Rapid light-dependent degradation of fluorescent dyes in formulated serum-free media†

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Chemically-defined serum-free media is increasingly used as a tool to help standardize experiments by eliminating the potential variability contributed by pooled animal serum. These media are formulated for the culture and expansion of specific cell types, maintaining high cell viability without the need for exogenous animal proteins. Use of formulated serum-free media could thus help improve viability and reduce variability during sample preparation for flow cytometry, yet a thorough analysis of how such media impact fluorochrome-antibody conjugates has not been performed. In this study, we expose fluorescent antibody-labeled cells or antibody capture beads to white light in the presence of various hematopoietic cell culture media and provide evidence that formulated serum-free media permit rapid light-initiated fluorescent dye degradation in a cell-independent manner. We observed fluorescence signal loss of several tandem dyes, which included fluorescence spillover into adjacent detectors. Finally, photostability of fluorescent dyes in formulated serum-free media is partially restored in the presence of either serum or vitamin C, implicating reactive oxygen species in observed signal loss. Thus, our data indicate that formulated serum-free media designed for standardized free cell culture are not currently optimized for use with fluorochrome-antibody conjugates, and thus extreme caution should be exercised when using these media in cytometric experiments.

Introduction

Cell culture using chemically-defined serum-free media represents a standardized and scientifically accepted alternative to the use of pooled animal sera (1-3). Immunological studies commonly use formulated serum-free media to ensure consistent culture and expansion of hematopoietic cells, such as primary human lymphocytes and chimeric antigen receptor (CAR) T cells (4-7). Varieties of formulated serum-free media are available commercially for many immune cell types, each including defined lot-to-lot combinations of amino acids, recombinant proteins, growth factors, and inorganics. While the development and use of formulated serum-free media aims to limit technical variability in experiments, the impact of such media on antibody-conjugated fluorescent dye stability has not been well studied.

In cytometry experiments, great lengths have been taken to standardize sample preparation (8), and the efficient conjugation and proper handling of fluorescently-labeled antibodies (9). While the properties of a dye are largely unchanged following antibody conjugation (10), tandem dyes, which rely on fluorescence resonance energy transfer (FRET) between donor and acceptor fluorophores, are susceptible to oxidation, leading to less efficient FRET (11). For example, Cy7-conjugated PE and APC fluorophores (12) may degrade in response to light, oxygen, or tissue fixation chemicals (11,13,14), resulting in loss of tandem dye signal intensity and introducing additional spillover into detector of the donor fluorophore. A quantitative assessment of PE tandem dyes in staining buffer revealed that increasing white light exposure resulted in proportionally elevated fluorochrome degradation, with the largest impact on Cy7 conjugates (11). The same study found a small but appreciable tandem dye signal loss during long-term temperature- and light-controlled storage, yet overall, shielding tandem dyes from light improved their long-term stability (13). Photon-induced fluorochrome degradation is thus a well-known issue, however an analysis of flow cytometry antibody conjugate stability in formulated serum-free media has not been performed.

In this study, we evaluated tandem-dye photostability in formulated serum-free media compared with traditional flow cytometry buffers. Our data demonstrate that serum-free media permit the rapid light-induced degradation of tandem fluorochromes in a cell-independent manner, and addition of serum or vitamin C can limit fluorescence signal changes. Thus, although formulated serum-free media help standardize cell culture and reduce experimental variability, we find these media in their existing formulations are unreliable for use during flow cytometry due to their negative impact on fluorochrome photostability following even brief light exposure.

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 chemically defined or partially defined, serum-free, xeno-free media were used in this study: X-Vivo™15

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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 for defined media were performed using RPMI 1640 medium with L-glutamine and phenol red (Thermo Fisher; Waltham, MA), or 1X calcium- magnesium-free PBS (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 and 3% FBS for 15-20 minutes at 37°C in the dark. Antibody capture beads, UltraComp eBeads™, (Thermo Fisher; Waltham, MA) were stained in the dark according to manufacturer instructions using 1X PBS. For fully-stained human samples, single color controls were generated using UltraComp eBeads™ and PBMC labeled with eBioscience Fixable Viability dye eFluor780 (Thermo Fisher; Waltham, MA). 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; Minneap., MN).

Strategy for modulating, measuring fluorescent signal loss

After staining, fluorochrome-antibody labeled human T cells, PBMC, or antibody capture beads were resuspended in the indicated media or buffers. Fluorescent measurements of stained samples were recorded in the dark immediately. The same samples were then exposed to either of two sources of white light: 1) the two manufacturer-installed, user-controlled, light emitting diodes (LED) contained within the sample injection chamber of a FACSAria™II or FACSAria™Fusion cell sorter, or 2) using ambient fluorescent light (Sylvania, 28W, 40 inch fluorescent tubes) on the laboratory benchtop (20-25°C), for a duration up to 1 hour. The fluorescence of samples exposed to the cell sorter LEDs was measured continuously in real-time during acquisition, for a duration of 3 to 7 minutes with 100 r.p.m. used for sample agitation. Where indicated, Hyclone FBS or vitamin C reagent grade L-ascorbic acid (Sigma Aldrich; St. Louis, MO) were added to the media after staining, but before exposure to light to determine their effects on fluorescent signal loss.

Fluorescent signal was assessed by measuring the change in mean fluorescence intensity over the course of sample acquisition and light exposure, and by monitoring increased spillover signal into adjacent detectors for which no staining was performed.

Data acquisition and analysis

Data acquisition was performed as indicated using either a four-laser FACSAria™II cell sorter (no ultra-violet laser), a FACSAria™Fusion cell sorter, or a BD™LSRII analytical cytometer (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). Cytometer calibration, and control fluorescent measurements were performed using Sphero™ rainbow calibration particles, 8-peak (Spherotech Inc.; Lake Forest, IL). 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

Fluorescence signal loss in defined serum-free media can occur rapidly in response to light in a cell-independent manner

Many published immunological studies now use specially formulated serum-free media to promote standardized hematopoietic cell culture and expansion (4-7), but a thorough characterization of the interaction between these media and antibody-fluorophore conjugates has not been performed. We therefore examined how different formulated serum-free media might impact the signal stability of fluorescent-antibody labeled cells.

We first labeled freshly isolated human $CD4^+$ T cells with a staining panel designed to discriminate $CD4^+$ naïve and memory T_{helper} subsets. Fluorescently labeled $CD4^+$ T cells were resuspended in the formulated serum-free hematopoietic media, X-Vivo15™, and their fluorescence intensity was recorded immediately in the dark (Fig. 1A, t=0m). These cells were then exposed to the LED within the sample injection chamber of the cell sorter for a minimum of five minutes and fluorescence intensity was recorded again (Fig. 1A, t=5m). All analysis was performed on live cells, with no change in viability following light exposure (data not shown). We observed an approximate 10-fold loss of staining intensity for CD45RA-AlexaFluor700. Degradation of CD127-PE/Cy7 was similar and included a reciprocal increase of signal into the detector for the PE donor fluorophore, which greatly impacted T_{helper} subset discrimination. Other dyes, such as the polymers BV605 and BV650, remained intact following exposure to the LED. These data suggest that formulated serum-free media reduces photostability of fluorochromes, even in response to very limited light exposure.

We next asked whether other serum-free hematopoietic media formulated for use with different immune cell populations could have a similar impact on photostability. For this, CCR6-PE/Cy7 and CD4-AF488 dual-labeled PBMC were resuspended in either staining buffer made of

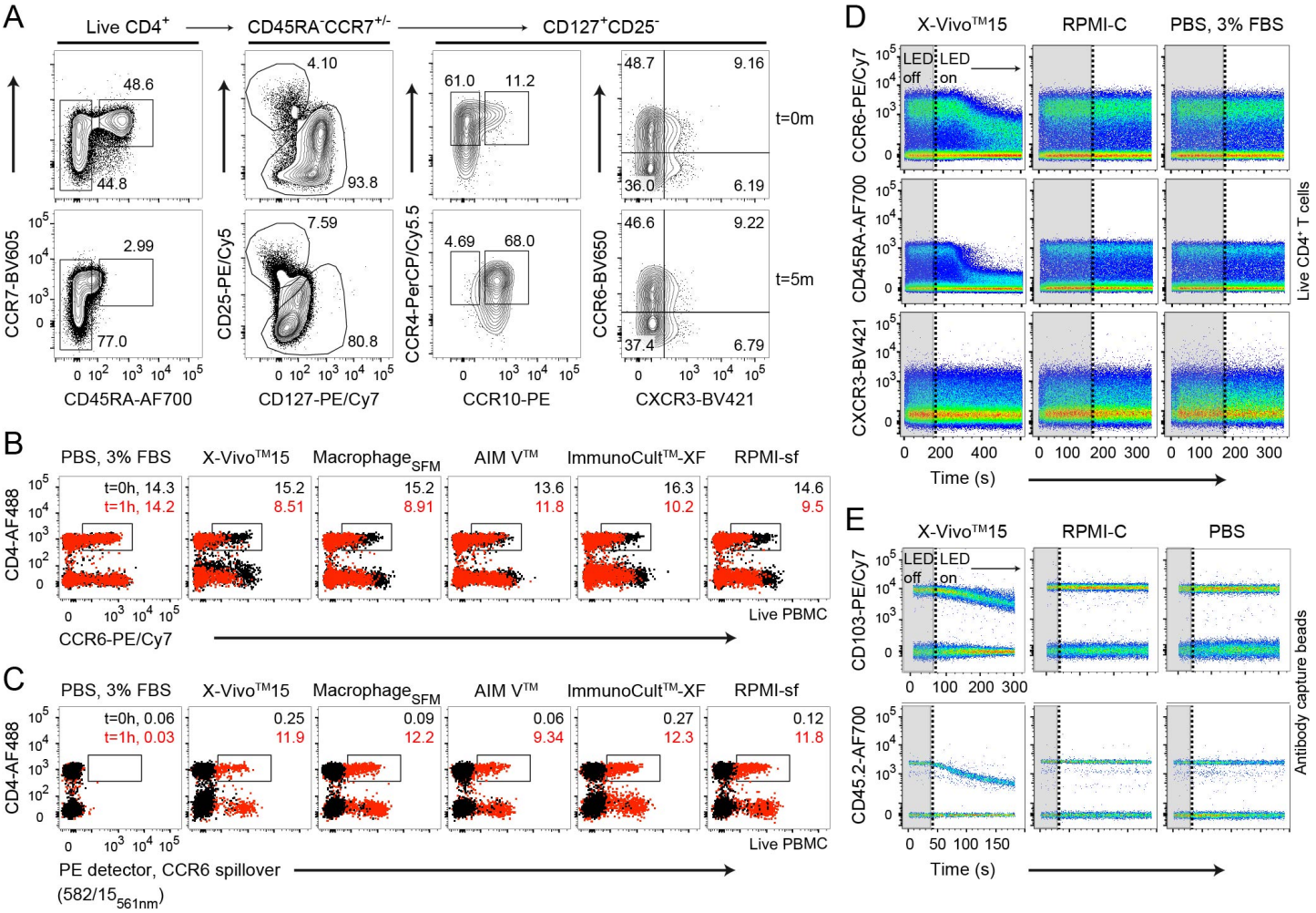

Figure 1. Rapid light-initiated, cell-independent fluorochrome degradation in formulated serum-free media. (**A**), Fluorescent measurements of labeled CD4+ T cells resuspended in formulated serum-free medium, X-Vivo™15, either before (t=0m) or after exposure to the LEDs (t=5m) in the cell sorter sample injection chamber. Cells gated as indicated above plots. (**B,C**), Fluorescent measurements of labeled, live gated PBMC resuspended in the indicated media were taken before (t=0h, black dots) or after (t=1h, red dots) exposure to ambient fluorescent light on the benchtop. The gated frequency at each time is shown. Simultaneous measurements were performed of (B) CCR6-PE/Cy7 into its primary detector and (C) spillover into the PE detector. The bandpass filter and laser line of the spillover detector are indicated. (**D,E**), Real-time fluorochrome photostability of either single-labeled (D) CD4+ T cells or (E) antibody capture beads was compared across the indicated media or buffer. Surface protein expression was measured first briefly in the dark (LED off, gray boxes ending in vertical dotted line), and then in response to light (LED on). Data are representative of at least 2 independent experiments.

PBS-FBS, one of four distinct formulated serum-free media, or unsupplemented RPMI (RPMI-sf). PE/Cy7 and AF488 fluorescence intensity (Fig. 1B, t=0h) and signal increase into the PE detector (Fig. 1C, t=0h) were measured immediately in the dark using an analytical cytometer. Cells were not stained with PE conjugates. Stained cells were then exposed to ambient light on the benchtop for 1 hour at room temperature. Measurements were then repeated for the tandem dye, PE/Cy7 (Fig. 1B, t=1h) and spillover into the donor fluorophore detector, PE (Fig. 1C, t=1h). When cells were placed in staining buffer, signal intensity remained constant for CCR6-PE/Cy7 and CD4-AF488 after 1 hour of white light exposure with no signal increase in the PE detector. By contrast, PE/Cy7 photostability was reduced by varying degrees following 1 hour of white fluorescent light exposure when antibody-labeled cells were stored in serum-free X-Vivo™15, Macrophage$_{SFM}$, AIM V™, ImmunoCult™-XF, or RPMI-sf (Fig. 1B). In some serum-free media, for example AIM V™, tandem dye signal spillover into the adjacent PE detector was extensive (Fig. 1C) despite a largely unchanged CCR6-PE/Cy7+ signal (Fig. 1B), indicating that increased donor fluorophore signal could occur in the absence of much noticeable loss of FRET. These data demonstrate that formulated serum-free media permit light-induced fluorochrome-antibody signal loss, which often included substantial spillover into adjacent detectors.

We next wanted to assess the rate of light-initiated fluorescence loss in formulated serum-free media. Fluorescence measurements of stained human T cells were recorded continuously, in real-time, on a cell sorter, first for three minutes in the dark, followed by an additional three to seven minutes of LED exposure in the sample injection chamber. We observed no signal loss on labeled T cells during the dark period, however drastic reductions to signal of PE/Cy7- and AF700-conjugates were detected within 30

seconds of LED exposure in formulated serum-free X-Vivo™15 media (Fig. 1D). By comparison, BV421-conjugates were unaffected by light exposure.

Tandem dye degradation can be impacted by metabolic processes and cell viability (14). We thus wanted to test whether the fluorescence signal loss we observed (Figs. 1A-1C) was cell-dependent. To do this, we measured the fluorescence stability of single-stained antibody capture beads in real-time for up to one minute in the dark, followed by three to five minutes of LED exposure in the cell sorter as described above. CD103-PE/Cy7 and CD45.2-AF700 labeled beads remained photostable in the dark, but a rapid signal reduction of both conjugates was observed after LED exposure (Fig. 1E). These dyes were comparatively photostable in complete RPMI containing serum. To investigate photostability over the full visible spectrum, we single-labeled antibody capture beads with 25 different fluorescent antibody conjugates, resuspended them in X-Vivo™15 serum-free media, and tested photostability in real-time (Fig. S1A). We observed varying degrees of light-induced signal loss to an array of tandem dyes including CCR6-PE/Cy7, as well as conjugates of APC/Cy7, BV785, and BV786, while base fluorophores such as PE, APC, and BV421, were photostable. PE/Cy7 and AF700 conjugates by far were the most susceptible to light-induced signal loss in formulated serum-free media, measured by decreasing MFI signal over time (Fig. S1B). These data collectively demonstrate that photostability of fluorescent antibody-conjugate labeled samples is reduced in formulated serum-free media, and that this is a cell-independent phenomenon.

Serum and vitamin C can limit light-induced fluorochrome degradation in formulated serum-free media

Our data indicate that fluorochrome are photostable in complete RPMI containing serum (Figs. 1D). In addition, a previously published study demonstrated that vitamin C could prevent APC tandem dye signal loss on stained cells (14). To test the impact of serum and vitamin C on cell-independent fluorescence signal loss, we generated single-stained antibody capture beads using PE/Cy7, APC/Cy7, or AF700 antibody conjugates. Stained beads were placed in formulated serum-free media alone, or in the presence of either 1mM vitamin C or 3% FBS, and exposed to ambient light on the benchtop for 1 hour and room temperature. We observed signal loss and adjacent detector signal increases in formulated serum-free medium alone following exposure to light (Fig. 2A). By comparison, signal loss was reduced by the supplementing media with either vitamin C or FBS.

To rule out that observed fluorescent signal changes are not artifacts of formulated serum-free media, antibody lot, or the light source used, and to confirm normal function of our cytometers, we measured the fluorescent signal of 8-peak rainbow calibration beads resuspended in PBS or medium. Because the calibration beads include highly stable fluorescent particles contained within the beads themselves, they should be immune to light-dependent fluorescent decay. Consistent with this notion, the rainbow calibration particles were entirely unaffected by exposure to 1 hour of ambient fluorescent light, irrespective of the buffer or media used, or the presence of serum (Fig. 2B).

Discussion

Chemically-defined serum-free media contributes to standardized cell-culture, circumventing lot-to-lot variability inherent to pooled human or animal serum. Cell type-specific formulations of serum-free media are now commonly used to permit immune cell culture without sacrificing cell viability or function. For example, these media have become a critical tool for the culture and expansion of CAR-T cells (6,7). Cytometry core facilities and technical manuals on cell sorting suggest using buffered, serum-supplemented media to help maintain cell

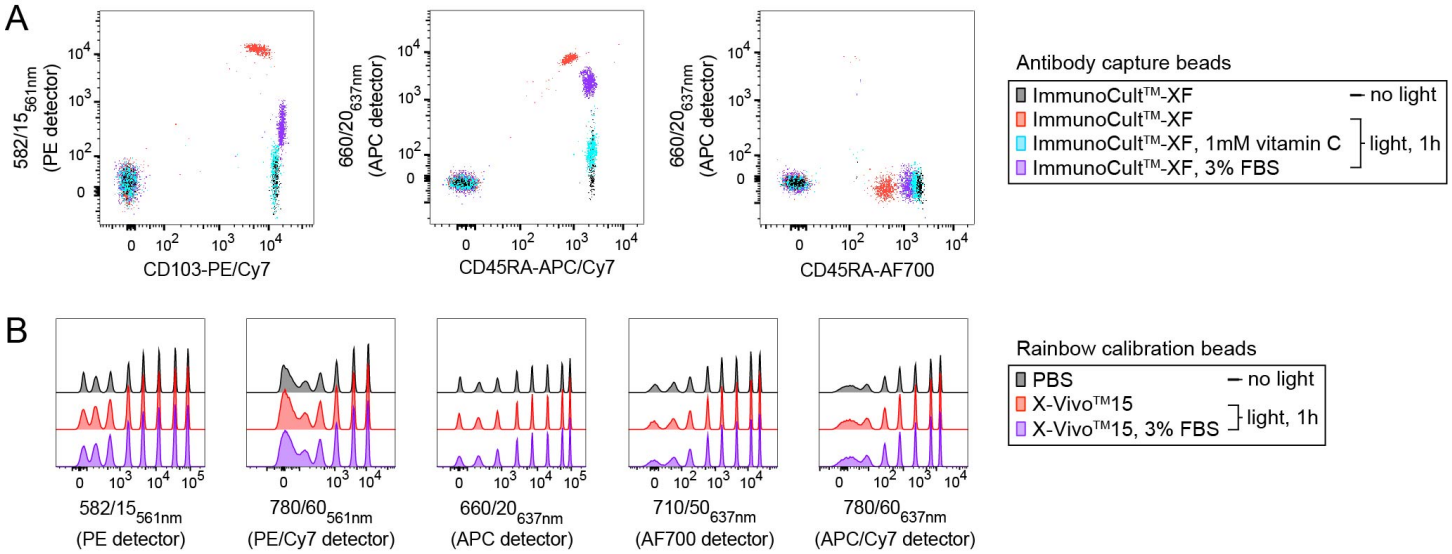

Figure 2. Serum and vitamin C limit light-initiated fluorochrome degradation in formulated serum-free media. (**A**), Antibody capture beads single-stained with the indicated conjugates were resuspended in formulated serum-free media, ImmunoCult™-XF. Fluorescent signal and spillover into adjacent detectors was measured immediately (no light), and again following exposure to ambient fluorescent light (light, 1h) with or without either vitamin C or FBS. (**B**) Rainbow calibration 8-peak beads were placed in PBS, or formulated serum-free media, X-Vivo™15 with or without FBS. Fluorescent signal was measured before and after light exposure as in (A) and for the same five detectors. Detector bandpass filter and laser line are shown. Data are representative of at least 2 independent experiments.

viability during the sorting and collection of live stained cells (15), but few, if any, manufacturer guidelines or publications address the use of formulated serum-free media for these procedures. Our study thus aimed to characterize the stability of fluorescent antibodies in formulated serum-free media.

Fluorescently-antibody conjugates, especially tandem dyes, are susceptible to signal loss from light exposure or reactive oxygen species (11,14). In this study, we found that white light caused rapid loss of PE- and APC-tandem dye FRET efficiency when stained samples were placed in chemically-defined or serum-free media (Fig. 1). Our data suggest this fluorescent signal loss is cell-independent, as it occurs similarly with both stained cells (Figs. 1A-1D) and antibody capture beads (Fig. 1E, Fig. S1). Importantly, this signal loss occurred even after brief periods of light exposure that might be encountered during normal sample preparation and handling.

Not all tandem dyes behaved similarly when exposed to light in formulated serum-free media. For example, antibody capture beads labeled with the polymer tandems BrilliantViolet™ 605 and 650 showed more photostability compared to BV785/6 (Fig. S1) in serum-free media. The reason for variable tandem dye stability remains unclear, but could be connected to differences in their specific chemistry. Surprisingly, we found rapid and substantial light-induced signal loss of multiple AF700 conjugates despite the known photostability of the Alexa Fluor family of dyes (16). No other Alexa Fluor dyes exhibited this effect, and it remains unclear why AF700, which is not a tandem dye, exhibited such a strong negative response to light in formulated serum-free media. Our understanding of how sample handling can impact fluorochrome degradation will be aided by the advent of spectral cytometers, which can measure the full fluorescent profile of dyes instead of only the areas confined to machine-specific bandpass filters.

Two recent studies showed that cell sorting can induce oxidative stress, altering the basal metabolic state of cells (17), and that the presence of photo-sensitive molecules within standard serum supplemented cell culture medium could drive morphological changes or cell death via oxidative stress (18). In each case, addition of serum or antioxidants reduced measurable cell stress. A third study showed that vitamin C, a scavenger of free oxygen radicals, was sufficient to prevent cell-dependent APC tandem dye degradation (14). We find that PBS or RPMI containing serum maintain photostability of stained cells (Figs. 1D,1E), while supplementing formulated serum-free media with serum or vitamin C can reduce, but does not eliminate signal loss and spillover into adjacent detectors observed using stained antibody-capture beads (Figs. 1D,2A). This beneficial effect of vitamin C suggests that free oxygen radical activity is likely to promote light-dependent fluorochrome degradation in formulated serum-free media.

Care must be taken before including vitamin C to sorting media as it has been shown to interact with components of cell culture to produce detrimental byproducts (19). 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 did not prevent light-induced fluorescent signal loss (data not shown). Additional work is therefore required to identify specific additives that can safely and repeatably address the issue of light-dependent fluorophore degradation in formulated serum-free media.

Collectively, our findings suggest that chemically-defined and formulated serum-free media in their current manufactured forms do not sufficiently support the photostability of fluorochrome-antibody conjugates. We conclude these media should be avoided during cytometric experiments, as even supplementing with serum did not fully restore dye photostability. Even collecting sorted cells into formulated serum-free media could prove problematic, as some sorters are equipped with lights near the collection tubes that cannot be disengaged by the user, and could thus impact the reliability of post-sort purity checks. Future modification of formulated serum-free media to support dye photostability would help reduce variability of flow cytometry sample preparation and collection by eliminating the need for pooled serum and other undefined additives that could impact downstream readouts.

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Disclosures

The authors have no financial conflicts of interest.

Author Contributions

P.A.M. and D.J.C. designed the experiments. P.A.M. developed the methods and wrote the manuscript. P.A.M. and S.M. performed experiments. P.A.M., S.M., and D.J.C. reviewed and edited the manuscript. D.J.C. supervised the project.

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Supplementary Data

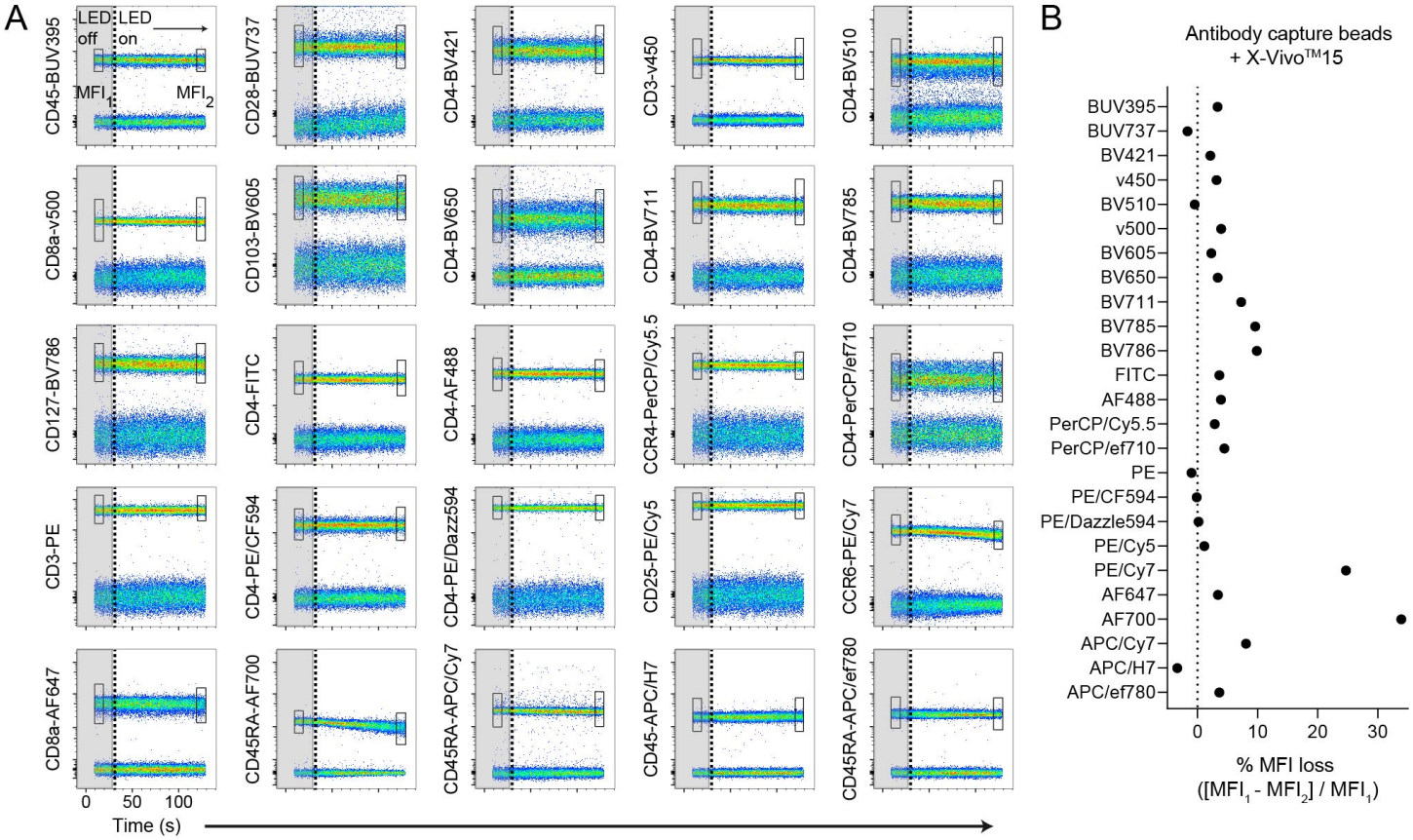

Figure S1. Broadly assessing fluorochrome photostability in formulated serum-free media. (**A**), Antibody capture beads single-stained with one of the 25 indicated antibody-dye conjugates were then resuspended in X-Vivo™15 serum-free media. Real-time fluorochrome photostability was measured, first briefly in the dark (LED off, gray boxes ending in vertical dotted line), and then in response to light (LED on). The MFI of the positively-stained bead population was calculated for the beginning (box "MFI_1", t=0s) and end (box "MFI_2", t=130s) of the time course. (**B**), Graph of percent MFI loss calculated from (A), between t=0s and t=130s.