Panel Design Cuidelines
Created by CC Flow Cytometry Shared Resource
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# The Cardinal Principles of Panel Design

With the arrival of flow cytometers designed to measure 10 or more fluorescent markers, flow cytometry panels require very careful planning and design to avoid some of the inherent problems of spectral overlap. These guidelines provide specific assistance for creating a new or modifying an existing multicolor panel on each of the Cancer Center FCSR flow cytometers. Page 5 also describes basic "backbone panels" of fluorochromes that generally work together well on each instrument.

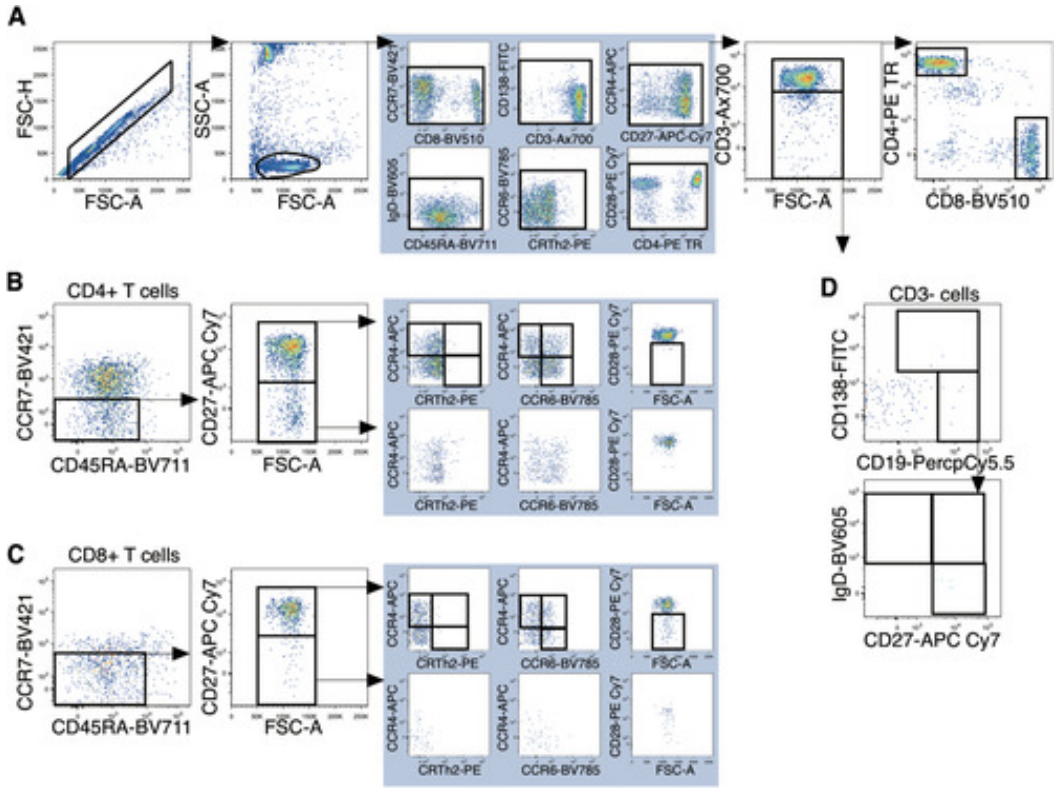
1. Choose fluorochromes that are spread across the spectrum of lasers and detectors. Selecting the first color on each laser path will create a panel needing very little, if any, compensation. For example, the Yeti has UV, 405, 488, 561, and 604 lasers, so choosing BUV395, BV421, FITC, PE, and APC for a five-color panel is the best choice. This panel will need a very small amount of compensation for PE spillover into FITC (<2%) yielding very little digital spread.

2. Understand the biology of your experiment and define your analysis objectives. Optimized Multicolor Immunofluorescence Panels (OMIP) can be a good place to start. These peer-reviewed panels are available in Cytometry Part A through the Wiley Online Library. The OMIPs provide a wide variety of well-designed analyses for various types of cells, but be sure to verify that color choices are best for your instrument of choice.

| | |
|---|---|
| Objective | Briefly describe the purpose of your experiment, e.g. "Measuring mouse PBMC subsets" |
| Species | Human, mouse, etc. |
| Cell types | List the cell type you will be using, for example PBMC, bone marrow, WBCs in tissue digest |

3. Sketch a gating scheme to identify marker relationships such as mutually exclusive marker sets and the relationships of low and high expression markers.

   Gating Example- OMIP-033: **https://doi.org/10.1002/cyto.a.22889**



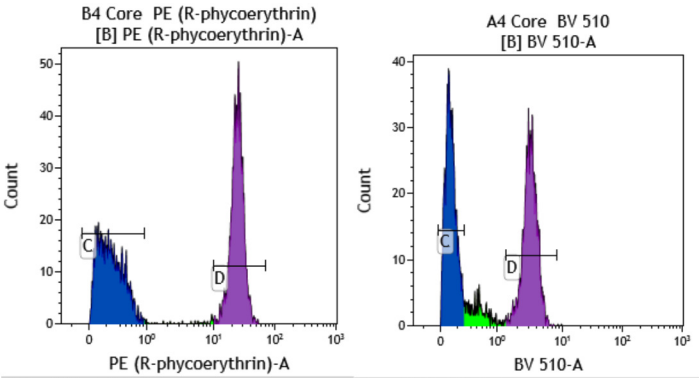

4. Evaluate the expression of each marker based these criteria (Mahnke YD, *Clin Lab Med.* 2007; 27:469-485)

   Primary- well characterized expression which is positive or negative.
   Secondary- mid-range expression or a continuum of expression.
   Tertiary- expressed at low levels, unknown levels, or is rare cell type.

   Manufacturers frequently provide expression levels on their websites. BioLegend is particularly helpful. www.biolegend.com

5. List the color choices available for each marker. Include what your lab has in stock, but be aware that these may not be the best choices for a large parameter panel. Making a table is useful here.

6. Evaluate the stain index of the available fluorochromes. Note that stain indices can vary between instrument models. High (primary ) expression markers should be used with low stain index flours and tertiary (low) expression should be on fluors with a high stain index.




**High Index** **Low Index**

Stain Index= (MED positive – MED negative) / 2 x SD negative

7. Evaluate the spillover behavior of each available fluorochrome using the Spillover Spreading Matrix (SSM). The SSM summarizes how fluors behave together by measuring the amount of signal spread into the other detector channels. The fluors in the Y column spread into the channels of the X columns. While SSM is directly related to the amount of compensation, it is independent of signal intensity. **Fluorochromes pairs that give and receive high spillover should be used for mutually exclusive markers. Low (tertiary) expression markers should be on fluors that don't receive spread.**

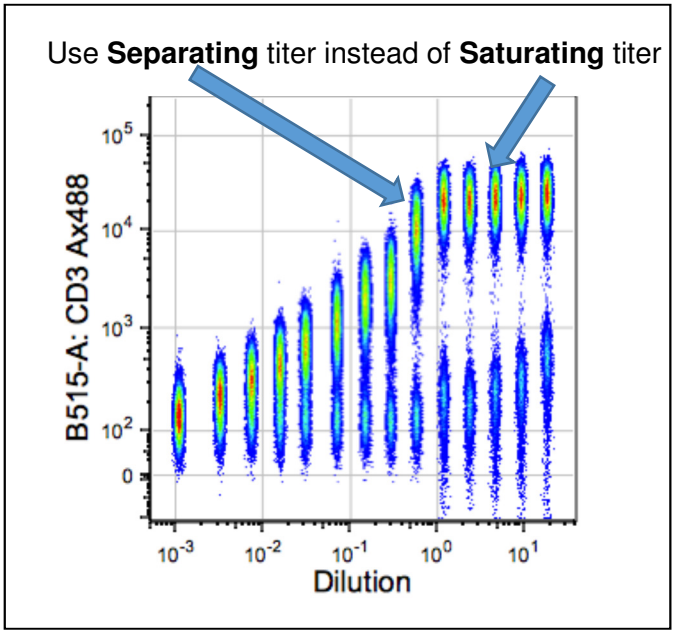
8. Use this information to create a table and assign color choices. See Table I.

**Table I: Example of summary information and fluorochrome choices for a simple panel.**

| Laser, Filter, & Common fluors | Marker | Fluor | Stain Index | Purpose | Expression<br>Primary<br>Secondary<br>Tertiary |
|---|---|---|---|---|---|
| 387/11 BUV395 | CD3 | BUV395 | high | T cells | primary |
| 420/10 BV421 | CD19 | BV421 | high | B cells | primary |
| 509/24 FITC/GFP | CD45 | FITC | low | WBC | primary |
| 577/15 PE | CD34 | PE | high | Hematopoetic progenitors | Tertiary (rare) |
| 670/30 APC/A647 | EpCAM | Alexa 647 | high | epithelial | primary |
| 775/50 APC Cy7 | Live-dead | NIR | low | Live-dead | Secondary |

9. At this point, we would be pleased to evaluate your panel. Please bring all of the above information, including the purpose, gating scheme, available reagents, and the summary information table.

10. Test your panel. Many manufacturers sell trial samples of antibodies for testing. Be sure to titer all antibodies and include FMO controls to delineate negative from positive. **Choosing the titer that gives a good separation rather than the highest signal/noise ratio will reduce spreading into other fluorochromes**



## Backbone panels of fluorochromes that generally work together well:

### Gallios Backbone Panels

| Laser (Filter) | 4 Color Panel | 8 Color Panel | Notes |
|---|---|---|---|
| 488 FL1 (525/40) | FITC or Alexa 488 | FITC or Alexa 488 | |
| 488 FL2 (575/30) | PE | PE | |
| 488 FL3 (620/30) | | | |
| 488 FL4 (695/30) | | PE Cy5.5 | |
| 488 FL5 (755LP) | | PE Cy7 | |
| 638 FL6 (660/20) | APC or Alexa 647 | APC or Alexa 647 | |
| 638 FL7 (725/20) | | | |
| 638 FL8 (755LP) | | APC Cy7 | Live/dead NIR, Zombie NIR |
| 405 FL9 (450/40) | BV421 or Pacific Blue | BV421 or Pacific Blue | |
| 405 FL10 (550/40) | | BV510 | |

### Yeti Backbone Panels

| Laser (Filter) | 6 Color Panel | 10 Color Panel | Notes |
|---|---|---|---|
| 355(387/11) | BUV395 | BUV395 | |
| 405(420/10) | BV421 | BV421 | |
| 488(509/24) | FITC | FITC | |
| 561(577/15) | PE | PE | |
| 641(670/30) | APC | APC | |
| 641(775/50) | APC Cy7 | APC Cy7 | Live/dead NIR, Zombie NIR |
| 405(525/59) | | BV510 | |
| 405(750LP) | | BV 711 | |
| 561(750LP) | | PE Cy7 | |
| 488(692/80) | | PerCP cy5.5 | |

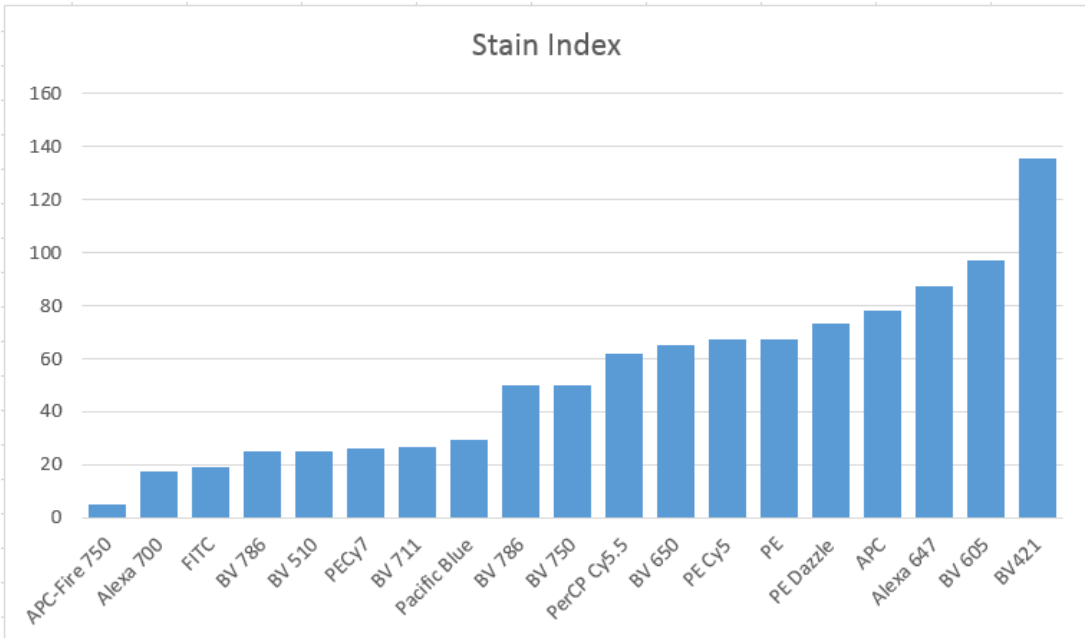
### Aurora Backbone 24 Color Panel (careful planning required)

| Violet | | Blue | Yellow | Red |
|---|---|---|---|---|
| BV421 | BV650 | BB515 | PE | APC |
| Super Bright 436 | BV711 | AF488 | PE DAZZLE 594 | AF647 |
| eFluor450 | BV750 | AF532 | PECy5 | A700 |
| BV480 | BV785 | PerCP Cy5.5 | PECy7 | APC Fire 750 |
| BV510 | | PerCP eFluor 710 | | |
| BV570 | | | | |
| BV605 | | | | |

**Yeti Experiment Summary Table**

| Laser, Filters, & fluors | Marker | Fluor | Stain Index | Purpose | Expression |
|---|---|---|---|---|---|
| 387/11 BUV395 | | | | | |
| 447/60 BUV496 | | | | | |
| 525/50 BUV563 | | | | | |
| 670/30 BUV661 | | | | | |
| 700LP BUV737 | | | | | |
| 420/10 BV421 | | | | | |
| 460/22 PACIFIC BLUE | | | | | |
| 525/50 BV510 | | | | | |
| 615/24 BV650 | | | | | |
| 670/30 BV650 | | | | | |
| 720/60 BV711 | | | | | |
| 750LP BV786 | | | | | |
| 509/24 FITC/GFP | | | | | |
| 549/15 YFP | | | | | |
| 692/80 PerCP | | | | | |
| 750LP PE Cy7** | | | | | |
| 577/15 PE | | | | | |
| 589/15 tdTomato | | | | | |
| 615/24 PE Dazzle | | | | | |
| 640/20 mPlum | | | | | |
| 670/30 PE Cy5 | | | | | |
| 720/60 PE Cy5.5 | | | | | |
| 750LP PE Cy7 | | | | | |
| 670/30 APC/A647 | | | | | |
| 720/60 A700 | | | | | |
| 775/50 APC Cy7 | | | | | |
| 800LP APC ef780 | | | | | |

**Yeti Stain Index**




**Yeti Spillover Spreading Matrix**

| | FL3 | FL5 | FL6 | FL7 | FL8 | FL11 | FL13 | FL14 | FL15 | FL16 | FL17 | FL18 | FL19 | FL20 | FL26 | FL27 | FL29 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | FITC | PECy7(488) | PCP-Cy5.5 | PECY7 | PECY5 | PE | PE-Dazzle | BV650 | BV711 | BV786 | PacBl | BV421 | BV605 | BV510 | Alexa700 | APC Fire750 | Alexa 647 |
| FITC | 1.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| PECy7 | 0.0 | 1.0 | 0.0 | | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.2 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.1 | 0.0 |
| PCP-Cy5.5 | 0.0 | 0.9 | 1.0 | 0.4 | 0.4 | 0.0 | 0.0 | 0.2 | 1.2 | 0.7 | 0.0 | 0.0 | 0.0 | 0.0 | 0.8 | 0.2 | 0.2 |
| PECY5 | 0.0 | 0.2 | 0.7 | 0.3 | 1.0 | 0.0 | 0.0 | 0.1 | 0.9 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.3 | 0.1 | 0.4 |
| PE | 0.0 | 0.0 | 0.1 | 0.0 | 0.1 | 1.0 | 0.8 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| PE-Dazzle | 0.0 | 0.0 | 0.1 | 0.0 | 0.2 | 0.2 | 1.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| BV650 | 0.0 | 0.0 | 0.0 | 0.0 | 0.1 | 0.0 | 0.1 | 1.0 | 1.4 | 0.6 | 0.0 | 0.1 | 0.1 | 0.0 | 0.2 | 0.0 | 0.2 |
| BV711 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 1.0 | 0.6 | 0.0 | 0.0 | 0.0 | 0.0 | 0.2 | 0.1 | 0.0 |
| BV786 | 0.0 | 0.1 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.3 | 1.0 | ..00322 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| PacBl | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 1604.0 | 0.0 | 1.0 | 0.1 | 0.0 | 0.5 | 0.0 | 0.0 | 0.0 |
| BV421 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.3 | 1.0 | 0.0 | 0.1 | 0.0 | 0.0 | 0.0 |
| BV605 | 0.0 | 0.0 | 0.1 | 0.0 | 0.3 | 1.7 | 2.3 | 0.8 | 1.2 | 0.5 | 0.3 | 0.8 | 1.0 | 0.1 | 0.0 | 0.0 | 0.0 |
| BV510 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.1 | 0.2 | 0.1 | 0.1 | 0.0 | 0.1 | 1.0 | 0.0 | 0.0 | 0.0 |
| Alexa700 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.1 | 0.1 | 0.0 | 0.0 | 0.0 | 0.0 | 1.0 | 0.3 | 0.0 |
| APC Fire750 | 0.0 | 0.0 | 0.0 | 0.2 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.2 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 1.0 | 0.0 |
| Alexa 647 | 0.0 | 0.0 | 0.0 | 0.0 | 0.2 | 0.0 | 0.0 | 4812.0 | | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.7 | 0.2 | 1.0 |

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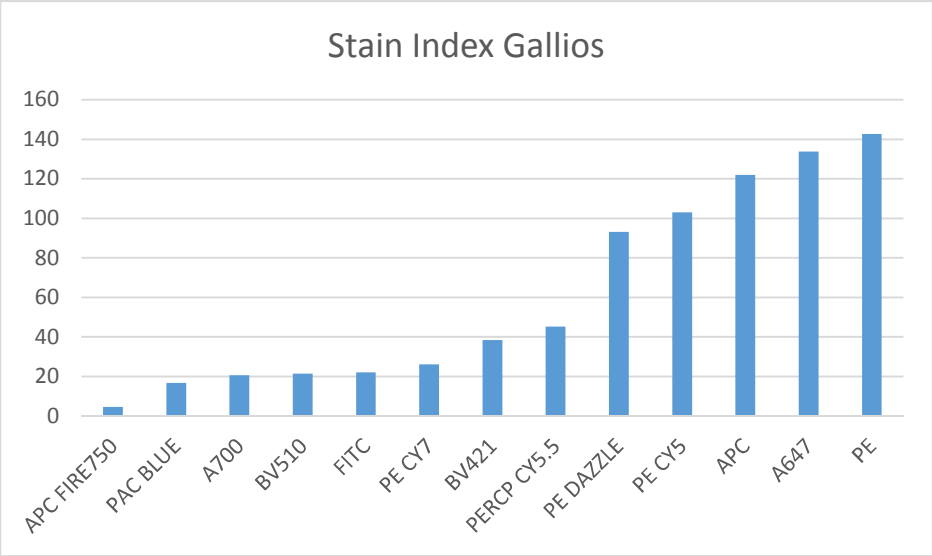
|---|---|---|---|---|---|---|
| 488 FL1 (525/40) | FITC or Alexa 488 | | | | | |
| 488 FL2 (575/30) | PE | | | | | |
| 488 FL3 (620/30) | PE-Texas Red, ECD | | | | | |
| 488 FL4 (695/30) | PE Cy5 or PE Cy5.5 | | | | | |
| 488 FL5 (755LP) | PE Cy7 | | | | | |
| 638 FL6 (660/20) | APC or Alexa 647 | | | | | |
| 638 FL7 (725/20) | Alexa 700 | | | | | |
| 638 FL8 (755LP) | APC Cy7 | | | | | |
| 405 FL9 (450/40) | BV421 or Pacific Blue | | | | | |
| 405 FL10 (550/40) | BV510 | | | | | |




**Gallios Spillover Spreading Matrix**

| SSM | FL1 FITC | FL2 PE | FL3 PE Dazzle | FL4 PE Cy5.5 | FL5 PE Cy7 | FL6 APC | FL7 A700 | FL8 APC FIRE750 | FL9 BV421 | FL10 BV510 |
|---|---|---|---|---|---|---|---|---|---|---|
| FL1 FITC | 1 | .125.4 | 0.08938 | 0.01229 | 0.00288 | 0 | 0 | 0.0003 | 0.00106 | 0.07678 |
| FL2 PE | 0.0123 | 1 | 0.83608 | 0.13055 | 0.02174 | 0 | 0 | 0 | 0.00059 | 0.19051 |
| FL3 PE Dazzle | 0.00231 | 0.17172 | 1 | 0.2096 | 0.03975 | 0.00101 | 0.00036 | 0 | 0.00146 | 0.03187 |
| FL4 PE Cy5.5 | 0 | 0 | 0 | 1 | 0.33902 | 0.0515 | 0.32253 | 0.10127 | 0.00193 | 0 |
| FL5 PE Cy7 | 0.00039 | 0.00343 | 0.00291 | 0.00118 | 1 | 0 | 0.0078 | 0.11654 | 0.00091 | 0.00084 |
| FL6 APC | 0 | 0 | 0 | 0.00227 | 0.00086 | 1 | 0.32144 | 0.07461 | 0.00129 | 0.00159 |
| FL7 A700 | 0 | 0 | 0 | 0.00443 | 0.00493 | 0.00288 | 1 | 0.22571 | 0.00393 | 0.00272 |
| FL8 APC FIRE750 | 0 | 0 | 0 | 0 | 0.01651 | 0.03764 | 0.06476 | 1 | 0.00552 | 0.00364 |
| FL9 BV421 | 0 | 0 | 0 | 0 | 0.00031 | 0 | 0 | 0 | 1 | 0.11775 |
| FL10 BV510 | 0.0001 | 0 | 0 | 0.0001 | 0.00031 | 0 | 0 | 0.00005 | 0.07195 | 1 |

**Cytek Aurora Panel Design**

The spectral fluorochrome signatures, lists of unique versus similar dyes, stain index tables, and spillover spreading matrices for the Aurora are available on the Cytek Biosciences website in the Data Sheets section of the Resources tab.

https://cytekbio.com/blogs/resources/tagged/data-sheets

All of the previously discussed Cardinal Rules of Panel Design apply to panels for the Aurora and spreading can still be a major problem. Careful attention to the details is very important. Panel evaluation should include inspecting the unmixing of each reference control by analyzing each control against each of the other colors in the panel. Build a worksheet with a dual-parameter plot for each of the fluorochromes, placing each fluor on a y-axis. Inspect each reference control by assigning that control fluorochrome to all of the x axes. Look for swoops and other odd angles that indicate over or under correction of the spillover. Slight corrections can be made by using the manual compensation option in the Aurora software.

For very large Aurora panels, breakdown the panel into plots of mini-panels and inspect the plots to verify that the data is biologically correct. For example, look at all of the T cell plots and examine them for odd populations which do not make biological sense or have other inconsistencies.