Compensation, Negative Controls and the Optimized Choice of Reagents for Multicolor Flow Cytometry
Overview

• Compensation
  ▪ Introduction
  ▪ Prevention of compensation related artifacts

• Negative Controls
  ▪ Fluorescence Minus One (FMO)
  ▪ Isotype controls
  ▪ Transfection controls

• Combination of Reagents for Multicolor Flow Experiments

• Characteristics of Fluorochromes
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• Characteristics of Fluorochromes
Compensation: Introduction

- Photons reaching a specific detector are coming from
  - the fluorochrome specific for this detector
  - optical background (especially cell-type specific auto-fluorescence)
  - photon spill over from all fluorochromes present in experiment
Compensation: Introduction

- Compensation is a procedure to subtracts from all photons that reach a detector the
  - 1) nonspecific electronic signal from cellular autofluorescence
    Application of “Instrument Settings” for one specific (type of) sample
  - 2) nonspecific electronic signal from fluorochrome spill over
Compensation: Introduction

- Compensation is a procedure to subtracts from all photons that reach a detector the
  - 1) nonspecific electronic signal from cellular autofluorescence
    Application of “Instrument Settings” for one specific (type of) sample
  - 2) nonspecific electronic signal from fluorochrome spill over
Compensation: Introduction

• 1) Generate sample-specific “Application Settings” according to SD\textsubscript{EN} given in CS&T Baseline Report
  ▪ Refer to Handout #3: Generation of Application Settings
  ▪ Refer to the Presentation: QC for Digital Instruments: BD CS&T\textsuperscript{TM}

• 2) Perform an Automated Compensation using the BD FACS Diva\textsuperscript{TM} software
  ▪ Refer to Handout #4: Automated Compensation
Compensation: Prevention of compensation related artifacts

• To avoid errors in compensation

  1) never change the PMT V of fluorescent parameters (Instrument Settings) after a compensation has been calculated

  2) only the “median-related” = “statistic-based” compensation will result in accurate compensation values: Never compensate “by eye”

  3) use only compensation controls that show in minimum equally high signal intensities than the highest signal you expect in your sample

  4) BUT, the signal intensities of the compensation controls have to lie within the maximum linearity range of the scales (refer to the CS&T baseline report)

  5) it is not recommended to re-use compensations if a tandem dye is included in the panel
Compensation: Prevention of compensation related artifacts

- Digital Compensation is a software-based compensation in that linear numbers are subtracted.

- But if each optimal compensation is a subtraction that results in “0”, why is a compensation value of 20% worse than 0.2%?

Uncompensated List-Mode Data

<table>
<thead>
<tr>
<th>Event 1</th>
<th>Time</th>
<th>FSC</th>
<th>SSC</th>
<th>FITC</th>
<th>PE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>60</td>
<td>120</td>
<td>5</td>
<td>23</td>
</tr>
<tr>
<td>Event 2</td>
<td>10</td>
<td>160</td>
<td>65</td>
<td>89</td>
<td>675</td>
</tr>
<tr>
<td>Event 3</td>
<td>30</td>
<td>650</td>
<td>160</td>
<td>39.271</td>
<td>30.621</td>
</tr>
</tbody>
</table>
Compensation: Prevention of compensation related artifacts

- High compensation values induce “data-spread” effects that reduce substantially the resolution between populations

<table>
<thead>
<tr>
<th>Uncompensated</th>
<th>Compensated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Compensation = 21.5%</td>
<td>Compensation = 0.2%</td>
</tr>
</tbody>
</table>

- CD45 - FITC
- CD3 – APC-H7
- CD56 – PE
- Gated on CD45 – FITC
- Gated on CD45 – APC
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Negative Controls: Fluorescence Minus One (FMO)

- Indicates the data-spread induced by “high” compensation values

compensation = 21.5%

Gated on PBMC

Sample

FMO
Negative Controls: Fluorescence Minus One (FMO)

- Fluorochrome-combinations with “high” compensation values?
  
- Get experience: compare of multicolor compensation matrices.

![Image of compensation matrices]
Negative Controls: Fluorescence Minus One (FMO)

- Does every fluorochrome combination with “high” compensation values requests an FMO Control?

- Not if the fluorochromes bind to different cells!

![Histograms showing the effect of CD45 and CD19-AmCyan with and without CD45 AmCyan](image)
Negative Controls: Isotype Control

- Indicates specificity of antigen-expression in unknown samples
- Indicates the nonspecific binding of antibodies to membranes

CD4 is THE T-helper cell marker

unstained
MFI = 48

IgG1 - PE
MFI = 102

IgG2a - PE
MFI = 156

IgG2b - PE
MFI = 163

0.025 μg antibody per 100 μl whole blood
Negative Controls:
Isotype Control

- Indicates specific binding of antibodies to very low and/or "smeared" expressed antigens

But (if available and known) "biological controls" are better
Negative Controls: Isotype Control

- Isotype controls HAVE to
  - show perfect match in isotype subtype
  - come from the same species
  - be labeled to the same fluorochrome
  - show equalized concentrations between specific antibody and isotype control
  - be specifically purified from free fluorochromes for intra-cellular staining
  - show comparable antibody to fluorochrome ratios
Negative Controls: Transfection Control (“Mock Control”)

- Transfection methods using Lipofectin and similar substances enhance the autofluorescence due to the binding of plasmid-DNA to cell membranes.
- Nontransfected cells are for this reason never a proper control!
- In this example the transfection-efficiency is 0% as the plasmid-promotor shows a deletion that prevents gene-expression!
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Combination of Reagents for Multicolor Flow Experiments

• Basic rule for the selection of antibody-fluorochrome-combinations:
  Match the fluorochrome brightness with the antigen-expression levels

• For further information see in Appendix 4:
  http://www.bdbiosciences.com/documents/Multicolor_AppNote.pdf
Combination of Reagents for Multicolor Flow Experiments

• Match fluorochrome brightness with antigen-expression levels
  ▪ Fluorochrome brightness?
    • The “Stain Index”
      \[ \text{Stain Index (SI)} = \frac{D}{W} \]
      \( D = \Delta \) between positive and negative peak medians
      \( W = \) Spread of the background peak

• Stain indices of BD specific fluorochromes obtained on a BD FACSCanto™ II

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Clone</th>
<th>Stain Index</th>
</tr>
</thead>
<tbody>
<tr>
<td>PE</td>
<td>RPA-T4</td>
<td>356.3</td>
</tr>
<tr>
<td>Alexa 647</td>
<td>RPA-T4</td>
<td>313.1</td>
</tr>
<tr>
<td>APC</td>
<td>RPA-T4</td>
<td>279.2</td>
</tr>
<tr>
<td>PE-Cy7</td>
<td>RPA-T4</td>
<td>270.5</td>
</tr>
<tr>
<td>PE-Cy5</td>
<td>RPA-T4</td>
<td>222.1</td>
</tr>
<tr>
<td>PerCP-Cy5.5</td>
<td>Leu-3a</td>
<td>92.7</td>
</tr>
<tr>
<td>BD Horizon™ V450</td>
<td>RPA-T4</td>
<td>90.0</td>
</tr>
<tr>
<td>Alexa 488</td>
<td>RPA-T4</td>
<td>75.4</td>
</tr>
<tr>
<td>FITC</td>
<td>RPA-T4</td>
<td>68.9</td>
</tr>
<tr>
<td>PerCP</td>
<td>Leu-3a</td>
<td>64.4</td>
</tr>
<tr>
<td>APC-Cy7</td>
<td>RPA-T4</td>
<td>42.2</td>
</tr>
<tr>
<td>Alexa 700</td>
<td>RPA-T4</td>
<td>30.0</td>
</tr>
<tr>
<td>AmCyan</td>
<td>RPA-T4</td>
<td>24.2</td>
</tr>
<tr>
<td>BD™ APC-H7</td>
<td>RPA-T4</td>
<td>18.0</td>
</tr>
<tr>
<td>BD Horizon™ V500</td>
<td>RPA-T4</td>
<td>12.0</td>
</tr>
</tbody>
</table>

Bright fluorochromes
Intermediate-bright fluorochromes
Dim fluorochromes
Combination of Reagents for Multicolor Flow Experiments

• Match fluorochrome brightness with antigen-levels
  ▪ Antigen Expression level?

Compare examples of PE-staining given in TDS

- CD3 - PE
- CD19 - PE
- CD80 - PE
- CD45 - PE
Combination of Reagents for Multicolor Flow Experiments

• Match fluorochrome brightness with antigen-levels
  ▪ Antigen Expression level?

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Antigen-Density</th>
<th>Expression Level</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3</td>
<td>80.000</td>
<td>++</td>
</tr>
<tr>
<td>CD4</td>
<td>100.000</td>
<td>+++</td>
</tr>
<tr>
<td>CD14</td>
<td>144.000</td>
<td>+++</td>
</tr>
<tr>
<td>CD19</td>
<td>18.000</td>
<td>++</td>
</tr>
<tr>
<td>CD25</td>
<td>3.000</td>
<td>+</td>
</tr>
<tr>
<td>CD45</td>
<td>200.000</td>
<td>+++</td>
</tr>
<tr>
<td>CD56</td>
<td>10.000</td>
<td>+</td>
</tr>
<tr>
<td>CD127</td>
<td>2.000</td>
<td>+</td>
</tr>
</tbody>
</table>

Antigen-expression High / Intermediate / Low:
+++ / ++ / +

Example for one proper Antibody-Fluorochrome-Match for a BD FACS Aria™
405nm / 688nm / 635nm

BD Horizon™ V450
FITC
BD™ APC-H7
PerCP-Cy™5.5
APC
BD Horizon™ V500
PE-Cy™7
PE
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• Characteristics of Fluorochromes
Characteristics of Fluorochromes

• Select fluorochromes according to instrument configuration

<table>
<thead>
<tr>
<th>Max Emission</th>
<th>Possible fluorochrome-excitation by</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>448nm or 452nm</td>
<td>BD Horizon™ V450 or Pacific Blue™</td>
<td></td>
</tr>
<tr>
<td>461nm</td>
<td></td>
<td></td>
</tr>
<tr>
<td>491nm</td>
<td>AmCyan or BD Horizon™ V500</td>
<td></td>
</tr>
<tr>
<td>519nm or 520nm</td>
<td>AF®488 or FITC</td>
<td></td>
</tr>
<tr>
<td>578nm</td>
<td>PE</td>
<td>PE</td>
</tr>
<tr>
<td>660nm or 668nm</td>
<td></td>
<td>APC or AF®647</td>
</tr>
<tr>
<td>667nm or 678nm or 695nm</td>
<td>Pe-Cy™5 or PerCP or PerCP-Cy™5.5</td>
<td>Pe-Cy™5 or PerCP or PerCP-Cy™5.5</td>
</tr>
<tr>
<td>723nm</td>
<td></td>
<td>AF®700</td>
</tr>
<tr>
<td>785nm or 783nm</td>
<td></td>
<td>APC-Cy™7 or BD™ APC-H7</td>
</tr>
<tr>
<td>785nm</td>
<td>Pe-Cy™7</td>
<td>Pe-Cy™7</td>
</tr>
</tbody>
</table>
Characteristics of Fluorochromes

- Select fluorescent dyes according to instrument configuration

<table>
<thead>
<tr>
<th>Max Emission</th>
<th>Violet 405nm</th>
<th>Possible fluorochrome-excitation by</th>
<th>Blue 488nm</th>
<th>Y-G 561nm</th>
<th>Red 635nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>461nm or 470nm or 477nm</td>
<td>Dapi or SYTOX-Blue or ECFP</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>505 or 510nm</td>
<td></td>
<td>CFSE or EGFP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>522nm or 524nm or 528nm</td>
<td></td>
<td>SYBR-Green or SYTOX-Green or EYFP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>562nm</td>
<td></td>
<td></td>
<td>mOrange</td>
<td></td>
<td></td>
</tr>
<tr>
<td>581nm</td>
<td></td>
<td></td>
<td>mTomato</td>
<td></td>
<td></td>
</tr>
<tr>
<td>586nm</td>
<td></td>
<td>dsRed</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>610nm</td>
<td></td>
<td></td>
<td>mCherry</td>
<td></td>
<td></td>
</tr>
<tr>
<td>617nm or 647nm</td>
<td></td>
<td>PI or 7-AAD</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>650nm</td>
<td></td>
<td></td>
<td>mPlum</td>
<td></td>
<td></td>
</tr>
<tr>
<td>658nm</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>SYTOX Red</td>
</tr>
<tr>
<td>683nm</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>DRAQ5</td>
</tr>
</tbody>
</table>
Characteristics of Fluorochromes

- BD-supported fluorochromes

```
Small Organic Molecules:
BD™ Horizon V450, Pacific Blue™, FITC,
Alexa Fluor (AF)® 350, 488 / 647 / 700

Tandem Conjugates
PerCP-Cy™ 5.5,
PE-Cy™5, PE-Cy™7,
APC-Cy™7, BD™ APC-H7

Large Proteins
AmCyan, PerCP, PE,
APC
```
## Characteristics of Fluorochromes: The “blue laser fluorochromes (I)”

<table>
<thead>
<tr>
<th></th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
</table>
| 1. FITC | - Most widely used  
- Stable, long-lasting conjugates  
- Easy conjugation (3-5 per Ig)  
- Cheapest fluorochrome | - One of the dullest fluorochromes  
- Photobleaching (relevant only for microscopy)  
- pH sensitive |
| 2. Alexa Fluor® 488 | - Very photo- and pH-stable (superior for microscopy) | - One of the dullest fluorochromes |
| 3. PE | - Bright reagents: IC/FCM  
- Low backgrounds: IC/FCM  
1st choice for intracellular protein/cytokine detection!  
- Good for quantification:  
1 PE per Ig (BD™QuantiBrite) | - Large size (240.000 D) is NOT a disadvantage! |
## Characteristics of Fluorochromes: The “blue laser fluorochromes (III)”

<table>
<thead>
<tr>
<th></th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>6. PE-Cy™5</td>
<td><strong>Bright fluorochrome</strong></td>
<td><strong>Unspecific binding to Fc Receptors</strong></td>
</tr>
<tr>
<td></td>
<td><strong>Useful additional fluorochrome for the LSR II</strong></td>
<td><strong>Very strong spill over to APC: has to be adjusted often by titration</strong></td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>Lot to lot differences in compensation</strong></td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>Light sensitive (just keep samples in the dark)</strong></td>
</tr>
<tr>
<td>7. PE-Cy™7</td>
<td><strong>Bright fluorochrome:</strong> Preferable above PE-Cy5</td>
<td><strong>Lot to lot differences in compensation</strong></td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>Light sensitive (just keep samples in the dark)</strong></td>
</tr>
</tbody>
</table>
## Characteristics of Fluorochromes: The “blue laser fluorochromes (II)”

<table>
<thead>
<tr>
<th>Characteristics of Fluorochromes: The “blue laser fluorochromes (II)”</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Advantages</strong></td>
</tr>
<tr>
<td>-----------------</td>
</tr>
<tr>
<td><strong>4. PerCP</strong></td>
</tr>
<tr>
<td>▪ Minimal Spill over to PE</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td><strong>5. PerCP-Cy™5.5</strong></td>
</tr>
<tr>
<td>▪ Intermediate bright fluorochrome (PerCP is dim)</td>
</tr>
<tr>
<td></td>
</tr>
</tbody>
</table>
### Characteristics of Fluorochromes: The “red laser fluorochromes (I)”

<table>
<thead>
<tr>
<th></th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
</table>
| 1. APC | ▪ Bright fluorochrome  
▪ Low background: 2nd choice for intracellular protein / cytokine detection! | ▪ Large size (106.000 D) is NOT a disadvantage! |
| 2. Alexa Fluor® 647 | ▪ Equivalent to APC, BUT  
▪ Very photo- and pH-stable (superior for microscopy) | / |
| 3. Alexa Fluor® 700 | ▪ Very photostable (superior for microscopy)  
▪ Additional fluorochrome for the red laser | ▪ Filter set not included in standard BD FACSArria™ configuration, but easy to obtain additionally |
# Characteristics of Fluorochromes: The “red laser fluorochromes (II)”

<table>
<thead>
<tr>
<th></th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
</table>
| 4. APC-Cy™7 | ▪ Additional red laser excited fluorochrome | ▪ Dim  
▪ Photo-instable  
▪ Fixative-sensitive  
▪ Lot to lot differences in compensation |
| 5. BD™ APC-H7 | ▪ Photo-stable  
▪ Fixative-stable: Preferable above APC-Cy7 | ▪ Dim  
▪ Lot to lot differences in compensation |
## Characteristics of Fluorochromes: The “violet laser fluorochromes (I)”

<table>
<thead>
<tr>
<th></th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Pacific Blue™</td>
<td>▪ Little Spill over to FITC</td>
<td>▪ dim</td>
</tr>
</tbody>
</table>
| 2. BD Horizon™ V450 | ▪ Little Spill over to FITC  
                          | ▪ Intermediate bright | /             |
## Characteristics of Fluorochromes: The “violet laser fluorochromes (II)”

<table>
<thead>
<tr>
<th></th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
</table>
| **3. AmCyan**  | ▪ Before BD Horizon™ V500 and BD Horizon™ V450 the only violet-excited fluorochrome from BD | ▪ dim  
▪ extremely strong spill over to FITC  
▪ Fixative-sensitive  
▪ Light-sensitive |
| **4. BD Horizon V500** | ▪ Little spill over to FITC: highly preferable alternative to AmCyan  
▪ Fixative-stable | ▪ Dim |
Characteristics of Fluorochromes

• Summary:
  - There are no “bad” fluorochromes!
  - Considering limitations, all fluorochromes are useful for designing multicolor flow experiments
  - But some fluorochromes are easier to handle for multicolor combinations than others!