impact signal stability to varying degrees, suggesting that certain components included in CDM may be more likely to cause dye uncoupling and should be avoided.

The susceptibility of tandem fluorophores to uncoupling is a well-documented phenomenon. Similar to many previous studies, we found light-mediated signal loss (~≥10%) on PE and some APC tandem dyes also occurs in the presence of CDM (Fig. 2). We found that the most substantial light-induced signal loss occurred to AF700

(≥30%), which is remarkable as the Alexa Fluor family of dyes are known for their photostability (10). As no other tested Alexa Fluor dyes exhibited this effect in our hands, further investigation will be required to determine the reason for this specific impact on AF700.

Although APC/Cy7 uncoupling was not always immediately apparent during data acquisition when viewing the primary detector for this fluorophore, an immediate impact of spillover into secondary detectors from tandem uncoupling was clear (Fig. 2). This observation not only reinforces the importance for careful sample handling and reagent selection, but also demonstrates that secondary detectors should be used during sample acquisition to monitor problematic changes to labeled samples that might otherwise go undetected.

Recent data from two independent studies showed that the process of cell sorting is sufficient to induce oxidative stress and alter the basal metabolic state of cells (12), and that the presence of photo-sensitive molecules within standard cell-culture medium could drive morphological changes or cell death via oxidative stress (13). In each case, addition of serum or antioxidants reduced oxidative stress. A third group showed that vitamin C, which can serve as a scavenger of free oxygen radicals, was sufficient to prevent cell-dependent APC tandem dye uncoupling (9). Consistent with these findings we found that the addition of serum or vitamin C to CDM was sufficient to protect against the majority of light-induced signal loss and spillover (Fig. 3). Care must be taken, however, with inclusion of vitamin C during stained cell collection or storage as it has been shown to interact with components of cell culture to produce detrimental byproducts (14). Other chemicals can also protect against oxidative stress, including

selenium and 2-ME (1), though we found that addition of 2-ME to X-Vivo™15 does not prevent light-induced fluorescent signal loss (data not shown). Collectively, data from our group and many others suggest that methodical selection and screening of media additives would improve fluorescence signal stability during cell sorting, as even brief exposure to light can rapidly impact certain fluorochromes.

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Disclosures

The authors have no financial conflicts of interest.

Figure legends

Figure 1. Cell-independent rapid fluorochrome degradation in chemically defined serum-free media. (**A**), $CD4^+$ T cells stained with the indicated conjugates were placed in X-Vivo™15 CDM and exposed to white light in the sample injection chamber. Fluorochrome intensity was recorded before and after light exposure. (**B**), Stained PBMC were resuspended in various media as indicated and exposed to light on the benchtop at room temperature. Fluorochrome stability was assessed before and after light exposure. Spillover detector bandpass filter and laser line are shown. (**C,D**), Fluorochrome stability to white light in the sample injection chamber was assessed over time using either stained $CD4^+$ T cells (C) or antibody capture beads (D) resuspended in X-Vivo™15. Where shown, gray boxes indicate samples first measured in the dark. Data are representative of at least 2 independent experiments.

Figure 2. Substantial fluorescence spillover following fluorochrome degradation in CDM. (**A**), Antibody capture beads were stained with indicated fluorochromes, placed in X-Vivo™15 chemically defined medium, and exposed to light as indicated. (**B**), Percent MFI loss from (A) calculated between t=0 seconds (s) and t=130 seconds. (**C-D**), Fluorescence spillover of CCR6-PE/Cy7 (C) and CD45RA-APC/Cy7 (D) after light-induced tandem dye uncoupling. Red text denotes the primary detector for indicated fluorochrome-antibody conjugate. Indicated detector bandpass filter and laser line are shown. Data in A and B are from a single experiment. Data in C and D are representative of at least 2 independent experiments.

Figure 3. Serum and vitamin C protect against light-induced fluorochrome degradation in CDM. (**A-B**), Antibody capture beads stained with indicated dyes (A), or 8-peak rainbow calibration beads (B) were placed in media and exposed to light for 1 hour. Primary detector fluorescence emission and spillover into indicated secondary detectors is shown. Indicated detector bandpass filter and laser line are shown. Data are representative of 2 independent experiments.

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Figure 1

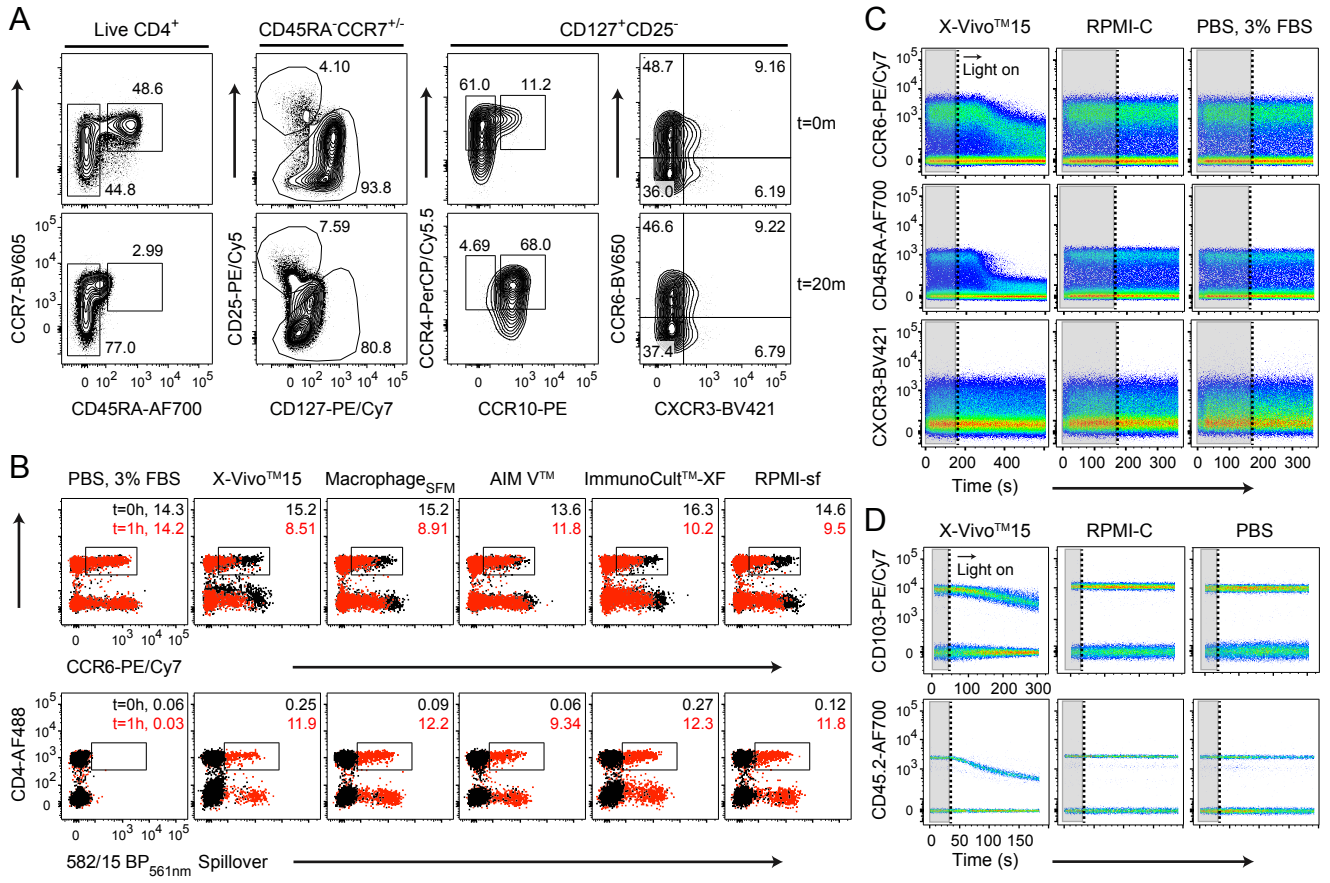
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Figure 2

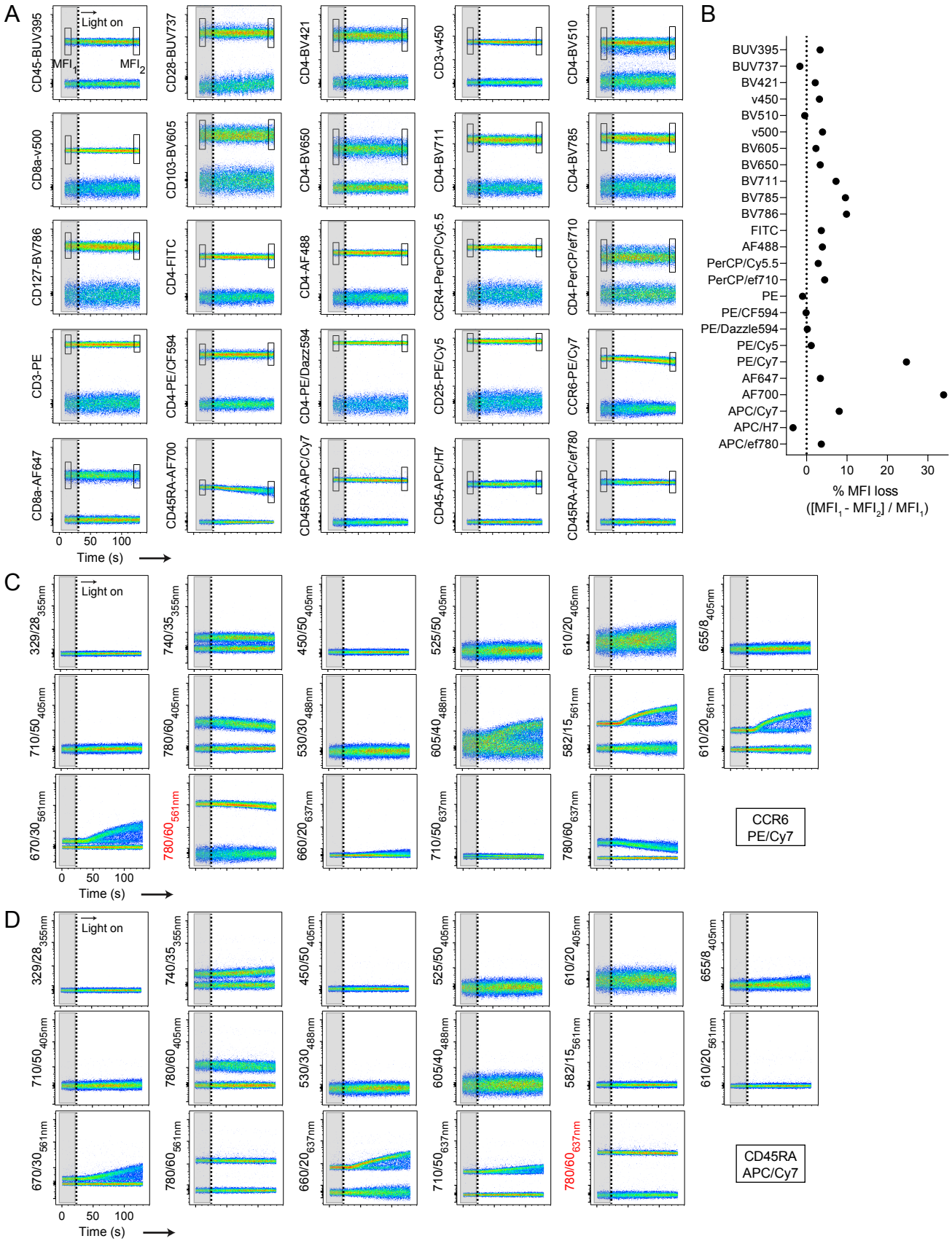
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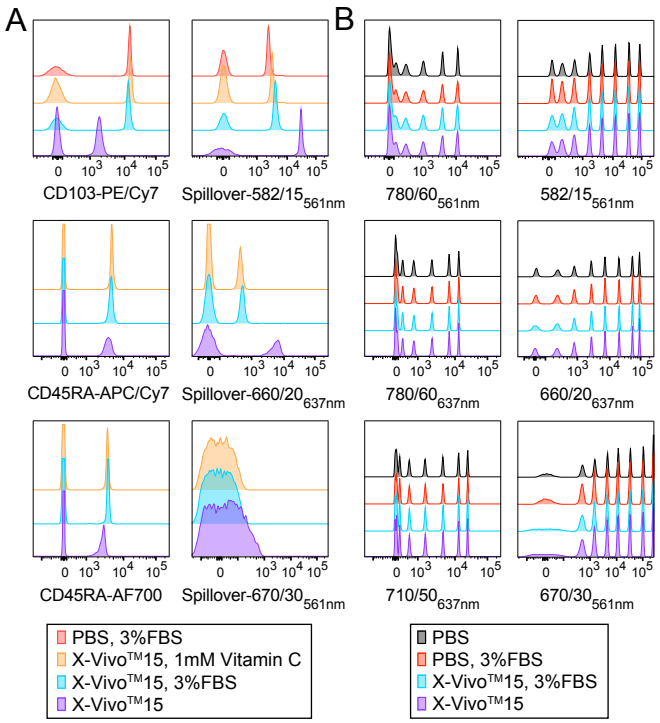
Figure 3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