

vFC™ Protocol 0.4 - Fluorescence Compensation using vCal™ nanoCal Beads

Objective

Determine the spillover and compensation matrices to account for spectral spillover in the relevant fluorescence channels.

Materials

- vCal™ nanoCal antibody capture nanobeads (four peaks, one negative, three positive, 800 nm diameter polystyrene, 1×10^7 /mL)
Note: nanoCal beads are available for each of mouse, rat, rabbit, and hamster antibodies.
- Fluorescent antibody conjugate(s) (10x)
- vFC™ Staining Buffer
- 1.5 mL microfuge tubes

Procedure


Prepare Beads

1. Briefly vortex vCal™ nanoCal Beads.
2. Add one drop (~50 uL) of nanoCal beads in a v-bottom plate or 0.65 mL microfuge tube for each marker to be calibrated.
3. Add 5 uL of each fluorescent antibody conjugate to be compensated/unmixed to a different well.
4. Incubate for 1 hour at RT.
5. Wash beads by two cycles of centrifugation and resuspension. To the stained beads add 1000 uL vFC™ staining buffer followed by centrifugation at 10,000 xg for 10 min (*note: there will not be a visible pellet*). Aspirate buffer carefully by removing buffer from the top of the tube and stopping when about 50uL remains. Repeat wash 1 more time. After second aspiration, add 200uL of vFC staining buffer to tube and resuspend beads by vortexing for 10 secs.

Measure Beads

6. Measure using the vCal Bead Template at same fluorescence detector gains as for vFC™ analysis.
7. Measure a Buffer-only well to measure the system background.
On conventional flow cytometers, including the CytoFlex and Aurora, lower the SSC trigger channel threshold until background noise events trigger detection. On the CellStream and ImageStream, measure the buffer as you would measure beads.
8. Save data files with informative names that include the data and antibody conjugate used to stain the bead (eg 210704 nanoCal CD9 PE.fcs, 210704 Buffer-unleaves, etc).

Data Analysis

1. Open the vFC™ Protocol 0.4 layout using FCS Express or FCS Express Reader
2. Load the data files from the stained nanoCal beads (plus the unstained bead control) into the Data List. Load the data files from vesicle spectral reference standards (vFRed-Lipo100, GFP-EV, plus buffer-only).
3. On the **nanoCal Tab**, use the bivariate plot of scatter parameters to identify and gate on the population of single beads. Load the single-color bead data into the appropriate individual histograms. Inspect the positive and negative populations in each histogram.
4. On the Gating Tab, load vFRed-Lipo 100 (and GFP-EV, if used) data into the appropriate histograms and inspect the data
5. Click on the **Tools** tab→**Transformations** group→**Compensation and Unmixing** command to open the **Compensation and Unmixing** navigator (Figure 1).
6. The **Compensation and Unmixing** navigator can be moved anywhere on the screen; it is [dockable and pinnable](#), and you can move it around the screen or close and reopen as needed.
7. Click on the blue plus button, , to **Add a new compensation** (Figure 1, red outline).
8. A text box highlighted in blue will appear with the text **New Compensation** (Figure 1).

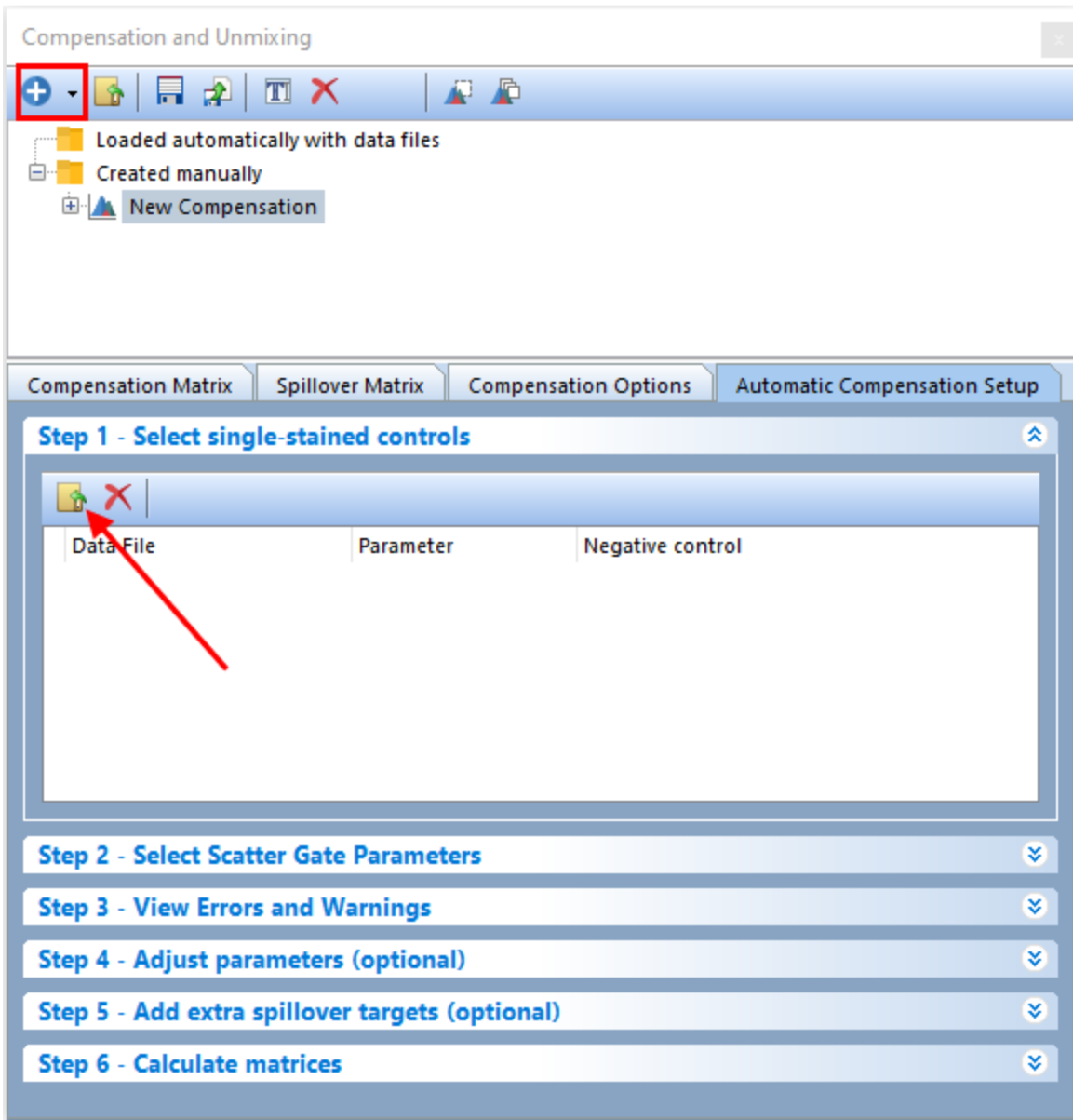

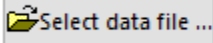


Figure 1. Compensation and Unmixing navigator: use to add new compensations, edit definitions, create Automatic Compensations, and unmix spectral signatures

9. Click on the Add data for compensation button, , in the **Automatic Compensation Setup**→**Step 1 - Select single-stained controls** window (Figure 1, red arrow).

10. The **Standard Open Data Dialog** will appear (Figure 2).

11. Note: based on your [User Options](#), the [Advanced Open Data Dialog](#) may appear. If

the **Advanced Open Data dialog** appears, please select the  button to access the **Standard Open Data Dialog**.

12. Select and load the single stain and negative control data files appropriate for your instrument (**Table 1**).

13. Click **Open file**.
14. Check that all of the single-stained control files and the unstained control file have been brought into the **Select single-stained control** window. In some cases, the **Parameters** will need to be set manually. Assign each single-stained control the appropriate **Negative Control (Table 1)**.

Data File	Parameter	Negative control
Buffer	Negative control	
Lipo100	vFRed-A	Buffer-only
Bead-FL1	FL1	Use self as negative
Bead-FL2	FL2	Use self as negative
Bead-FL3	FL3	Use self as negative
Bead-FLn	FLn-A	Use self as negative

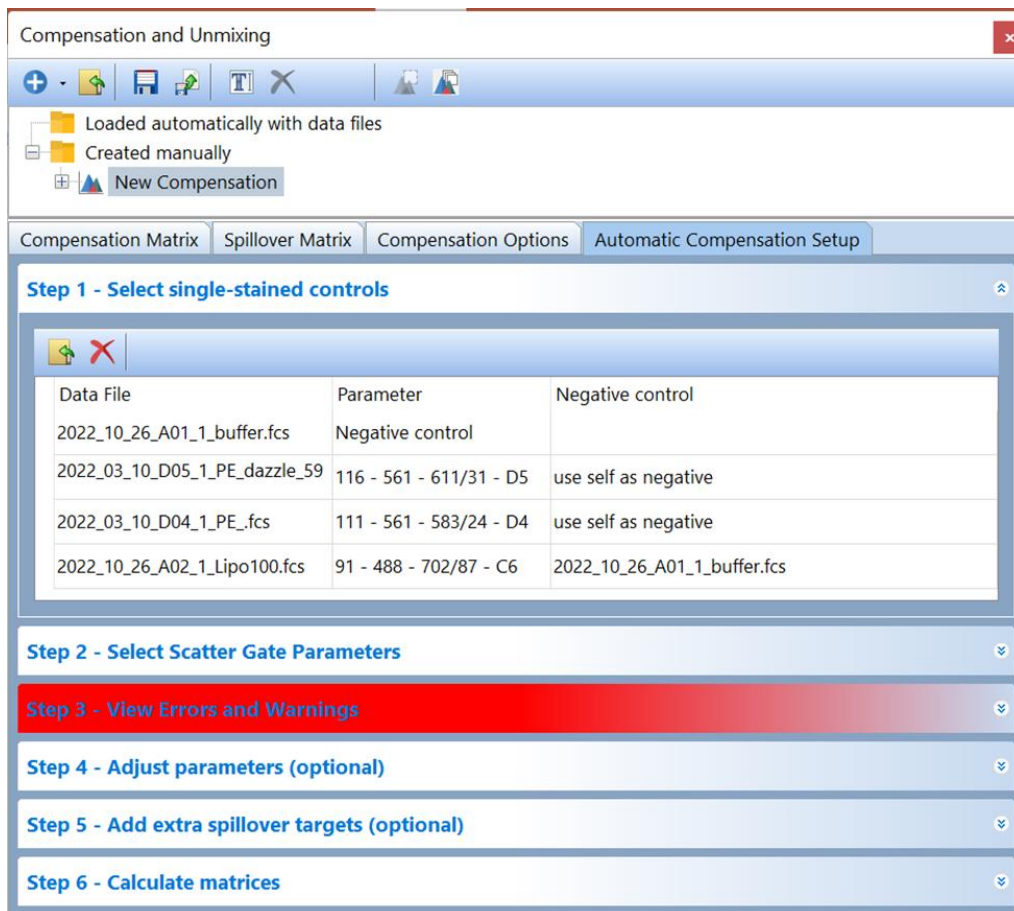


Figure 2. Files for controls have been added to Step 1 in the Automatic Compensation Setup and are typically matched automatically to their respective parameters. Note: For some instruments this may need to be done manually (See instruments specific protocol 0).

15. Set the Scatter Gate Parameters (Step 2) to SSC-H and VSSC-H (Figure 3). FCS Express may regenerate charts each time something changes but this shouldn't affect the compensation results.

- a. CytoFlex: SSC-H vs VSSC-H
- b. CellStream: Raw Max Pixel SSC - 773/56 - A1 vs SSC - 773/56 - A1
- c. ImageStream: Raw Max Pixel MC CH12 vs Intensity MC CH12

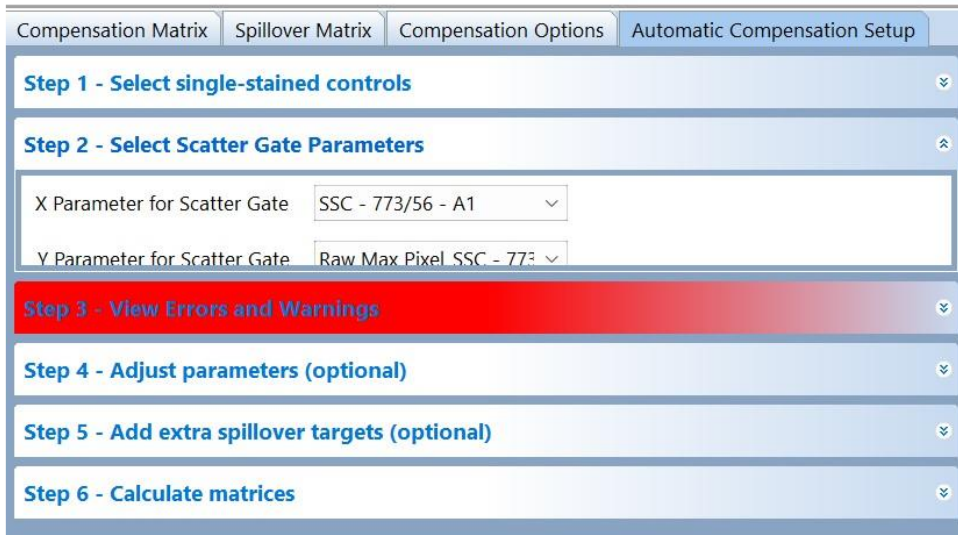
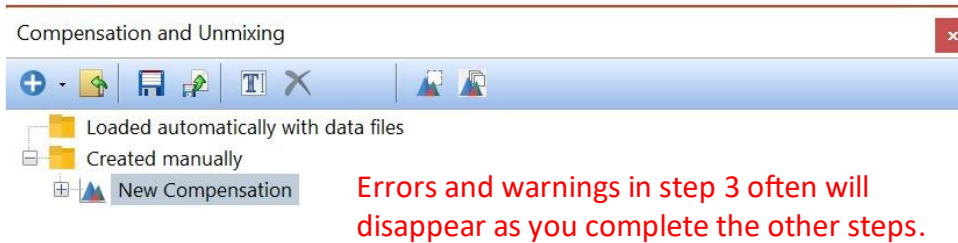
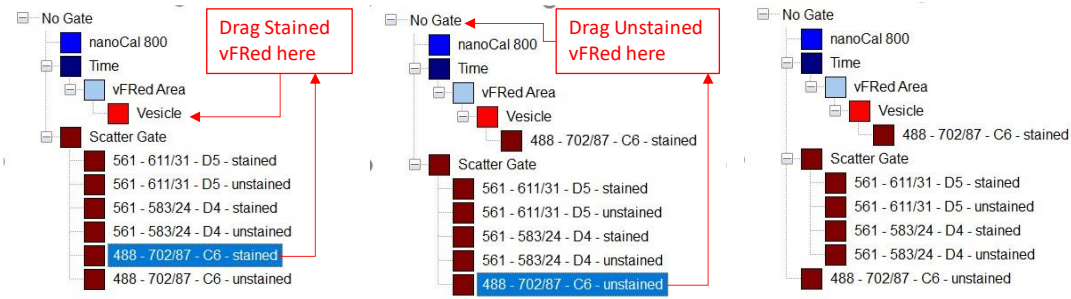


Figure 3. Select scatter gate parameters

16. Once Step 1 and Step 2 of the FCS Express Compensation wizard are completed, charts and gates will be automatically generated for the compensation.
17. Step 3 View Errors and Warnings – Don't worry about this step until the end. The errors should be resolved by the end of the protocol.
18. In the gate hierarchy (on gating tab) adjust so that the positive and negative control for vFRed are not gated (See diagram below).



19. Inspect the plots on the newly created pages (Figure 4). Inspect and adjust the Scatter Gate to select the population of single stained beads. For the antibody-stained beads, confirm that the positive and negative populations are correctly identified. Adjust positive and negative gates/markers as needed. The software should identify the brightest of the four peaks in the nanoCal bead set. The vFRed control charts will need to have their gate adjusted as shown below (Figure 4). The chart for the stained vFRed control should be set to use the vesicle gate while the chart for unstained vFRed should be set to no gate.

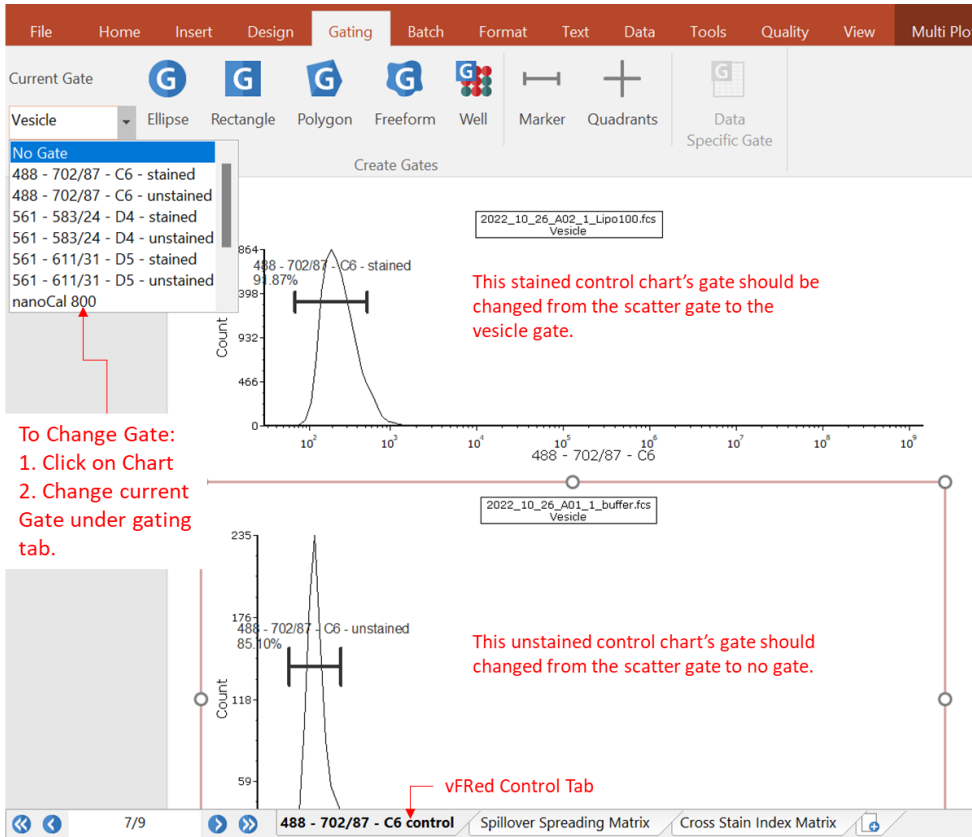
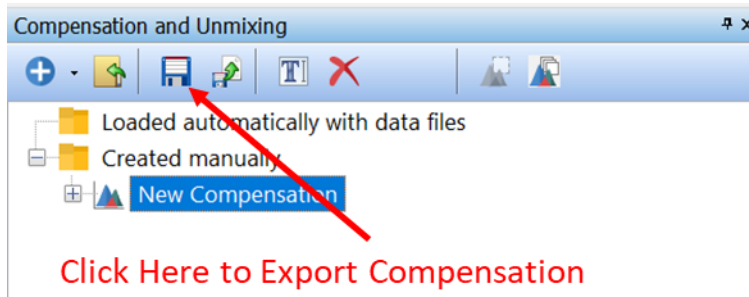


Figure 4. Adjust markers and gates on generated compensation plots (vFRed charts shown).

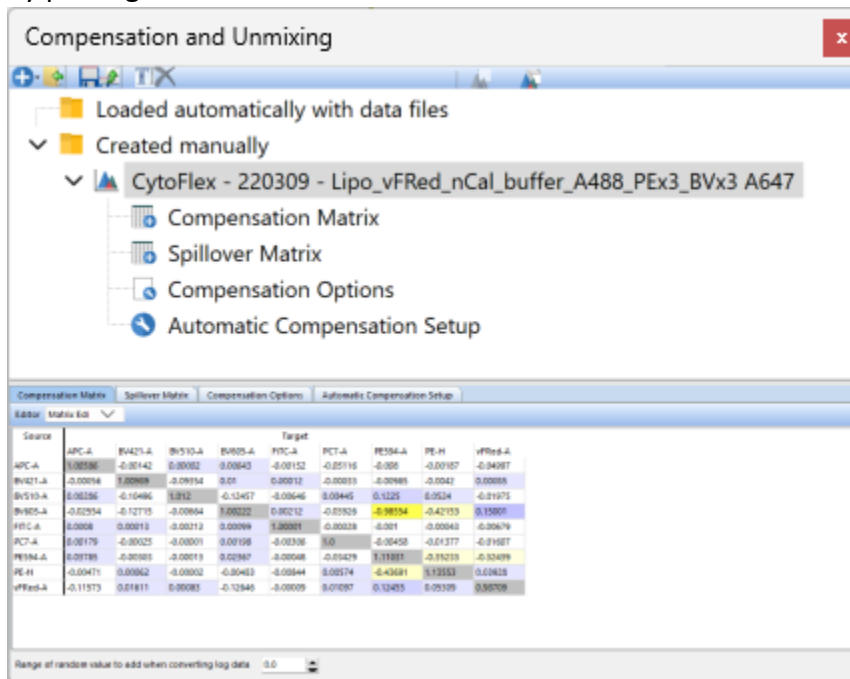
20. Click Calculate Matrices (Step 6) using the median fluorescence intensity values. The Spillover and Compensation matrices will be calculated (Figure 5).

Figure 5. The calculated Compensation matrix.

- Export the compensation matrices as a *.compensation file with an informative name (eg. Protocol 0.4 – 220309 – Lipo100_vFred_nanoCal_buffer_Ax488_PEx3BVx3_A647.compensation, describing the particles and fluorophores used).



- Copy the name of this file, and rename the New Compensation (right click, F2, Rename) by pasting.



- Save the Layout with a similarly informative name (eg Protocol 0.4 – 220309 – Lipo100_vFred_nanoCal_buffer_Ax488_PEx3BVx3_A647.fey) as documentation of the calculations that produced the Spillover and Compensation matrices.

Notes

For more information about using FCS Express for Compensation and Spectral Unmixing, see the FCS Express manual, Tutorials, and videos at denovosoftware.com.

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