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A Setup System for Compensation:

BD CompBeads plus BD FACSDiva Software

Introduction

Compensation in multicolor flow cytometry is most accurately determined by measurement of spectral overlap values and their subsequent conversion to compensation values by matrix algebra (Bagwell and Adams, 1993; Roederer et al, 1997; Verwer, 2002). Spectral overlap values (or spillovers) are simply the fluorescence values (above background) of a given fluorophore in all detectors relative to the primary detector for that fluorophore. For example, the fluorescence of a PE-stained sample is defined as 100% in the PE detector, and its spectral overlap into shoulder detectors could be 1% in the FITC detector, 20% in the PerCP detector, and 0% in the APC detector.

To determine compensation values, spectral overlap values are measured for all fluorophores, via single-color controls, in every detector. An array of spectral overlap values (of all colors in all detectors) is inverted by matrix algebra to yield compensation values. This matrix algebra essentially represents the simultaneous solution of the equations for the contributions of the spectral overlaps of each of the colors into every detector. Compensation values (not spectral overlap values) are used by the flow cytometer to subtract out the contributions of non-primary colors overlapping into a given detector. The result is that only the fluorescence of PE is seen in the PE detector, for example, with the exclusion of overlaps from all other fluorophores, as is required.

Measurement of spectral overlap values requires particles that are stained with only a single color. BD CaliBRITE™ beads, single-stained cellular controls, and now single-stained BD™ CompBeads can be used for such purpose. The advantage of the last two controls is that spectral overlap determination can be done using fluorophores identical to those used in the experiment. This has become more important with the increasing use of tandem fluorophores such as PE-Cy5*, PE-Texas Red®, PE-Cy7*, and APC-Cy7*. Because the photon transfer efficiency from donor to acceptor in tandem pairs tends to be different each time the conjugation chemistry is performed, the resultant spectral overlaps vary from lot to lot (Stewart and Stewart, 1999). BD CompBeads provide a convenient method of presenting single-stained controls to the flow cytometer for spectral overlap determination.

BD CompBeads are beads that have been coupled to an antibody specific for the Kappa light chain of Ig, from mouse, rat, or rat/hamster. These beads are easy to stain and have a robust fluorescence signal regardless of the Kappa antibody conjugate. They can be used to capture different lots of a given tandem fluorophore to enable their separate measurements. In contrast, spectral overlap determinations can be compromised using cellular single-stained controls, where the fluorescence signal could be very dim or the antigen could be expressed on a rare cell, resulting in inaccurate values. Spectral overlap values determined from single-stained BD CompBeads have been shown to closely match those from single-stained cells (Crowther et al, 2002).

BD FACSDiva™ software (version 2.2 or later) provides a convenient method to automatically read in the spectral overlap values from single-color compensation controls. The software reads the fluorescence of each single-color control (single-stained BD CompBeads, single-stained cellular controls, or BD CaliBRITE Beads) in sequential acquisitions. The software can accommodate two or more different controls for the same color. For example, CD4 APC-Cy7 and CD45 APC-Cy7 could be run, where there might be a difference in spectral overlaps between the two because of lot differences between the tandem fluorophores. After each control is acquired, the user must identify the negative and positive populations for each color. Spectral overlap values are automatically calculated by the software. All of the spectral overlaps are stored as a named setup, which can be recalled later.

BD FACSDiva software provides easy and automatic access to the stored spectral overlap values. For a given experimental Tube, the user specifies in the Experiment Layout or in the Tube Inspector's Labels tab which colors are contained in the tube, including CD4 APC-Cy7 vs CD45 APC-Cy7, for example. The software automatically accesses the appropriate spectral overlaps, constructs a spectral overlap array, solves it for compensation values, and sends the compensation values to the flow cytometer. This process is repeated for each Tube, calculating compensation from the specified spectral overlaps. This new feature for automatic determination of spectral overlap builds upon the existing matrix algebra for compensation that was introduced with the new digital electronics (Verwer, 2002).

The following instructions are provided in brief. More details can be found in the *BD CompBeads* package insert and in the *BD FACSDiva Software User's Guide.*

Equipment

1. BD Falcon™ disposable 12 x 75-mm polystyrene test tubes with no cap (BD catalog no. 352052), or equivalent

2. Vortex

3. Tabletop centrifuge, same as what would be used for washing stained cells

4. Micropipettor with tips

5. BD FACS™ brand flow cytometer, running BD FACSDiva software version 2.2 or later

Refer to the appropriate instrument user's guide and the software user's guide for detailed information.

Reagents

Reagents not provided by BD Biosciences

1. Wash buffer: PBS with 0.5 mg/mL bovine serum albumin and 0.1% sodium azide, or equivalent

2. 1% paraformaldehyde in PBS, or equivalent (optional)

BD Biosciences reagents for compensation determination

1. BD CompBead sets

   Contains separate vials, respectively, of either anti-mouse Ig Kappa (catalog no. 552843), anti-rat Ig Kappa (catalog no. 552844), anti-rat/hamster Ig Kappa (catalog no. 552845), and negative control beads.

2. Fluorochrome-conjugated monoclonal antibodies (those being used for your experiment)
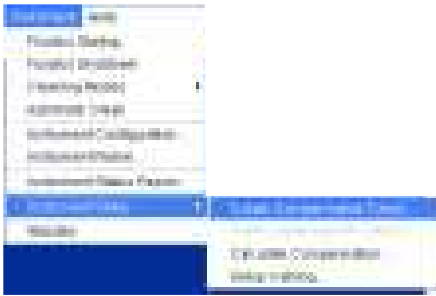
Figure 1

BD FACSDiva Instrument Setup menu for automatic compensation calculation

Figure 2

Compensation Tubes and worksheet created for six-color Experiment

Figure 3

Creating label-specific Tubes for APC-Cy7

Procedure

Staining beads

1. Add 20 μL antibody conjugate (one test) to a test tube. Add one drop of BD CompBeads (approximately 60 μL).

   Use the anti-mouse Kappa capture bead for most BD Biosciences Immunocytometry Systems antibodies. Use the appropriate species specificity for other antibody conjugates. NOTE: Resuspend the beads by gently vortexing the vial before use.

2. Vortex and incubate 15 minutes at room temperature while shielding the tube from light.

3. Add 1 mL of wash buffer, vortex, and centrifuge at 2,000 x *g* for 5 minutes.

4. Remove supernatant, and add 1 mL of wash medium or paraformaldehyde solution.

5. Add 1 drop of BD CompBeads negative control bead to each of the tubes containing stained beads. Alternatively, leave the stained beads as they are, and make a separate tube with 1 drop of negative control bead plus 1 mL wash medium or paraformaldehyde. BD FACSDiva software will accommodate either situation.
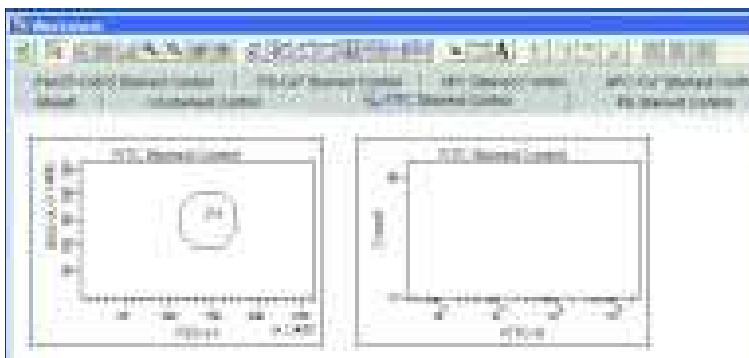
Recording stained beads with BD FACSDiva software

1. Create a new Experiment and specify appropriate parameters (detectors) for the fluorochromes used.

   After creating the Experiment, select the Experiment-level instrument settings in the Browser and use the Inspector to delete any unnecessary parameters. The remaining parameters determine which compensation Tubes will be created in step 2.

   NOTE: If a detector you need is not displayed in the Parameters tab, add it to the current Instrument Configuration or select a more appropriate configuration, and then create the Experiment.

2. Create compensation control Tubes.

   Choose Instrument > Instrument Setup > Create Compensation Tubes (Figure 1). A Tube is added to the Browser for each parameter specified in step 1 (Figure 2).

3. Create label-specific Tubes, if needed.

   If you need to compensate different reagents of the same color, select the corresponding Stained Control Tube in the Browser, and choose Instrument > Instrument Setup > Create Label-Specific Tube(s) (Figure 1). A dialog box appears where you can add Tubes and define labels for the selected fluorochrome, such as CD4 (Figure 3).

4. Adjust PMT voltages as needed with either the Unstained Control Tube (if used) or with the first Stained Control Tube.

   PMT voltages must be set before recording single-stained controls.

5. Record each single-stained BD CompBead Tube in turn, using the PMT voltages set in step 4.

   NOTE: Voltages for color detectors (not for scatter) must be kept constant for the remaining compensation Tubes. You can adjust scatter, if needed.
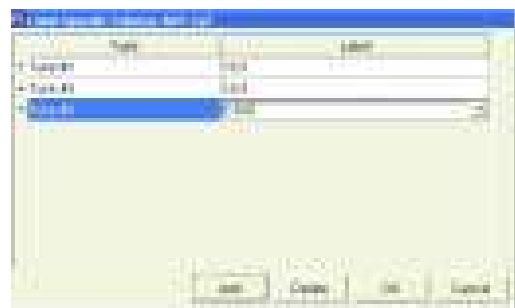
Calculating spectral overlaps

1. On the dot plot for each single-stained control, adjust the region with BD FACSDiva 4.0 to encompass the appropriate population or you can right-click on the gate and select "Apply to All Compensation Controls" to copy the gate to other control tubes.

2. On the histogram for each single-stained control, create gates around the negative and positive populations.

   Use the Auto-Interval tool to quickly create an Interval gate around each population (Figure 4). Adjust the gate boundaries, if needed. Note that the gate on the negative population needs to go past the left axis of the histogram to include all events.
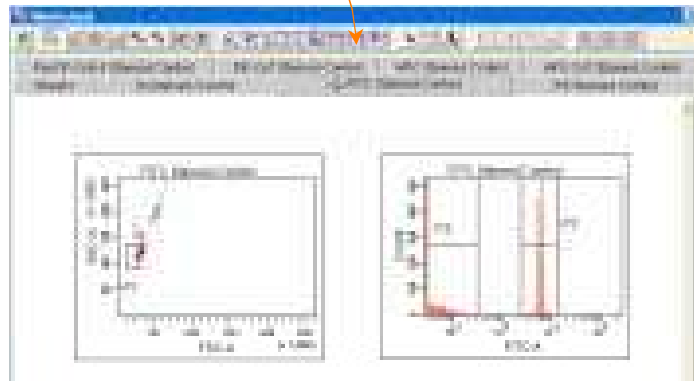
   NOTE: Create negative populations only if you added the BD CompBeads negative control to each tube containing stained beads. If you put the negative control beads in a separate tube, create only positive populations. The software first looks for a negative population within each fluorophore's single-stained control. If no negative population has been defined, the software uses the negative population from the Unstained Control Tube.

Figure 4.

Setting gates for spectral overlap determination.

3. Calculate the compensation.

   After all histograms have been gated, choose Instrument > Instrument Setup > Calculate Compensation. Spectral overlaps are automatically calculated and stored. You will be prompted to name your setup.

Using spectral overlaps in an Experiment

   After Spectral Overlap values are calculated by the software, all Tubes and Specimens in the Experiment that do not already have their own instrument settings will inherit the instrument settings of the Experiment, which are linked to the named Setup. You can also link the named Setup to other Experiments, or to Specimens or Tubes within another Experiment.

1. Create a new Experiment or open an existing Experiment.

2. Right-click the Experiment- or Specimen-level settings and choose Link to Setup.

   A Setup Catalog appears, listing all saved Setups.

3. Select your saved Setup from the Setup Catalog, and click Link.

   Spectral Overlap values from your Setup are copied to the current instrument settings (Figure 5).

4. If your linked Setup contains label-specific Tubes, specify which Spectral Overlap value to apply to new Tubes in one of the following ways:

   • Use the Experiment Layout dialog box to label all Tubes in the Experiment at once.

   • Use the Labels tab in the Inspector to label one Tube at a time (Figure 6).

Compensation is automatically calculated for each Tube based on the corresponding single-stained control or labeled control in the saved setup. For example, if your saved setup contained label-specific spectral overlaps for CD4 APC-Cy7 and CD8 APC-Cy7, compensation will be calculated using the CD4 value for CD4-labeled Tubes, or using the CD8 value for CD8-labeled Tubes. If your setup contained an unlabeled FITC spectral overlap determination, you can still assign labels to FITC Tubes in the linked Experiment, for example CD16 and CD11a, but the Spectral Overlap values calculated from your single-stained FITC control will be applied to both CD16 and CD11a.

Figure 5.

Spectral overlap values (from Figure 4) after linking

Figure 6.

Assigning labels in the Inspector

Conclusions and Notes

A system has been described to easily compensate multicolor stained samples. BD CompBeads provide a convenient method of capturing the experiment's antibody conjugates. The Instrument Setup feature within BD FACSDiva software (version 2.2 or later) automatically determines the spectral overlap of each of the captured conjugates. Additionally, the feature allows measurement of different lots of the same fluorophore, which could have different spectral overlap values. Within an experiment, the correct spectral overlap is applied merely by designating the fluorophore's Label name in either the Tube's Label Tab or the Experiment Layout. Use of the system for determining compensation is exemplified by the properly compensated stained cells in Figure 7.

Some notes on the feature follow.

(a) Single-stained controls. Stained BD CompBeads, stained cells, or a fluorophore bead such as BD CaliBRITE Beads can be used as controls. The stained cell or bead must have only one fluorophore on it, so that measured spectral overlap values are due only to that single fluorophore.

(b) Autofluorescence. It is not necessary that the autofluorescence of the BD CompBeads match that of the experimental sample. Spectral overlap is calculated as the difference between positive and negative populations of the control, so that autofluorescence disappears from the calculation.

(c) Gating the negative population. The Interval gate should go below the axis to include the entire negative population. Populations can gain a valley in the center of the population due to display on a log scale, especially if the population is raised very far above the first decade. Extension of the gate below the axis ensures inclusion of the bottom half of the population in the measurement.

(d) Area versus Height. Spectral overlap values will be slightly different for Height versus Area. If only Area or Height is chosen, the feature will calculate spectral overlap values for that parameter. If both are chosen, calculations are done for Area by default. Note that Area Scaling, Window Extension, and Delay values all alter Area measurements and, consequently, the spectral overlap values measured in Area. Therefore, these values should be entered before PMT voltages are set.

(e) Correct compensation. The Instrument setup feature aligns the positive population with that of the negative population in the overlap direction. When overlaps are applied to the fluorophore Stained Control itself, alignment should be nearly identical. When applied to another sample, there can be small mismatches. This is not unexpected, and could be due to small differences in the overlaps of fluorophore Stained Control versus sample for biological or carrier reasons, or to statistical variation in the populations.

(f) Troubleshooting if spectral overlap values are inaccurate. Contamination of a given single-stained control with a small amount of fluorophore carried over from the previous control can compromise the spectral overlap calculation. To assess this possibility, on the worksheet for the problematic single-stained control, display a dot plot of the primary parameter versus that of the possible contaminating color. If contamination is noticeable, make a fresh tube and re-run.

(g) PMT voltages and spectral overlap. Changing a voltage changes spectral overlap values. Therefore, voltages should be set before spectral overlap is determined, and all tubes in the Experiment should use those voltages. As a subtlety, it is actually the fluorescence brightness that needs to be the same. In cases where the instrument drifts a little, a slightly different voltage can be used to regenerate the original brightness; however, spectral overlaps determined before the drift would still be accurate.

* Patents: PE and APC: US 4,520,110; 4,859,582; 5,055,556; Europe 76,695; Canada 1,179,942 PerCP: US 4,876,190 Cy: US 5,268,486; 5,486,616; 5,569,587; 5,569,766; 5,627,027 PE-Cy7: US 4,542,104 APC-Cy7: US 5,714,386
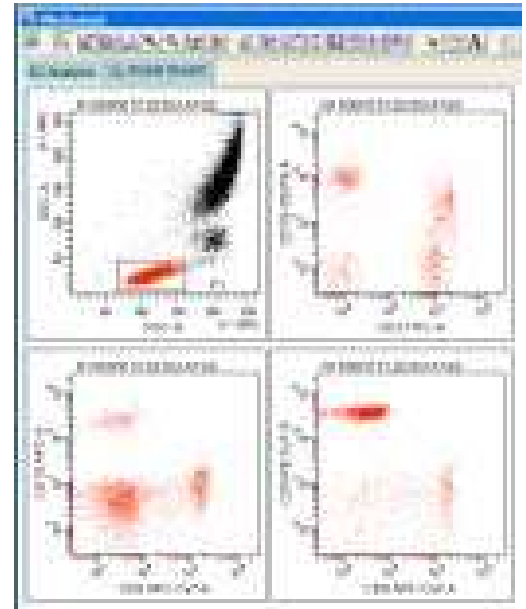

Figure 7.

Compensation calculation from the spectral overlap feature successfully applied to stained cell sample

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