Use of Surface Modified Microspheres for Setting Compensation in Multicolor Flow Cytometry

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INTRODUCTION

Compensation is a mathematical process which corrects multiple parametric flow cytometry data for spectral overlap. Setting compensation properly requires the use of right controls. The traditional method of performing compensation is to use a series of single-color stained cells, each stained with a single fluorescent marker for every color used in the experiment.

Recent advances have introduced antibody capture beads as a substitute for cells in setting compensation. Antibody capture beads can be stained with antibodies, just like cells, and provide a bright uniform signal, regardless of how bright an antibody would stain. We compare two methods of setting compensation, using cells and using antibody capture beads, with both human lymphocytes and mouse splenocytes. The compensation assays are performed with the same set of fluorochrome labeled antibodies, and the data indicated that the antibody capture beads performed comparably.

Because of the consistent nature of bead scatter and high surface antibody binding capacity, using capture beads allows more consistent and accurate setting of compensation for any combination of fluorochrome-labeled antibodies. In addition, valuable clinical samples could be saved for additional therapeutically significant assays instead being used for spectral overlap corrections.

It is essential that a dead cell discriminator be included in each sample, because antibody conjugates bind nonspecifically to dead cells and may lead to erroneous results. Using a dead cell dye has presented a challenge for setting proper compensation. We study the need for using a dead cell discriminator in three types of assays: surface immuno-phenotyping, intracellular testing requiring fixation, and a functional assay. We found that excluding dead cells from that analysis improved the results. We then used the amine-reactive beads along with the antibody capture beads for setting compensation in a multicolor experiment, and found both types of compensation beads used together provide a consistent, accurate, and easy-to-use technique for the setting of flow cytometry compensation.

MATERIALS AND METHODS

Each Antibody Compensation Beads Kits, AbC anti-Mouse Bead Kit and AbC anti-Rat/Hamster Bead Kit, contains two types of specially modified polystyrene microspheres, the AbC™ Capture Beads (Component A), that bind all isotypes of mouse, rat or hamster immunoglobulin; and the AbC™ Negative Beads (Component B), that have no antibody binding capacity.

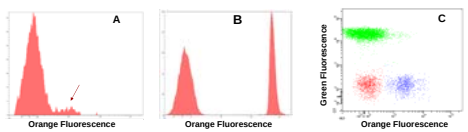
The Antibody Compensation Bead Kit provides a consistent, accurate and simple-to-use technique for the setting of flow cytometry compensation when using fluorochrome-conjugated mouse antibodies.

After incubation with a fluorochrome-conjugated mouse antibody, the two components provide distinct positive and negative populations of beads that can be used to set compensation. Compensation is performed using the same fluorochrome-labeled antibody to be used for cell staining.

Because of the consistent nature of bead scatter and high surface antibody binding capacity, this allows the investigator to more consistently and accurately set compensation for any combination of fluorochrome-labeled mouse antibodies

RESULTS

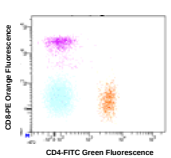
Figure 1. Compensation using AbC™ anti-Mouse Bead Kit



Obtain proper compensation settings can be difficult, especially in situations where the antigen of interest is expressed at a low levels, such as CD56. Using AbC™ anti-Mouse Bead Kit could be a better approach.

In this example, human lymphocytes were labeled with both phycoerythrin-conjugated mouse anti-human CD56 antibody (Invitrogen #MHCD56044) and FITC-conjugated mouse anti-human CD3 (Invitrogen # MDCH03014). When labeled human lymphocytes were used as single-color control for compensation setting, only a weak CD56-positive signal was generated (A). By using the AbC™ anti-Mouse Bead Kit, Capture Beads generated bright signal (B), and a proper compensation setting was obtained shown as dual parameter plot (C) with gated human lymphocytes populations labeled with anti-human CD56-PE and anti-human CD3-FITC antibody conjugates respectively.

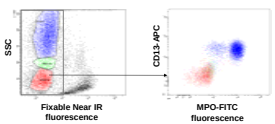
Figure 2. Compensation Using AbC™ anti-Rat/Hamster Antibody Compensation Bead Kit



Dual parameter plot showing gated mouse spleenocytes labeled with both FITC-conjugated rat anti-mouse CD4 and Phycoerythrin-conjugated rat anti-mouse CD8a and using compensation settings obtained using the AbC™ anti-Rat/Hamster Bead Kit. To obtain proper compensation, FITC-conjugated rat anti-mouse CD4 (Invitrogen Cat. No. MCD0401) labels the AbC™ RH capture Beads for a positive signal and the negative beads providing a negative signal. Phycoerythrin-conjugated rat anti-mouse CD8a antibody (Invitrogen Cat. no. RM2204) labels the AbC™ Rat/Hamster Capture Beads for a positive signal and the negative beads providing a negative signal.

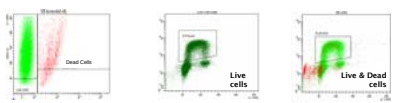
Figure 3. Eliminate Dead Cells in Immunophynotyping and Intracellular Staining

It is essential that a dead cell discriminator be included in assays because antibody conjugates bind nonspecifically to dead cells and may lead to erroneous results. We study the need for using a dead cell discriminator in three types of assays: surface immunophenotyping, intracellular testing requiring fixation, and a functional assay. The data indicated that excluding dead cells from that analysis improved the results.



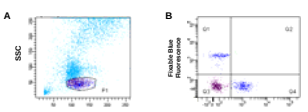
Two day old whole blood was ammonium chloride lysed. Cells were first stained with CD13-APC conjugate. Cells were washed, stained with LIVE/DEAD® Fixable Near IR dead cell stain, washed, fixed with Caltag Fix and Perm reagent A. Cells were then washed, permeabilized with Caltag Fix and Perm reagent B, then stained with MPO-FITC conjugate, and washed. Cells were analyzed on a BD™ LSRII flow cytometer. Suggested method of using live cell gating to eliminate dead cells is shown; further subgating is then recommended to get the most accurate results.

Figure 4: Use Dead Cell Discriminator in a Click-iT® EdU Cell Proliferation Assay



Jurkat cells were treated with 10uM EdU for one hour, harvested, and washed. Cells were then stained with LIVE/DEAD® Fixable Violet stain & washed again. Cells were then paraformaldehyde fixed & permeabilized with a saponin buffer. Labeling with the Click-iT® reaction mixture using AlexaFluor® 488 azide was followed by a wash before DNA content labeling with FxCycle™ Far Red stain. Dead cells were identified in a SSC vs. dead cells plot. Click reaction vs DNA content show gating on either live cells only, or on all cells; Red dots represent dead cells.

Figure 5. Compensation Using Both AbC™ anti-Mouse Kit and ArC™ Bead Kits

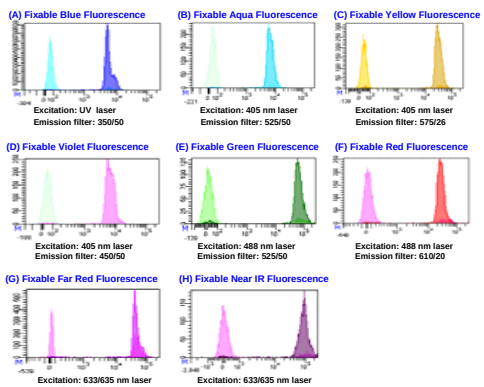


A mixture of live and Heat-treated (60°C, 20 min) human lymphocytes (P1) were labeled with CD4-Pacific Blue™ and the LIVE/DEAD® Fixable blue stain (A). Compensation was achieved using a combination of AbC anti-mouse beads stained with anti-mouse CD4-Pacific Blue™ and ArC beads stained with the blue-fluorescent dead cell stain (B). The Negative Beads in the ArC kit was used as negative control.

Figure 6. Staining of ArC ™ Amine Reactive Compensation Bead with different LIVE/SEAD® Fixable Stain

The **A**mine **R**eactive **C**ompensation Bead Kit, or **ArC**™ Kit is designed to facilitate compensation when using any of the eight LIVE/DEAD® fixable dead cell stains, all amine-reactive dyes. This kit provides two types of specially modified polystyrene microspheres, the ArC™ reactive beads that bind any of the amine-reactive dyes, and the ArC™ negative beads, that have no reactivity. Using the two kit components with any amine reactive dye will provide distinct positive and negative populations of beads that can be used to set compensation, either manually or using instrument software.

The AbC™ Compensation Bead Kits can be used together with the ArC™ Amine Reactive Compensation Bead Kit to calculate compensation in multicolor immunophenotyping experiments that incorporate antibody labeling and a LIVE/DEAD® fixable dye for dead cell identification, providing a complete solution for compensation controls.



CONCLUSION

1. AbC™ Bead Kit (for mouse or for rat/hamster antibodies) provides a consistent and accurate technique for the setting of flow cytometry compensation when using fluorochrome-conjugated antibodies.

2. ArC™ Bead Kit is a useful tool for correction spectral overlap when using LIVE/DEAD® amine-reactive fixable stains.

3. The AbC™ and ArC™ Bead Kits can be used together to generate a complete solution for compensation controls.

REFERENCES

1. J Immunol Methods 313, 199 (2006); 2. J Virol doi: 10.1128/ JVI.01083 (2008);

3. Stem Cells 26, 1009 (2008); 4. Blood 111, 750 (2008); 5. Blood 111, 3155 (2008).