nanocellect Biomedical, Inc.

White Paper

WOLF G2 cell sorter compensation guidelines

1. What is Compensation?

Fluorescent compensation is a mathematical method to correct for fluorochrome spillover by creating a spillover matrix. "Spillover" or spectral "cross talk" occurs when the emission spectrum of a fluorochrome is sensed by a detector that is assigned to another fluorochrome (**Figure 1.1 A-C**). While emission spectra exhibit a maximum value at well-defined wavelengths of light, they actually emit light within a range of wavelengths. Part of this emission peak may fall into the neighboring light detectors meant to capture the maxima of other colors. This spectral cross-talk is measured by analyzing individual fluorochromes (single-stained controls).

1.1 Compensation controls

Every experimental plan should include a panel of single-color controls, stained with one fluorochrome only. These controls allow the software to assess the emission wavelengths falling within non-specific detector channels (for example, FITC signal in the FL2 and FL3 detectors), and then to correct for this signal by subtracting these values from the non-specific detectors. The values are computed to generate a spillover matrix that will show the fraction or percent of each fluorochrome bleeding into other channels. This is essentially a visual representation of the spectral overlap within your panel of fluorophores (**Figure 1.1 D**). A compensation matrix, generated by inverting the spillover matrix, is then applied to the sample fluorescence intensity values in each channel to correct for spillover mathematically. See section 3 for more details on compensation controls.

Compensation should be employed whenever an experiment contains 2 or more fluorochromes. The workflow involves first analyzing an unstained or parent population, followed by single-color controls for each active detection channel to allow automatic calculation of the spectral overlap for each detector.

1.2 Why worry about compensation?

In multicolor flow cytometry assays, compensation is needed to prevent spectral overlap of fluorochrome emissions into neighboring filters. Whenever more than one color is involved, compensation is necessary and should be planned for. Uncompensated samples may give user a skewed or incorrect view of the markers expression in a given sample.

2. WOLF G2 fluorescent panel design

The WOLF G2 is available in three different configurations; upon purchase, users will decide which of the three configurations best fits their experimental needs (see details in the compensation section of the WOLF G2 User Manual).

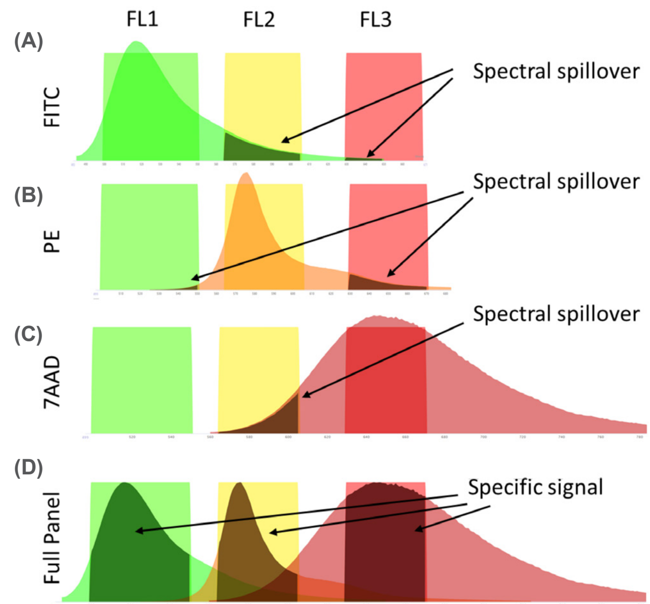

Figure 1.1. Visualization of spectral spillover: Y axis indicates fluorescence intensity (peak height), and X axis represents visible light wavelengths (~499 to 780 nm). The solid color blocks are bandwidth filters that divert fluorescent signal to FL detectors. **(A)** FITC emission spectra are detected specifically in FL1 detector (green). However, a small percentage of it will "spill over" into FL2 and FL3. **(B)** PE emits in FL2, however, neighboring detectors FL1 and FL3 may capture part of the spectrum. **(C)** Same is true for 7AAD DNA stain that emits in FL3 but can also be detected to a lesser degree in FL2. **(D)** All three fluorophores can still be combined into a 3-color panel, since compensation will substract the spillover to only display specific signal, shaded here.

2.1 Fluorescent panel design

When designing a flow cytometry experiment, it is important the user answers a series of questions that would lead to a successful panel design:

What markers am I looking for? How many colors do I need?

Understanding the experiment, the hypothesis and the expected outcomes is very important for a successful panel design. Not only should the user understand what is the target marker, but users should also know what they do not want – and make sure that they can label cells that are not of interest, to avoid sorting unwanted targets.

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What fluorochromes (antibodies, dyes) are available to me? What do I expect to find?

If the experiment is designed from scratch, users may need to do some research to find the best markers and corresponding antibodies. Refer to manufacturer's instructions for initial antibody concentration; antibody titration may need to be performed for optimal results.

Typically, users can predict the expression of a particular marker or the frequency at which the marker will be present on a subset of cells within the sample. The typical wisdom of panel design holds that **the most highly expressed markers should be paired with the dimmest fluorochrome, and the rarest markers with the brightest fluorochrome**. Following this advice can improve detection of weakly expressed markers. The instrument can generally detect the change in fluorescence with just <200 molecules of the fluorochrome. But generally the more, the better for that rare protein of interest to show up clearly.

2.2 WOLF G2 considerations when designing a fluorescent panel

From a technical point of view, the lasers are modulated (alternated) within the system, and each detector will be assigned two colors at a time (one from each laser). While the detectors will be able to detect and discriminate signals from the two lasers based on the timing of the signal, the gain of each individual detector may be shared between the two lasers. **It is essential to pair fluorochromes of similar intensities to be detected by the same detector** to make sure that the gain will match for both fluorochromes. If two fluorochromes of very different intensities are paired on the same detector, adjusting the gain to work for both may be challenging.

3. Best Practices and Sample Preparation for successful compensation

To successfully compensate a sample, proper negative and single-color fluorescent controls are needed. One control is needed for each fluorochrome; the more complex your experiment, the more single-color controls will be needed. The WOLF G2 software offers the possibility to automatically compensate your sample. This feature should always be used for optimal compensation results.

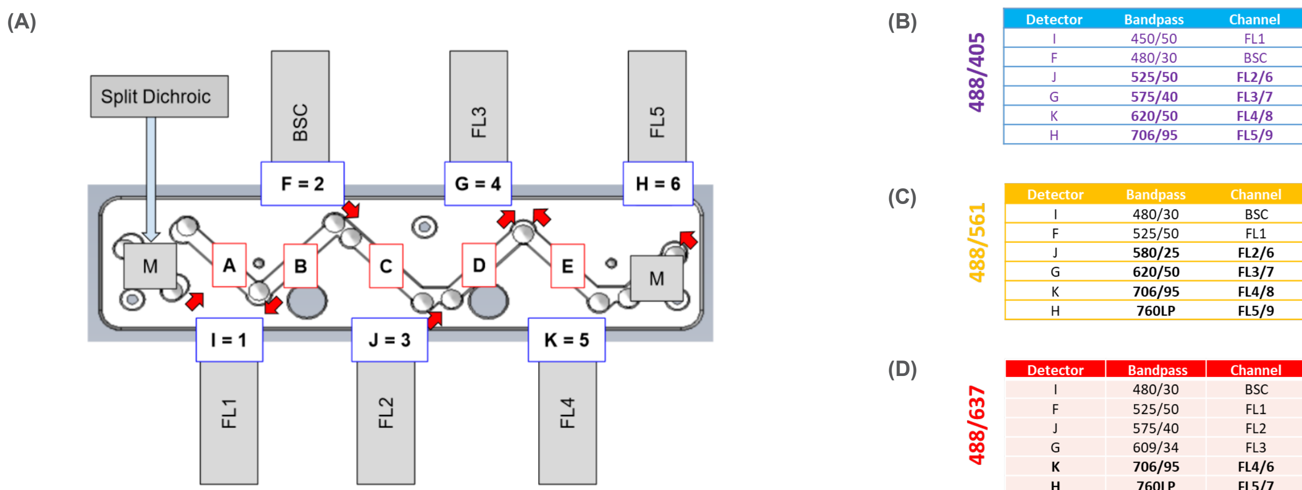
To Prepare Compensation Controls

1. Prepare your fluorescent compensation controls (1 for each color) by labeling your cells with each individual fluorochrome.

   It is recommended that the brightness of your fluorescent controls be at least as bright as, but ideally brighter than your sample. This can be achieved by using compensation beads or cells known to express high amounts of the target marker. **The fluorescent compensation controls must be uniform throughout the whole experiment – either cells or beads, but not both.**

2. Label your cells or beads with each individual fluorochrome. Use the same fluorochrome that is used in the sample you wish to compensate (do not use AF488 to compensate FITC).

   If you are working with a new antibody, a titration may be recommended to determine the best concentration for your sample.



Detector	Bandpass	Channel
I	450/50	FL1
F	480/30	BSC
J	525/50	FL2/6
G	575/40	FL3/7
K	620/50	FL4/8
H	706/95	FL5/9

Detector	Bandpass	Channel
I	480/30	BSC
F	525/50	FL1
J	580/25	FL2/6
G	620/50	FL3/7
K	706/95	FL4/8
H	760LP	FL5/9

Detector	Bandpass	Channel
I	480/30	BSC
F	525/50	FL1
J	575/40	FL2
G	609/34	FL3
K	706/95	FL4/6
H	760LP	FL5/7

Figure 2.1. Fluorescent detector set for each available WOLF G2 configuration: (A) Detector setup: the fluorescence detectors may be shared between two fluorophores if the two are excited by different lasers. "M" indicates mirrors. A-E indicates pre-filters. F-K indicates filters (Bandpass in tables). **(B)** 488/405 nm WOLF G2 configuration will combine detection of FL2 and FL6, FL3/FL7, FL4/FL8, FL5/FL9 on the same detectors. **(C)** Analogous representation of 488/561 nm configuration. **(D)** Chart for the 488/637 nm configuration.

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3. Prepare an unstained control.

   Your WOLF uses a universal negative control. This means beads and cells cannot be used together to calculate compensation. Autofluorescence, which is much different between beads and cells, can affect compensation and should be the same across all the compensation controls.

4. Analyze the negative control to establish the negative and positive gates in all channels. Acquire at least 10 000 events in the gates used for compensation to obtain accurate median values.

5. Analyze the positive controls. Acquire at least 10 000 events in the gates used for compensation to obtain accurate median values. Establish optimal detector gains for each fluorochrome to avoid saturating signals (events against the axes walls). **The values of the gains for each detector must stay constant across all the compensation controls**. If a gain needs to be modified, all the compensation controls will need to be re-acquired for the compensation to work properly. Use the same detector gains for your sample of interest.

6. **We recommend acquiring your fully stained control and adjusting detector gains to appropriate levels before moving on to acquire all your single stained controls. This will save you time.**

7. Once all controls are acquired, you can calculate the compensation matrix and apply to your sample of interest. The experimental conditions of your sample of interest (fluorochromes, gains) must be the same as those used to acquire the compensation controls.

The WOLFViewer auto-compensation algorithm processes the data of every control and automatically calculates the appropriate compensation for each fluorochrome combination. Currently, WOLFViewer facilitates generating automatic compensation matrices as well as manual editing of existing matrices.

We recommend using automatic compensation algorithms to correct for spillover with high accuracy. The automatic compensation feature will analyze the single stained and unstained controls to generate the values for the compensation matrix without further user input. If results after applying a matrix are not as expected, the user should look at redesigning the controls, or adjusting the matrix using the Edit Compensation Matrix functionality. WOLFViewer offers the option to adjust a compensation matrix by editing the associated spillover matrix in the wizard. The user may choose to do this to correct a mathematical error resulting from mis-matched controls or another unexpected experimental error. To adjust particular fields in a matrix, the user will need to know how to read it.

See the Compensation section of the User Manual for a step-by-step guide on how to navigate the compensation feature in the WolfViewer software.

For more information, visit nanocellect.com or email info@nanocellect.com

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