Impact of fixative type on flow cytometry fluorochrome fluorescence

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Abstract

Fixation of cells prior to acquisition is a common practice of many flow cytometry users. Fixing cells allows users to analyze cells at their convenience and is required for the analysis of intracellular antigens. In response to hardware advances and the advent of spectral flow cytometry, new fluorochromes, such as DNA-based fluorescent reporters have been developed. Although the fluorescence of certain dyes, such as PE, are altered by some fixation methods, the impact of various methods of fixation on DNA-based fluorescent reporters is unknown. **Purpose:** To determine the stability of DNA-based fluorescent reporters, in comparison to traditional fluorochromes for flow cytometry, when stored in paraformaldehyde, a formaldehyde based proprietary fixative solution, and methanol. **Methods:** DNA-based fluorescent reporters and a selection of tandem and small molecule fluorochromes were used to stain human peripheral blood mononuclear cells (PBMCs) against CD4 and CD8a. Unfixed PBMCs were acquired immediately post-staining. PBMCs were fixed with either eBioscience™ IC fixation buffer (2% paraformaldehyde), Foxp3 fixation buffer (proprietary formula), or methanol (100%) were fixed for 30 minutes. Cells were stored in fixative and were acquired 30 minutes, 3 days, and 5 days post-fixation. **Results:** Compared to unfixed cells, fluorescence of PE and tandem dyes was reduced by methanol fixation at 30 minutes. For samples fixed in methanol, no other time points had noticeable differences in fluorescence compared to cells fixed for 30 minutes. The fluorescence of PE and tandem dyes was not different following 5 days of storage in methanol compared to 30 minutes post-fixation. In comparison to unfixed cells, fluorescence of DNA-based fluorescent reporters, small molecule dyes, and FITC were not impacted by any form of fixation. Long-term (5 days) storage of cells in fixative did not alter the fluorescence of any dyes, apart from PE, PE-Cyanine7, and APC-Cyanine7. **Conclusion:** Fluorescence of DNA-based fluorescent reporters was not altered by any method of fixation. Methanol fixation reduces brightness of PE, PE-Cyanine7, and APC-Cyanine7 in comparison to non-fixed samples, while 2% paraformaldehyde and Foxp3 fixation buffer do not impact the fluorescence of any dyes. In comparison to 30 minutes post-fixation, long-term storage of cells in the fixation buffers tested does not further impact fluorescence. Flow cytometry users should carefully select the combination of dyes and fixation methods used in their applications to obtain optimal results.

Introduction

Flow cytometers are critical tools frequently used for immunophenotyping and analysis of single cells. Fixation of cells prior to acquisition is a common practice of flow cytometry users, which allows cells to be stored and analyzed at the user's convenience. The brightness of some conventional dyes, such as phycoerythrin (PE), is reduced by methanol fixation. In addition, emission spectra of conventional dyes may be altered by the method of fixation, thereby impacting compensation. Furthermore, both brightness and emission spectra can be altered by storage of cells over multiple days. Therefore, the development of dyes that are not altered by fixation and are stable during storage would reduce the burden of users and increase the accuracy of flow cytometry data. Recently, a line of DNA-based fluorescent reporters has been developed for use in flow cytometry. Currently, the impact of fixation and long-term storage in fixative on DNA-based fluorescent reporter brightness and emission spectra is unknown. Therefore, the purpose of this investigation was to determine the stability of DNA-based fluorescent reporters, in comparison to traditional fluorochromes for flow cytometry, when stored in either a paraformaldehyde-based fixative (IC fix), a formaldehyde based proprietary fixative solution (Foxp3 fixation), or methanol.

Materials and methods

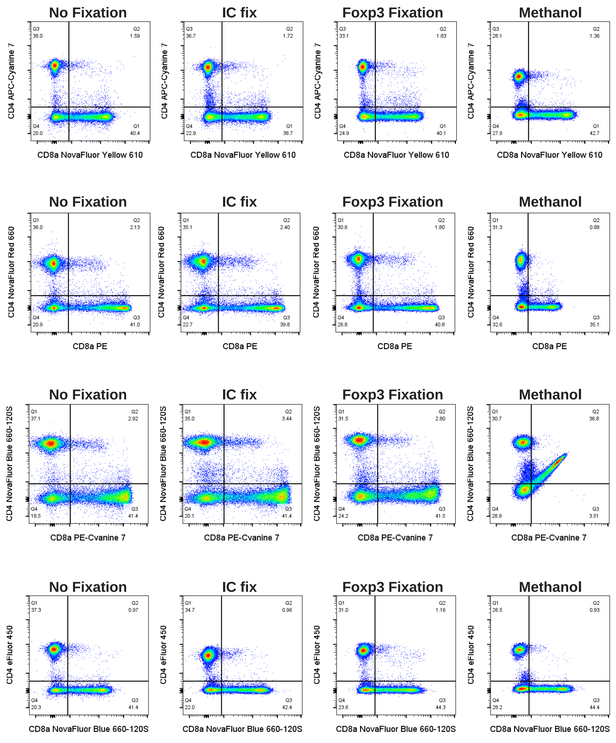
Frozen human peripheral blood mononuclear cells (PBMCs) from an apparently healthy donor were thawed and plated at a concentration of $10x10^6$ cells/mL. Briefly, PBMCs were washed twice using flow cytometry staining buffer and stained in duplicate using primary conjugated antibodies against CD4 (SK3) and CD8a (OKT-8). The conjugate combinations used were, NovaFluor™ Red 660 CD4+ PE CD8a, NovaFluor Blue 660-120S CD4 + PE-Cyanine 7 CD8a, APC-Cyanine 7 CD4 + NovaFluor Yellow 610 CD8a, and eFluor™ 450 CD4 + NovaFluor Blue 660-120S CD8a. All samples were stained for 30 minutes, on ice, protected from light and fixed for 30 minutes. Cells were split and a set of cells were stored in fixative solution for 3 and 5 days prior to washing and analysis. The remaining cells were washed, resuspended, and analyzed immediately afterward. Samples were analyzed using the Attune™ NxT V4. All compensation was performed using single color controls, fixed in the same manner as primary samples, acquired on the first day.

Results

Table 1. Conjugates

Fluorochrome	Antigen	Fluorochrome family
eFluor 450	CD4	Organic
NovaFluor Blue 660-120S	CD4	DNA-based fluorescent reporter
NovaFluor Blue 660-120S	CD8a	DNA-based fluorescent reporter
PE	CD8a	Protein
NovaFluor Yellow 610	CD8a	DNA-based fluorescent reporter
PE-Cyanine 7	CD8a	Protein-tandem
APC-Cyanine 7	CD4	Protein-tandem
NovaFluor Red 660	CD4	DNA-based fluorescent reporter

Figure 1. Methanol reduces MFI and creates compensations. Pseudocolor plots showing CD8a vs CD4 expression on freshly stained human PBMCs. Methanol fixation reduced fluorescence of tandem dyes and PE. Methanol fixation created large PE-Cyanine 7 compensation errors.



Results (continued)

Figure 2. DNA-based fluorescent reporters are stable during long-term storage. Pseudocolor plots showing CD8a vs CD4 expression 3 days after staining. Fluorescence was similar to freshly stained cells for each method of fixation. Methanol fixation continued to elicit large PE-Cyanine 7 compensation errors. Storage in IC fix produced small PE-Cyanine 7 compensation errors.

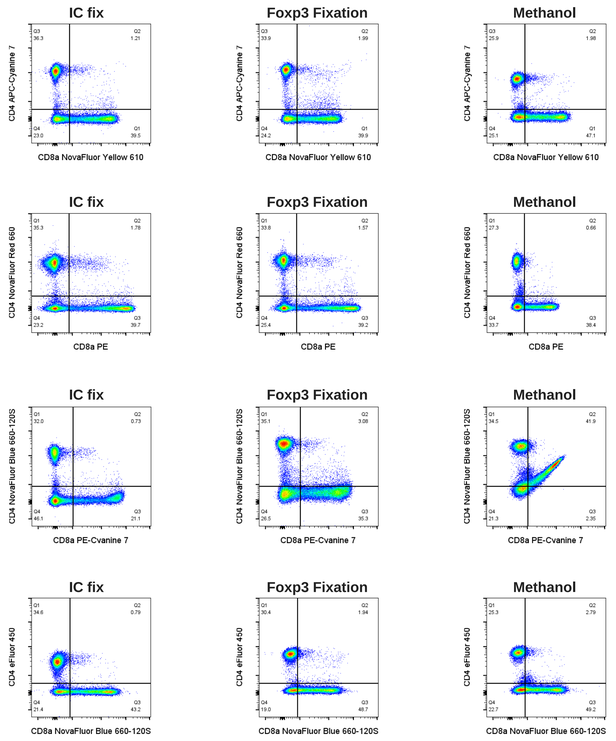
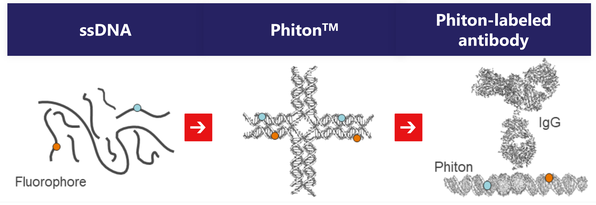
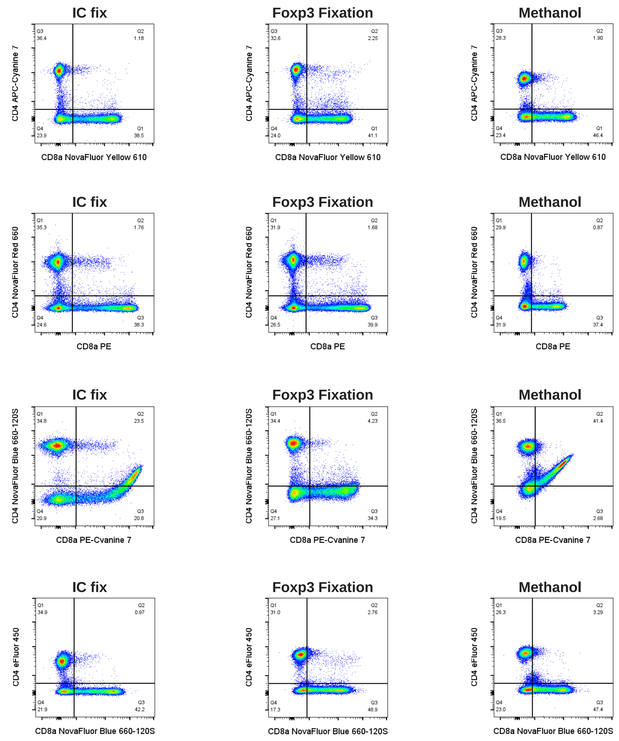


Figure 3. Generating a Phiton-labeled antibody. Dye selection and precise placement allows for control of excitation and emission. Phiton conjugation to an antibody enables consistent dye stability in fixatives.



Results (continued)

Figure 4. PE-tandem dye instability during long-term storage. Pseudocolor plots showing CD8a vs CD4 expression 5 days after staining. Fluorescence of dyes stored in IC fix was similar to freshly stained cells. For cells stored in Foxp3 fix, except for PE + NovaFluor Red 660, the percentage of $CD4^+CD8a^+$ events was increased compared to cells fixed in the same buffer on day 1. 5-day storage in IC fix produced large PE-Cyanine 7 compensation errors.



Conclusions

- DNA-based fluorescent reporters are not altered by the method of fixation or by long-term storage in fixative.
- Methanol reduces the brightness of protein and protein-tandem dyes.
- Methanol fixation and long-term paraformaldehyde storage (IC fix), may impact FRET efficiency of PE-Cyanine 7, creating compensation errors.

Taken together, these results suggests that flow cytometry users should carefully select the combination of dyes and fixation methods used in their applications to obtain optimal results.

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