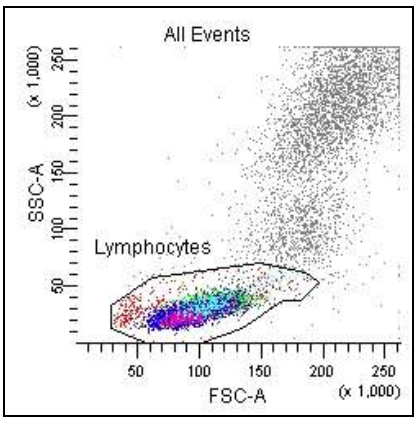
Go With the Flow

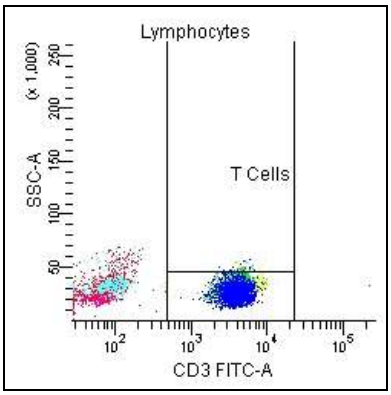
Flow Cytometry Optimization

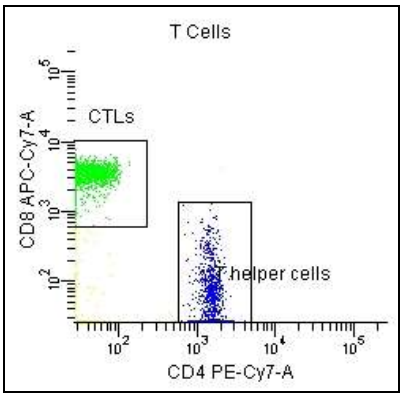
Following is a list of recommendations for optimizing the data in your flow cytometry experiments. It is not all-inclusive, but should help in planning and obtaining the optimal data possible.

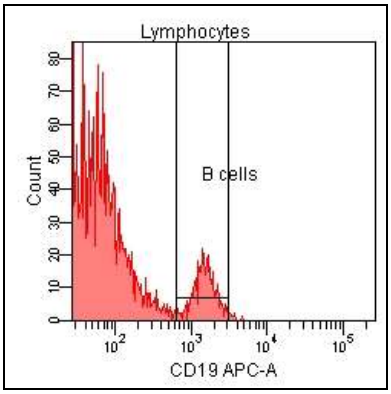


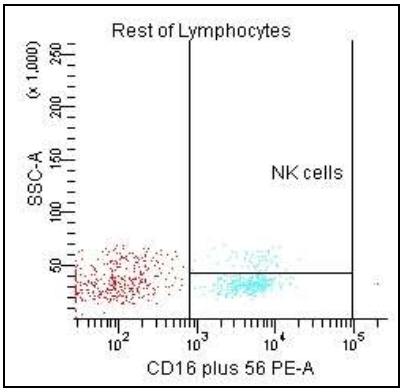
Tube: 3/16+56/45/4/19/8/

Population	#Events	%Parent	%Total
All Events	10,000		100.0
Lymphocytes	5,490	54.9	54.9
T Cells	4,409	80.3	44.1
CTLs	1,997	45.3	20.0
T helper cells	2,248	51.0	22.5
B cells	372	6.8	3.7
Rest of Lymphocytes	711	13.0	7.1
NK cells	332	46.7	3.3









Representative Flow Data

Designing Your Experiment:

Step 1

- What are you trying to prove?
  - Biological hypothesis

Step 2

- Which subsets do you need to identify?

Step 3

- Choose antigens needed to identify subsets
  - Primary, Secondary, Tertiary
    - Primary – Well-characterized, identify broad subsets of cells, expression is usually on/off. (e.g. CD3, CD4, CD8, CD14, CD19, CD20)
    - Secondary – Well-characterized, bright expression patterns, expression levels can be a continuum (e.g. CD27, CD28, CD45RA/RO, gIFN)
    - Tertiary – Low expression levels or uncharacterized (e.g. CD25, CCRs, ?)
  - Use a Dump channel (label antigens on negative cells)

Step 4

- What Fluorochromes are available?
  - What lasers are available? (405, 488, 633)
  - What fluorochromes can the machine detect? Which filter sets are installed? (See your friendly core facilitator…)
- What conjugates are available? (Consider using Biotin/Streptaviden staining to "buy" another color)

Step 5

- Design staining combinations (panels)
  - By Antigen Density
    - Match high Quantum Efficiency (bright – e.g. PE, APC) fluorochromes with low antigen density
  - By Compensation considerations
    - Minimize compensation by using fluorochromes with widely spaced emission peaks
    - See www.bdbiosciences.com/spectra for a spectral overlap tool

Controls, Controls, Controls

(Which controls and when?)

Instrument Setup

- Unstained cells to make sure cells are on scale and to set PMT voltages based on background fluorescence

Compensation Controls

- Single stained controls (cells) for each fluorochrome
    - Use a 'bright' control – dim cells cannot be distinguished well from autofluorescence, so compensation is only valid for samples that are duller than the control
- Antibody capture beads plus antibody-fluorochome conjugate for each parameter
    - These work very well for dim or weak expressing antigens
- Compensation is specific for the fluorochrome, not the cell type, so it does not matter whether you use beads or cells, as long as autofluorescence is the same in the positive and negative populations you are lining up.

Gating Controls (FMO – Fluorescence Minus One)

- Unstained cells or complete isotype control stains are improper controls for determining positive vs. negative expression in multi-color experiments. The threshold for positivity may depend on the amount of fluorescence in other channels. The best control is to stain cells with all reagents except for the one of interest – Fluorescence Minus One.
- FMO controls should be used whenever accurate discrimination is essential or when antigen expression is relatively low.

Isotype Controls

- Used to identify Non Specific Binding
- Match to the isotype of the primary antibody
- Do not use cells stained with all isotype controls to set the gates!
- Add the one isotype reagent to the appropriate FMO that determines where to set gates for that fluorochrome
- Warning! Do not rely on them too heavily! No longer considered essential
- They are not the perfect control – they are not necessarily matched to the concentration of your reagent

Experimental Controls

- Normal samples or untreated cells

Optimization Steps

Area Scaling Factor

- For the FACSAria, this adjustment needs to me made. Because of the design of the instrument, the width of the laser beam is only 9 um. So depending on cell size, the whole cell may not be seen at once as it passes through the beam. In order for the instrument to properly calculate the area of the pulse it measures, the area scaling factor must be set to fully include the whole pulse area. If the whole pulse area is not measured, small or dim populations may me missed. This is done by turning on the height parameter and adjusting the ASF until the height and area are the same. This must be done for each laser used. It will vary from experiment to experiment and should be set for each experiment. This should be the first step of setting up the experiment (before compensation), and then the height parameter should be turned off (to save on data space.)

Viability Dyes

- Dead cells non-specifically bind antibodies and masquerade as viable cells. They bind all kinds of markers. Not all dead cells can be gated out by use of scatter properties. By using a viability dye, the dead cells can be gated out and more accurate discrimination of populations can be determined. This is essential when considering small or weak expressing populations.

Titrating Reagents

- Needed to determine the appropriate staining concentration in order to saturate all antigens on cell. Concentration of reagents varies from lot to lot. Titrating improves accuracy of staining, which is critical if comparing mean fluorescence intensities. It saves money and avoids non-specific binding caused by high concentration of reagent. Further explanation and a protocol can be found at www.microbiology.emory.edu/altman/f_protocols/f_flowCytometry

Compensation

- This is a whole subject by itself! The tip here is: if you are doing more than three colors, use the autocomp feature. It is much more accurate than manual methods. Relying on setting compensation by visualization is not an option on the Aria due to many things including analog to digital data conversion, log scale distribution, and fluorescence measuring error from multiple parameters. The Autocomp Beads are highly recommended and work very well when there are dim or small populations.

Miscellaneous Flow Tips

Tandem Dyes

- PE-Cy5 (Cychrome) can non-specifically bind to monocytes.
- APC-Cy7 doesn't work well with fixatives. It dissociates, causing more compensation in the APC channel.
- Exposure to light and temperature can affect tandem dyes. Follow manufacturer's recommendations for temperature storage. Watch expiration dates closely. When working with tandems, just make sure all tubes and dyes are kept in the same conditions – same amount of time in the light, temp, etc.
- Different lots of tandems can require significantly different amounts of compensation.

Bright – Off Scale Values

- If possible, use a different fluorochrome.
- Try adding a little unconjugated antibody, before adding the conjugated antibody to take up a few of the antigen sites.

Green Fluorescent Protein (GFP)

- If too bright (off scale) try leaving FITC voltage at normal setting and reading the fluorescence in the PE channel or even another higher channel. Turning the FITC voltage way down to accommodate the GFP can increase the amount of compensation in other channels.

Doublet Discrimination

- Cells can stick together and cause erroneous data as they are passed through the laser. This is especially a problem for sorting, where purity is desired or in cell cycle analysis where it is imperative to look at one cell at a time. By turning on the width parameter, it is easy to gate out doublets and aggregates, as they will be apparent (they will have a larger width.)

Linear vs. Log

- As a general rule, always use linear parameters for FSC and SSC. Use log parameters for fluorescent parameters. Only use log scale for scatter when looking at very small particles such as bacteria or platelets. Linear scale for fluorescence is used for cell cycle studies.

Helpful Resources

Web Resources:

- www.bdbiosciences.com/spectra for viewing spectral overlaps to help choose fluorochromes
- www.facs.scripps.edu/recovery.html for a calculator to help estimate sort recovery
- www.jcsmr.anu.edu.au/facslab/statistics.html for a good tutorial on when to use which statistic for analysis (can be accessed from the Scripps website as well)
- www.microbiology.emory.edu/altman/f_protocols/f_flowCytometry/p_titering_Abs.html for an explanation of how and why to titer antibodies
- www.drmr.com for protocols on making your own conjugated antibodies

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Feel free to contact these specialists about your specific applications, reagent questions or protocol advice.