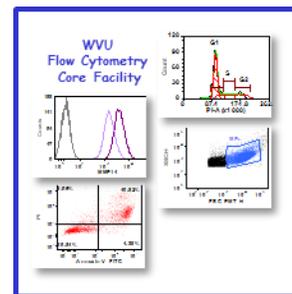


WVU FLOW CYTOMETRY CORE FACILITY



Newsletter Volume 1 issue 3

January 2015

A very Happy and Prosperous 2015 to all of you! In the current issue, we highlight the benefits of using multiple fluorescent molecules in a single experiment and tell you how to optimize the setup and analysis of such an experiment. Due to the complexity and importance of this topic, we will not be featuring an instrument in this issue. Also, the frequently asked questions (FAQ) on page 4 has been replaced by announcements about data organization associated with the LSRFortessa. The instrument highlight and FAQ will return in the next issue.

Issue highlight: Multicolor analysis

Recent innovations in flow cytometry instrumentation, fluorochrome reagents and software algorithms have led to a new era in multicolor flow cytometry. One can assess the functional state of any cell type with respect to cell differentiation status, cell cycle status, cell signaling, effector function and other cell fate. Newly developed cell fixation and permeabilization buffers enable the concurrent detection of multiple cell surface markers as well as intracellular cytokines; transcription factors, phosphoproteins, DNA and RNA. Multiparametric marker analysis has also become routine in clinical settings, facilitating the diagnosis, classification, staging and monitoring of many disease states.

The numbers and types of lasers and optical filters in a flow cytometer determine the multicolor combinations that can be analyzed. For example, the recently acquired BD LSRFortessa in our facility equipped with four lasers (violet, blue, yellow-green and red) and 14 bandpass filters, can detect 13 different colors/markers in its current configuration. While the power of single cell analysis grows with each additional laser and set of filters, the experimental design for such analyses calls for more stringent controls.

Below is a list of [pre-experiment preparations and experimental controls](#) that are [key](#) to obtaining meaningful data using multicolor analysis.

Antibody titrations: While most reputable companies that sell marker specific antibodies provide a suggested dilution factor for use with a given number of target cells, the density target marker varies with cell type and therefore, it is up to the user to find the antibody concentration/dilution that is optimal for their cells of interest. Titrating antibodies has a couple of advantages. First, in many cases good quality staining can be obtained with less antibody than what is recommended by the manufacturer, resulting in a cost savings. Second, too much excess antibody can cause high background and make compensation of spectral overlap (explained below) as well as data analysis difficult.

Fc receptor blocking: Antibodies bind through their non-specific tail end

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(the Fc region), via Fc receptors on many types of cells. With the development of bright fluorochromes and higher fluorochrome to antibody conjugation ratios, it has become important to minimize such Fc-mediated binding, to avoid misquantitation of target cell/epitope frequency. If you are staining human or mouse cells, pre-incubation of cells with human IgG or mouse IgG, respectively, for 15-20 minutes will block Fc receptor binding during subsequent staining steps.

Single color controls: Most commonly used fluorescent dyes emit fluorescence over a broad range of the spectrum when excited by commonly used lasers. This property creates a spectral overlap between different dyes. This causes the fluorescence from one channel to bleed into that of another fluorochrome channel on the flow cytometer, resulting in false signal. This spectral overlap (or bleed) can be compensated for, by appropriate subtraction algorithms built into the acquisition software of the cytometer. In order to accomplish this, one must have control samples that are stained with a single fluorochrome, for each fluorochrome used in the experiment.

Autocompensation: Once you start using more than 4 fluorescent molecules in your flow cytometry experiments, performing manual compensation for spillover into the other detectors can be quite daunting and time consuming. The FACS Diva acquisition software has an autocompensation tool to assist you. This tool requires that your single color control contains a positively stained population clearly distinct from the unstained cells. If you don't have good separation between the positive and negative populations, you can use commercially available compbeads from vendors like eBiosciences, Spherotech and Bangs laboratories. You incubate the beads with your antibody and acquire data using the same voltage settings as for your cells. The FACS Diva software will automatically calculate and subtract any spillovers. Compensation can also be accomplished using the FCS 4 Express analysis software. See page 3 of this newsletter for more details.

Fluorescence minus one (FMO) controls: Simultaneous staining of cells with different fluorescent dyes sometimes results in fluorochrome interactions that result in a combined spillover into non-target detection channels. This phenomenon is usually not a problem when assessing discreetly stained populations. However, when assessing dim or non-discreet markers, setting the correct boundaries between negative and positive populations during analysis is greatly facilitated with the use of FMO controls. Each FMO control is a mix of all fluorochromes used in the multicolor experiment, less (minus) one. There needs to be one FMO control for each fluorochrome used.

Please contact us if you need assistance planning a multicolor flow cytometry experiment. We will guide you in selecting the best fluorochrome reagents, proper controls and data analysis.

To log in and reserve a flow cytometer, AutoMACS, MSD or analysis computer, please point your browser to the following URL



Core Ordering & Reporting Enterprise System

<https://cores-wvu.mis.vanderbilt.edu/login.cfm>

Instruments in the Facility

FACSAria Cell Sorter/Analyser

Operator: Facility Staff

Lasers: 488 nm Sapphire(SS)
633 nm HeNe
407 nm Violet(SS)

Detection Parameters:

Forward Scatter, Side Scatter, simultaneous detection of up to 11 fluorochromes

Applications:

Cell Sorting (Aseptic)
Cell phenotyping
Cell Viability
FISH, FRET, SPA

FACSCaliber Analyser

Operator: User

Lasers: 488 nm Argon
633 nm Red Diode

Detection Parameters:

Forward Scatter, Side Scatter, simultaneous detection of up to 4 fluorochromes

Applications:

Cell phenotyping
Cell cycle analysis

AutoMACS Magnetic

Bead separator

Operator: User

Application:

single marker (extracellular) sorting, depletion sorting

LSR Fortessa

Operator: User

Lasers: 405 nm
488 nm
561 nm
628 nm

Detection Parameters:

Forward Scatter, Side Scatter, simultaneous detection of up to 12 fluorochromes

Applications:

Cell phenotyping
Cell Viability
Cell Cycle analysis
FISH, FRET, SPA

Analysis Tips

Data acquired on LSR Fortessa and FACSAria can be adjusted for **compensation** of spectral overlap during acquisition, using the FACS Diva software or following acquisition using either the FACS Diva or the FCS Express software during subsequent analysis. The latter is particularly useful if you are re-analyzing files that you or someone else acquired some time ago.

Compensation can be achieved **manually** or **automatically** in FCS Express. In this issue, we show you how to compensate flow data manually. Manual compensation can sometimes introduce **digitization errors**; explanation of how to correct such errors follows. Automatic compensation will be discussed in a future issue.

Manual compensation of data in FCS Express

You will first need to create a new layout in FCS Express and open your data file(s) to which you wish to apply compensations, in the appropriate 2D dot/contour/density plots, and draw and apply appropriate live cell gates. Then perform the following steps:

- 1 Right-click on the plot where you wish to apply compensations to bring up the associated pop-up menu.
- 2 Select **Compensation > Create New** from the pop-up menu. A **Create New Compensation** dialog will open.
- 3 Type a name in the **Enter a name for the new compensation** field.
- 4 If you will perform batch analysis, then make sure that the box underneath the name field where it says **Use this compensation on new plots** is checked, so that the same compensation is applied to all files that will

be loaded into this plot. If you are not performing batch analysis, uncheck this box.

- 5 Click **OK**. An **Edit Compensation** dialog will now appear.
- 6 Use the sliders to adjust the plots. Note that moving the slider to the right causes the population to move in the direction of the arrow in the image to the left of the respective slider. Be careful not to overcompensate.
- 7 Click **OK** to accept the compensation.
- 8 Perform steps **1-7** for each new plot where you wish to apply compensations for spectral overlaps.
- 9 If these steps introduce digitization errors in the compensated plots, follow these steps:

Correction of digitization error

- 1 Click on the plot that contains digitization error(s) to select it.
- 2 Select **Data > Data Info > Compensations** command.
- 3 Click on **Create Manually** folder to open it, then on **Digitization Error**.
- 4 Click on the up or down arrows to change the numbers in the **Range of random value to add when converting log data** field. The plot will change in real time to reflect the change.
- 5 Close the window when the error has been acceptably corrected. Repeat for each plot.
- 6 Proceed to batch analysis if this is your goal.

Note to users:

Please acknowledge the WVU Flow Cytometry Core Facility when reporting your flow cytometry data, using the appropriate phrase(s):

FACSAria users: Flow Cytometry experiments were performed in the West Virginia University Flow Cytometry Core Facility, which is supported by the National Institutes of Health equipment grant number RR020866 and the Institutional Development Award (IDeA) from the National Institute of General Medical Sciences of the National Institutes of Health under grant numbers P30GM103488 (CoBRE) and P20GM103434 (INBRE).

LSRFortessa users: Flow Cytometry experiments were performed in the West Virginia University Flow Cytometry Core Facility, which is supported by the National Institutes of Health equipment grant number S10OD016165 and the Institutional Development Award (IDeA) from the National Institute of General Medical Sciences of the National Institutes of Health under grant numbers P30GM103488 (CoBRE) and P20GM103434 (INBRE).

What's New for 2015

As the new year starts we would like to make our LSR Fortessa users aware of some changes that have been made to data organization in order for the software and instrument to operate more smoothly.

1. All experiments from 2013 have been archived onto the WVU server and removed from the desktop as well as the FACS Diva database on the associated computer.
2. All users will find a new folder labeled "2015" under their named folder. Please use this folder for all experiments you run this year. If you want to use an experiment setup from 2014 folder there are two options.
 - A. You can export the experiment in the same manner as you export files except you choose the "experiment template". A box will open up and you can give it your lab name and a name for the experiment. Once you do this it will be there any time you want to use that set up. To open the experiment in the 2015 folder just right click on the folder, select new experiment, find your experimental template in the list and click open. Now you are set to go.
 - B. You can duplicate the experiment in the 2014 folder as you normally do then drag it into your 2015 folder.

Email us with questions / topics you would like to see included here in future issues!

Upcoming Events

March 30-31 & April 1	Kathy out of town	Facility open to experienced users, sorting limited to Fridays
January 19	Martin Luther King Day	Facility Closed
April 3	Spring Holiday	

Fee Schedule (2014-2015 hourly rates)

	WVU user	Non-WVU user
Data acquisition by User	\$33.00	\$48.84
Facility assistance for data acquisition	\$17.00	\$25.16
Data acquisition by Facility Staff	\$50.00	\$74.00
Sorting	\$74.00	\$109.52
Data Analysis (unassisted)	0.00	0.00
Data Analysis by Facility Staff	\$50.00	\$74.00

New User Guide

Hands-on training for FACSCaliber and LSRFortessa is **mandatory** for all new users and must be scheduled by consultation with facility director.

Sorting as well as data acquisition on FACSAria is by facility staff only.

Training will initiate with user's first experiment. Due to the complexity of the instruments and software, facility staff will fully assist with the acquisition of the first dataset and will continue with additional assistance on a "needs" basis until users are comfortable operating the instrument on their own.