FLOW CYTOMETRY: ANALYSIS, SAMPLE PREP, INSTRUMENT AND REAGENTS
• Survey Results

• “Background”
  – analysis, sample prep, instrument and reagents
DIAGNOSTICS

More colors do not provide more info. Less colors does not provide enough info.

RESEARCH

2, 3, 4

6, 7, more
7-MARKER ANALYSIS

7-color assay

• 1x sample:
• 1x unstained control
• 7x singly labeled controls
• 7x FMO controls

Total 16 Tubes

2-color assay

• 6x sample:
  – CD1/CD2
  – CD1/CD3
  – CD1/CD4
  – CD1/CD5
  – CD1/CD6
  – CD1/CD7
• 1x unstained control
• 7x singly labeled controls

Total 14 Tubes
7-MARKER ANALYSIS

3-color assay

• 3x sample:
  – CD1/CD2/CD3
  – CD1/CD4/CD5
  – CD1/CD6/CD7
• 1x unstained control
• 7x singly labeled controls
• 9x FMO controls

Total **20** Tubes
HOW MANY ‘TUBES’ IN AN ASSAY?
How much does flow cytometry contribute to variability in your data?

- Similar to other aspects of process: 43%
- Less: 15%
- More: 32%
- Don’t know: 10%

Clinical: 39%
Industry/Biotech: 27%
Academic: 34%
What aspect of flow cytometry contributes most to variability?

![Bar chart showing the percentage of respondents in each category. Clinical (39%), Industry/Biotech (27%), Academic (34%), Similar (29%), Analysis (19%), sample prep (17%), Operator (11%), Setup (8%), Reagents (3%), Acquisition (1%), Other (6%), don't know (5%).]
Variability-related FCM publications
Background Fluorescence

AUTOFLUORESCENCE
- optical configuration
- excitation source
- biological conditions
  - cell type
  - cell activation/cell death
- physiological conditions
  - labeling conditions
  - sample prep conditions

SPECTRAL OVERLAP
- optical configuration
- excitation source
- spectral compensation
- fluorescence intensity
  - antigen expression level
  - choice of fluorochrome

UNDESIRED Ab BINDING
- antibody specificity
  - cell type
  - cell activation/death
  - physiological conditions
  - clone/affinity
- binding through fluorochrome
  - choice of fluorochrome
- binding through Fc region
- labeling conditions
  - Ab amount
  - Ab concentration

Hulspas R, O'Gorman MRG, Wood BL, Gratama, JW, Sutherland DR.
Considerations for the control of background fluorescence in clinical flow cytometry.
Cytometry Part B 2009;76B:355–364
Autoﬂuorescence

- Different excitation wavelengths result in different levels of autoﬂuorescence.

- Naturally occurring cellular components (Flavins, NADPH).

- Biological and physiological conditions.
Autofluorescence Due to Sample Preparation Procedure

- Fixation procedure can cause increase in autofluorescence
- Increase most pronounced when measured around 530 nm
- Cell type specific
Spectral overlap

- Minimize spectral overlap
  - Spectrally separated fluorochromes
  - Narrow bandpass filter
  - Separate excitation wavelengths for individual fluorochromes
- Feng Shui in panel design
FENG SHUI

CD3-PE-TxRd

CD3-PE-TxRd

CD45RO-PE-Cy7

CD45RO-PE

CD45RO PC7 PE
CD27 PE PC7
CD3 ECD ECD
CD8 PC5 PC5
Clones and conjugates

![Flow cytometry plots of Clones and conjugates](image-url)
Correcting for spectral overlap

Generalized Unmixing Model for Multispectral Flow Cytometry Utilizing Nonsquare Compensation Matrices.
<table>
<thead>
<tr>
<th></th>
<th>Accurate</th>
<th>Inaccurate (systematic error)</th>
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<tbody>
<tr>
<td>Precise</td>
<td><img src="image1" alt="Precise Accurate Target" /></td>
<td><img src="image2" alt="Precise Inaccurate Target" /></td>
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<tr>
<td>Imprecise</td>
<td><img src="image3" alt="Imprecise Accurate Target" /></td>
<td><img src="image4" alt="Imprecise Inaccurate Target" /></td>
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</table>
Increasing reproducibility

$N = 40$
$C6s = 15$
Increasing reproducibility

SpC:
CFSE = 12.4%
FITC = 7.5%
GFP = 6.0%
Emission leakage in tandem dyes

Thematic workshop on fluorescence compensation settings in multicolor flow cytometry.
PE-tandem dyes in ambient light

Keep tandems in the dark

Degradation of tandem dyes

![Graph showing degradation of tandem dyes](image-url)

**PE-TxRd**

**PE-AF750**

**PE-Cy5**

**PE-Cy7** (improved packaging)

**rel. 'PE-leakage' (%)**

**time (days)**
Undesired Ab Binding

Specific Binding

antigen d
(not of interest)

antigen b
(Fc-receptor)

fluorochrome-conjugated anti-"C" (epitope C1)

antigen c
(antigen of interest)
Undesired Ab Binding

NON-SPECIFIC BINDING

antigen a (not of interest)

antigen b (Fc-receptor)

antigen c (antigen of interest)

fluorochrome-conjugated anti-"C" (epitope C1)
The IgG isotype as ligand

<table>
<thead>
<tr>
<th>FcγRI</th>
<th>(CD64)</th>
<th>high</th>
<th>Monocytes</th>
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<tr>
<td>FcγRIIA</td>
<td>(CD32)</td>
<td>low</td>
<td>Dendritic cells</td>
</tr>
<tr>
<td>FcγRIIB1</td>
<td>(CD32)</td>
<td>low</td>
<td>Macrophages</td>
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<tr>
<td>FcγRIIB2</td>
<td>(CD32)</td>
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<td>(CD16a)</td>
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<td>FcγRIIIB</td>
<td>(CD16b)</td>
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<td>FcRn</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>B cells</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>NK cells</td>
</tr>
</tbody>
</table>
Some vendors already include blocking reagents in product.
An isotype control is an antibody of the same isotype as a primary antibody with no relevant specificity to the target antigen. Isotype controls are used as negative controls to help differentiate non-specific background signal from specific antibody signal.

An isotype control is another primary antibody! — .... raised against an epitope generally not present on the target cells (e.g. against keyhole limpet hemocyanin)
Antibody titration

• Typical manufacturer’s recommendations:
  \[ X \mu\text{L per 1E6 cells (in 0.2 mL)} \].
Antibody titration

\[
\text{Staining Index} = \frac{\text{Mean}_{\text{positive}} - \text{Mean}_{\text{background}}}{2 \times \text{S.D.}_{\text{background}}}
\]
Ab concentration vs Ab amount

- 200 µg/mL
  - 198 µL sample
  - 2 µL Ab
  - 2.0 µg/mL
  - 400 ng Ab
    - (~1.6E+12 molecules)

- 200 µg/mL
  - 19.8 µL sample
  - 0.2 µL Ab
  - 2.0 µg/mL
  - 40 ng Ab
    - (~1.6E+11 molecules)

Note: a human T lymphocyte contains about 1.2E+05 CD3 molecules.
• Many more cells can be labeled with ‘standard’ amount of antibody

\[
\text{Ag} + \text{Ab} \rightleftharpoons_{k_1}^{k_2} \text{Ag-Ab}
\]

antigen + antibody \rightarrow \text{antigen-antibody complex}

0.1 \mu g/ml: T=150'
T=30'

Labeling one Billion Cells

mouse IgG anti-human CD4

rel. fluorescence intensity

1000

100

10

1

1E6

1E7

1E8

1E9

total number of cells in assay

10 \mu g/ml
5 \mu g/ml
1 \mu g/ml
0.1 \mu g/ml
0.01 \mu g total
1 \mu g total

1 \mu g/ml
0.1 \mu g total
0.01 \mu g total
Minimizing non-Specific Binding

Titration of fluorochrome-conjugated antibodies for labeling cell surface markers on live cells.

Hulspas R. Curr Protoc Cytom, 2010, Chapter 6: Unit 6.29
Non-specific binding of low affinity antibody

Increasing antibody amount in set assay volume (0.5 mL)

- no antibody
- 10 ng/mL
- 20 ng/mL
- 100 ng/mL
- 200 ng/mL
- 1 μg/mL

Decreasing assay volume with set antibody amount (10 ng)

Hulspas R. Curr Protoc Cytom, 2010, Chapter 6: Unit 6.29
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- unlabeled controls
- FMO controls
- (single) positive controls
- unlabeled controls
  - internal negative controls
  - isotype controls
  - isoclonic controls

INTERPRETATION OF FLOW CYTOMETRIC DATA

- Three positive?
- Two positive?
- One positive?
- No positive?
Multi-parameter analysis

unstained human PBMCs