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How to set up a good protocol or Polychromatic flow cytometry: Advantages and pitfalls

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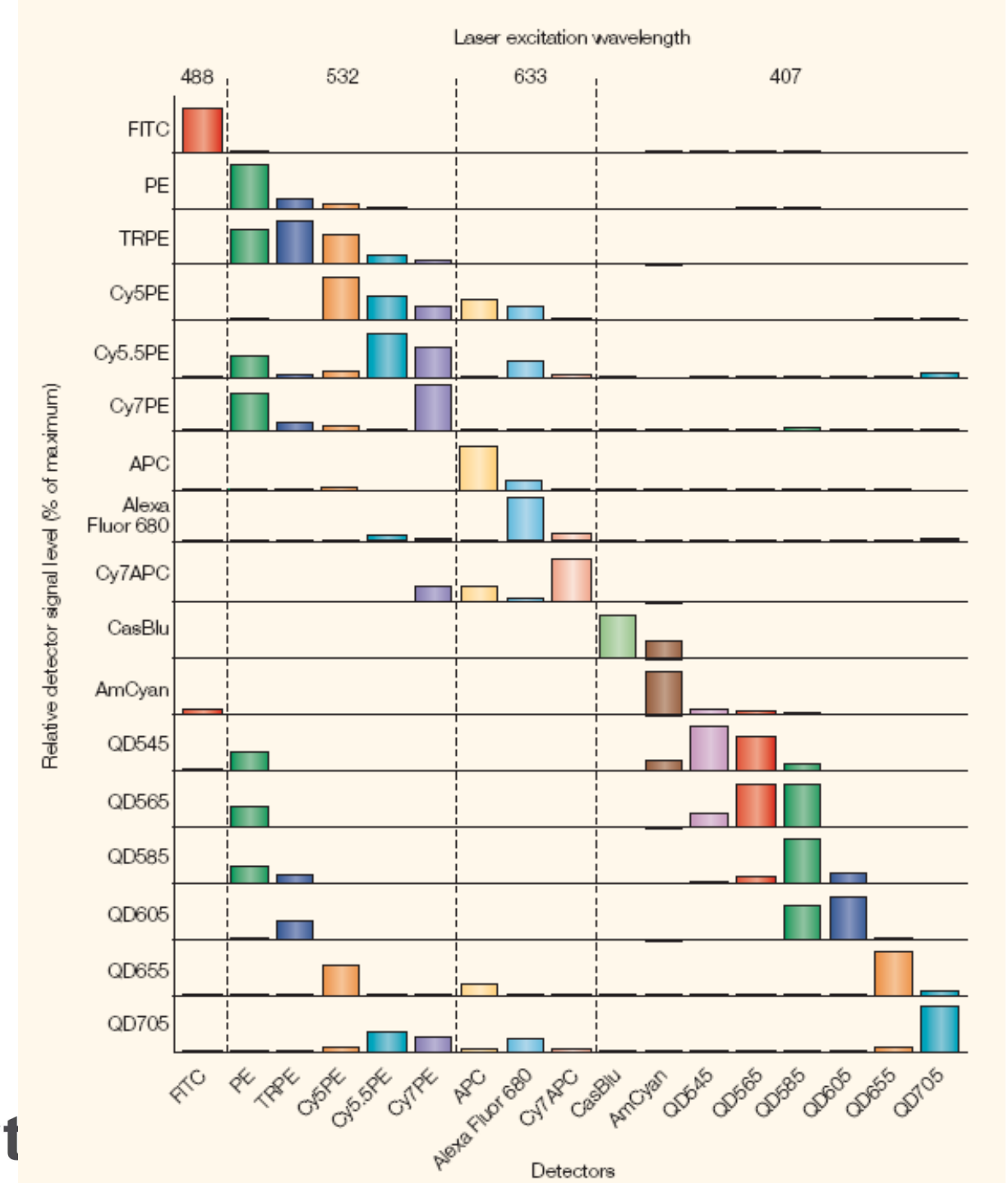
Thanks to: Prof. M Roederer, NIH, Bethesda USA

Cytometry PART A

ISAC International Society for Analytical Cytology

What is polychromatic flow cytometry and why is it needed?



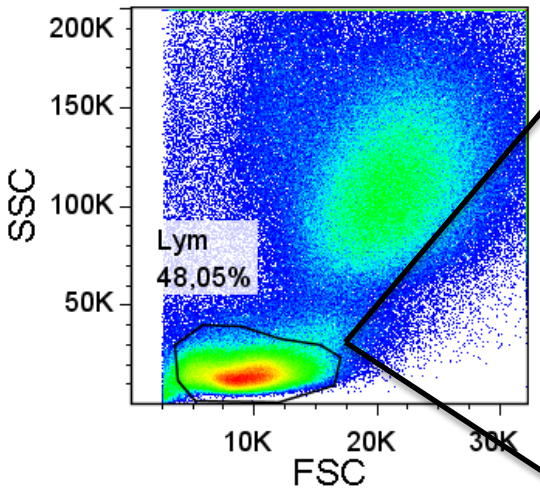
17-Color Flow Cytometry

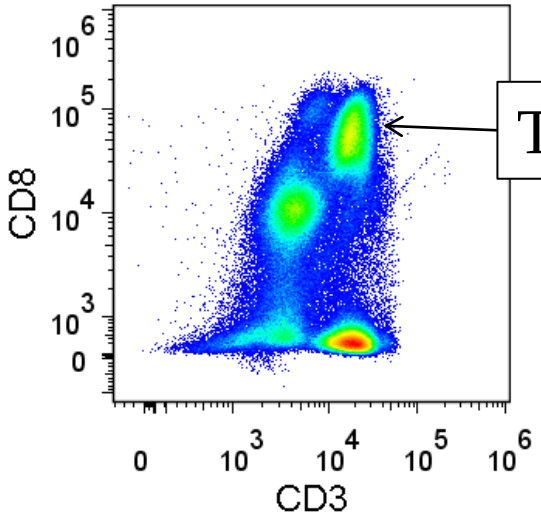
Roederer, Nature Rev - Immunology 2004

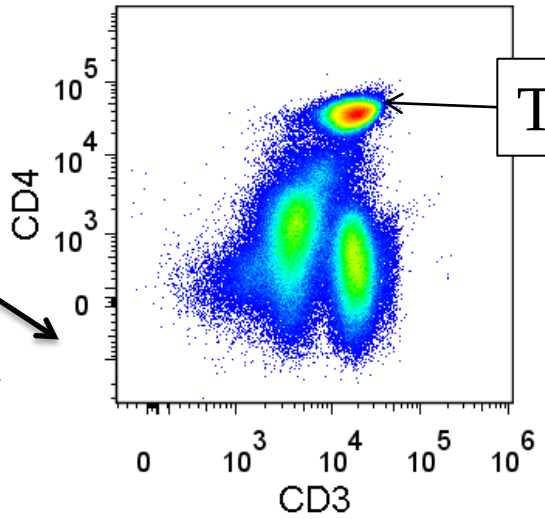
Cyt

ISAC
International Society for Analytical Cytology

2-colors =
2 tubes 2 cell types

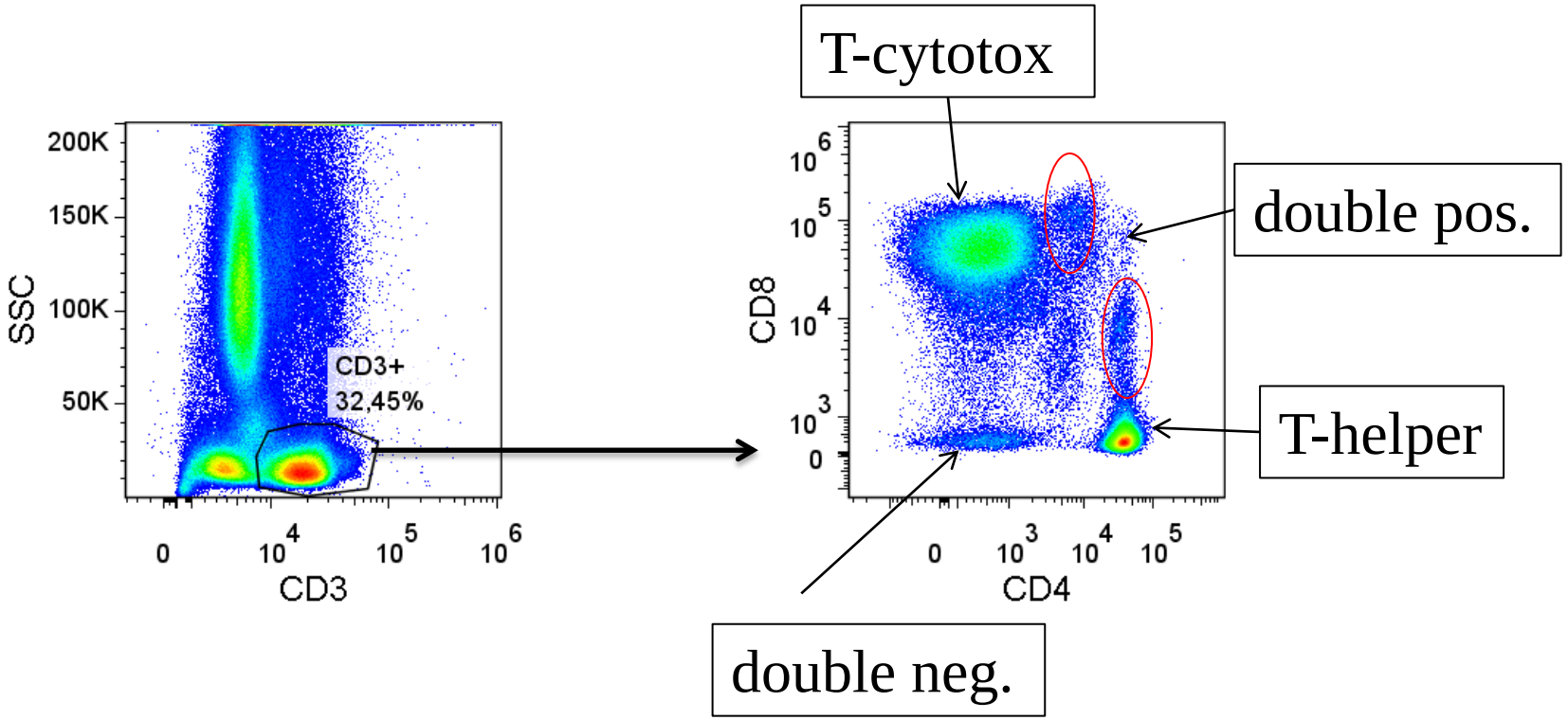




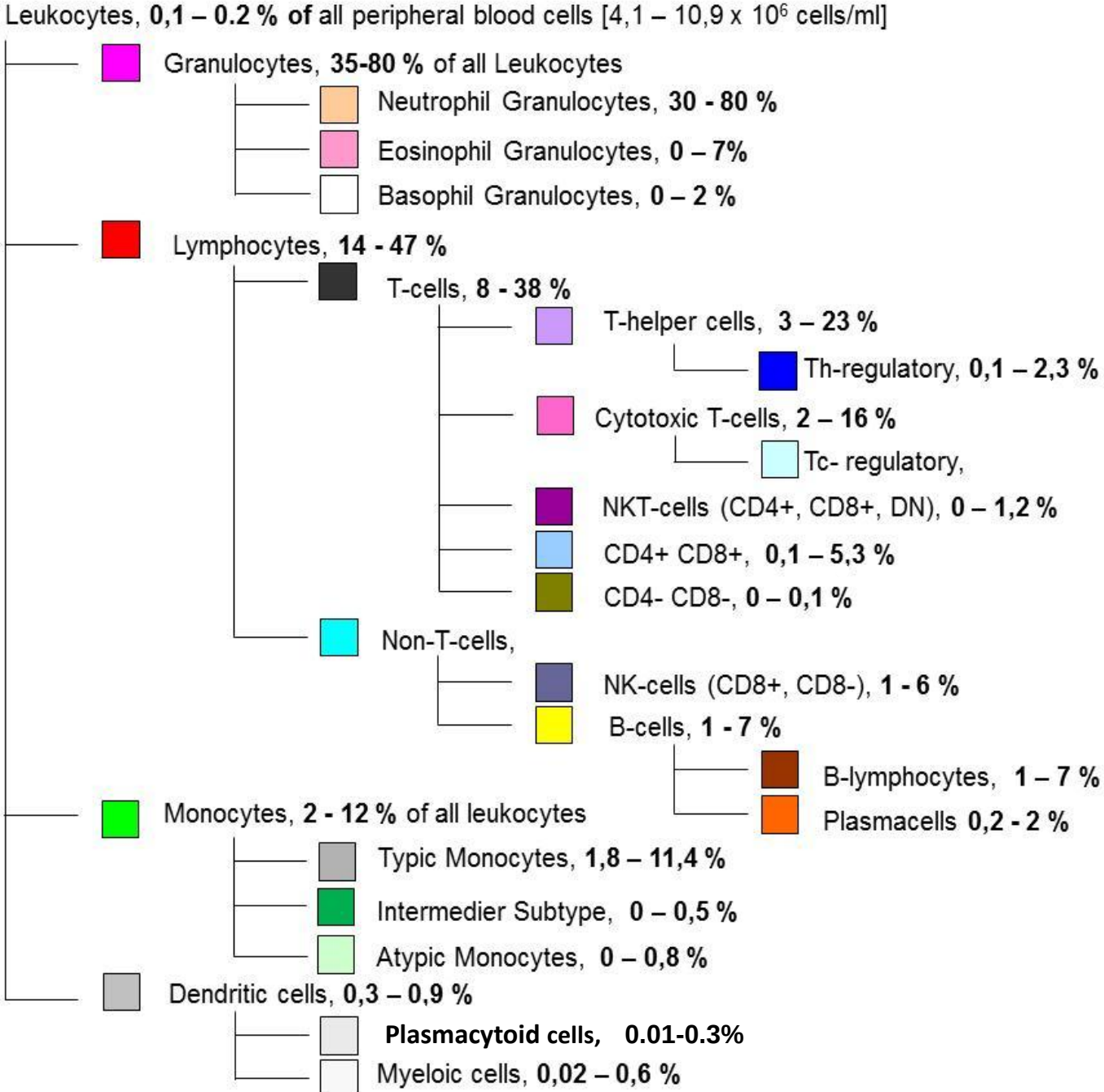


Adding more colors increases depth of information and sensitivity.

3-colors = one tube > 7 populations



Complete differential blood picture and normal distribution of different sub-sets



Cyto

Markers	Category	Parent population	Subset name
all CD45+			
CD3+	T cells	lymphocytes (CD45+, low SSC)	T cells
CD3+,CD4+, CD8-	T cells	T-cells (CD45+,CD3+) gated region	T helper
CD3+,CD4+, CD8+	T cells	T-cells (CD45+,CD3+) gated region	Double positive
CD3+,CD4-, CD8+	T cells	T-cells (CD45+,CD3+) gated region	T cytotoxic
CD3+,CD4-, CD8-	T cells	T-cells (CD45+,CD3+) gated region	T immature
CD3+,CD4+,CD8-	T helper cells	(CD45+,CD3+) gated region	T helper
CD3+,CD4+,CD8-,CD127+	T helper cells	(CD45+,CD3+, CD4+,CD8-) gated region	IL7 r on T helper cells (activated and Treg)
CD3+,CD4+,CD8-,CD25high+,CD127low+	T regulatory cells	(CD45+,CD3+, CD4+,CD8-) gated region	Treg
CD3+,CD8+,CD4-	T cytotoxic cells	(CD45+,CD3+) gated region	T cytotoxic
CD3+CD8+CD4-CD25+	T cytotoxic cells	(CD45+,CD3+,CD8+,CD4-) gated region	Activated T cytotoxic CD25+
CD3+CD8+CD4-CD25high, CD127low	T cytotoxic reg. cells	(CD45+,CD3+,CD8+,CD4-) gated region	Tcreg

How to set up a comprehensive polychromatic panel.

Designing a Multicolor Panel

Considerations:

1. **What do you want to identify?**
   - Minimum set of necessary markers
   - Multiple panels vs. single panel
2. **What do you want to exclude?**
   - Dump channel
   - Negative markers
3. **What additional markers might you use?**
   - Rank: Is it useful, or is it luxury?

How Many Markers to Use?

It is always tempting (and in fact desirable) to use as many markers as possible.

However, this must be balanced against the overriding tenet of multicolor flow cytometry

The more colors you use, the more problems you will have

Problems include:

- Loss of sensitivity (from spectral crossover)
- Unwanted FRET
- Reagent interactions

How Many Markers to Use?

Divide your potential reagents into three groups:

(1) Absolutely necessary

(2) Important

(3) Luxury

Always **consider splitting panels** if the information content not overlapping (for example, if you are separately interrogating B cells and T cells).

You will optimize in same order as your list, being careful to validate each step against the previous.

Selection of Marker/Color Combinations

All colors are not created equal.

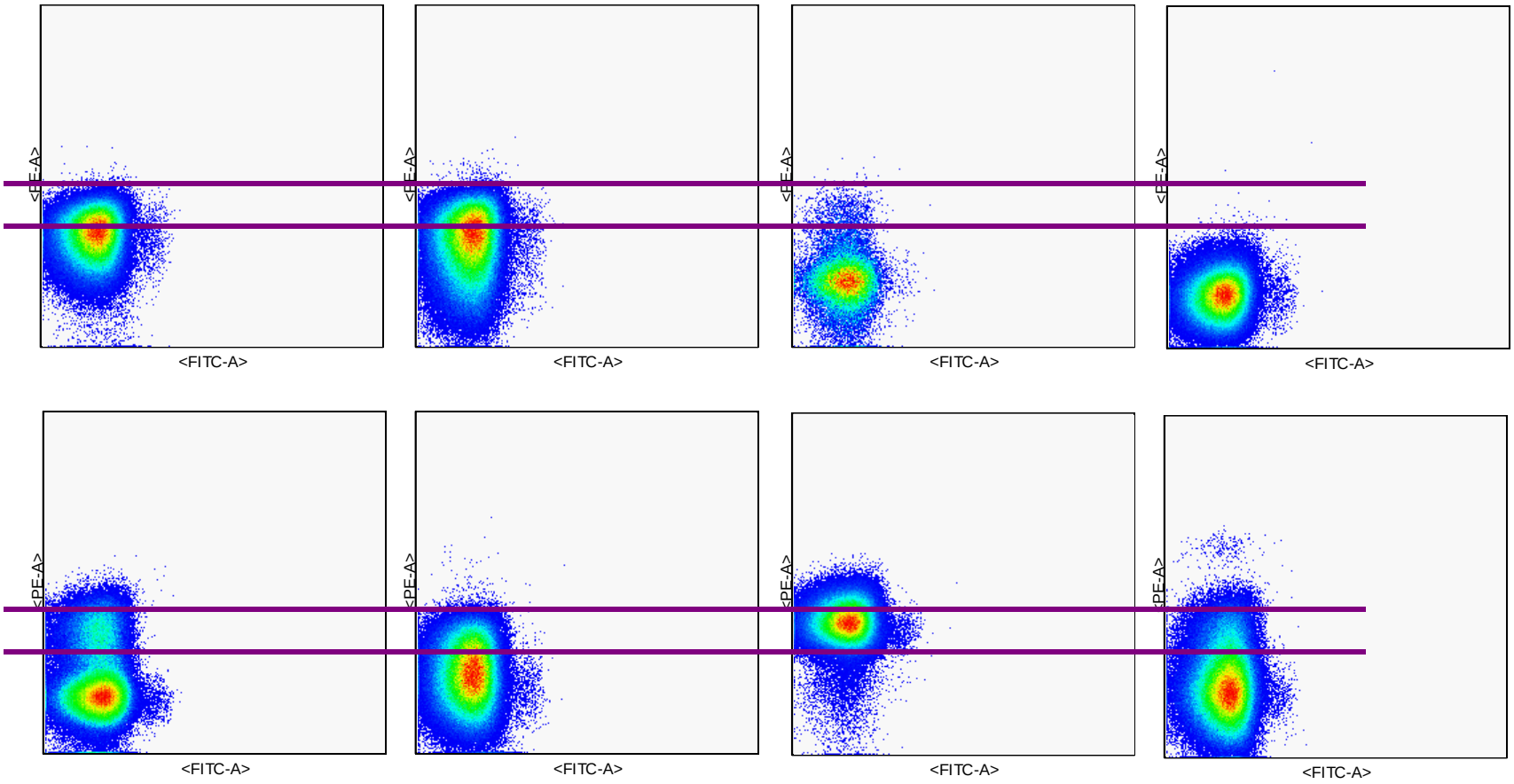
Same monoclonal antibody conjugated to FITC, PE, Cy5PE, APC, Cy7APC can show apparently different distributions on singly-stained cells.

Two facets contribute to this:

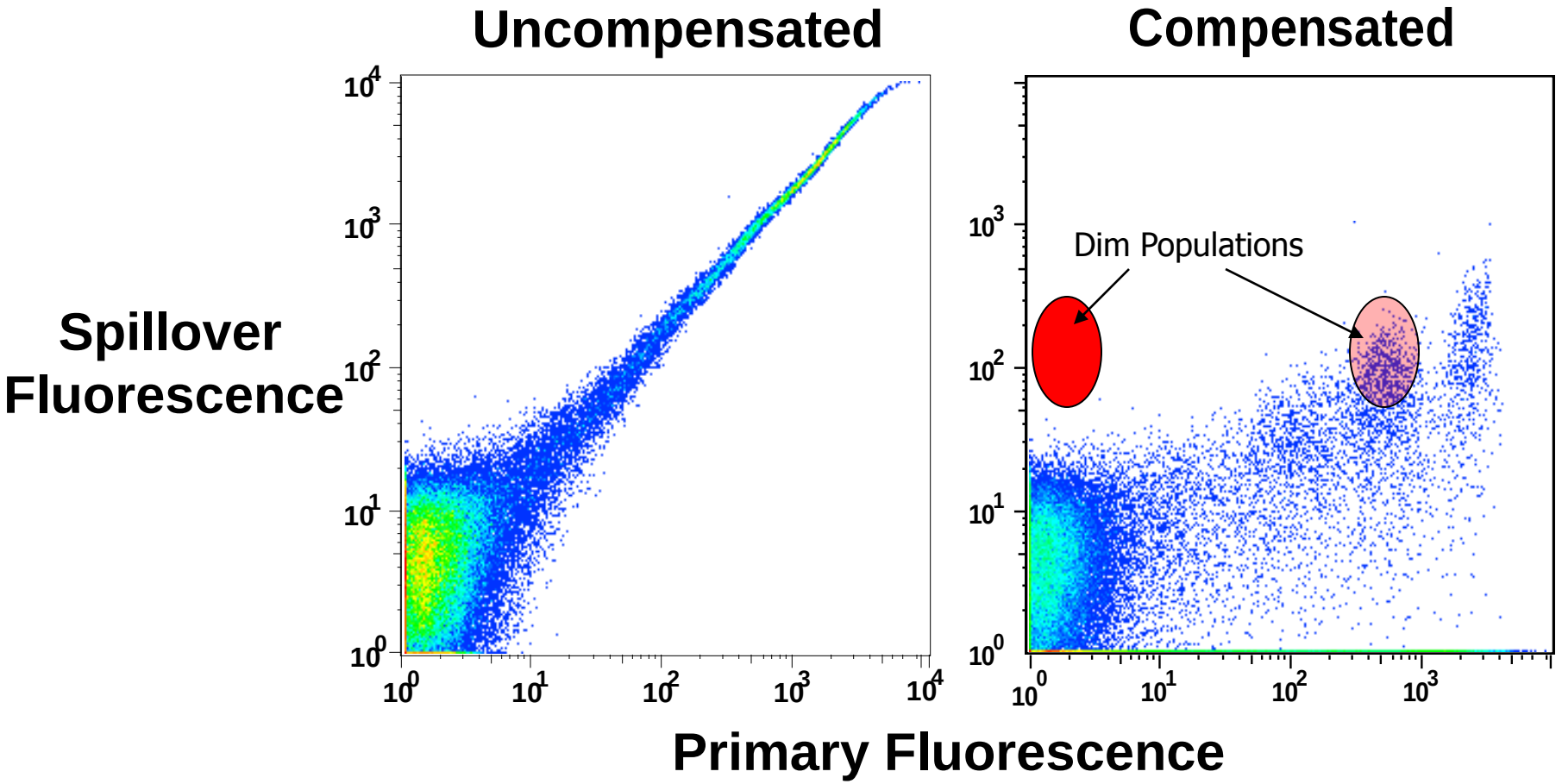
Reagent brightness: Compared to autofluroescence, dimly stained cells may resolve with some colors but not others (combination of brightness, AF, sensitivity)

Absolute signal: PE yields many more photons per antibody-conjugate than Cy7PE, hence the *width (CV)* of distributions is narrower, providing better separation even for brightly-stained cells.

Sensitivity for FITC, PE



Panel Development: Effect of Spreading Error



Spreading error makes it difficult to detect dimly-staining populations

Selection of Marker/Color Combinations

Given the difficulty in predicting how color selection for each reagent will perform in the final panel, it is **necessary to perform panel optimization *empirically* and *iteratively***.

The iterative process should be **performed *step-wise***: begin with a subset of the reagents in the panel, and then add the other reagents one or two at a time.

At each step, validate the combination to make sure the performance is what you expect.

Fortunately, this process is not pure guess-work…

Selection of Marker/Color Combinations

We divide reagents into three categories:

"Primary" Well-characterized, identify broad subsets of cells, expression is usually on/off. **Fluorochrome selected: Lowest**

e.g., CD3, CD4, CD8, CD14, CD19, CD20
Typically used as "parent" gates in analysis

"Secondary" Well-characterized, bright expression patterns

e.g., CD27, CD28, CD45RA/RO, γIFN, perforin
Expression levels can be a continuum. **Fluorochrome: Medium**

"Tertiary" Low-expression levels or uncharacterized.
Fluorochrome : Best

e.g., CD25, CCRs, "X"

Reagent Inventory

In order to test multiple combinations and **iteratively improve** your panels, you will need to have **multiple colors of each conjugate** available!

This is **expensive**. (Hopefully, the reagent manufacturers will help).

Our approach is to have as many combinations of Primary reagents as possible, less for Secondary, and only one or a few for Tertiary.

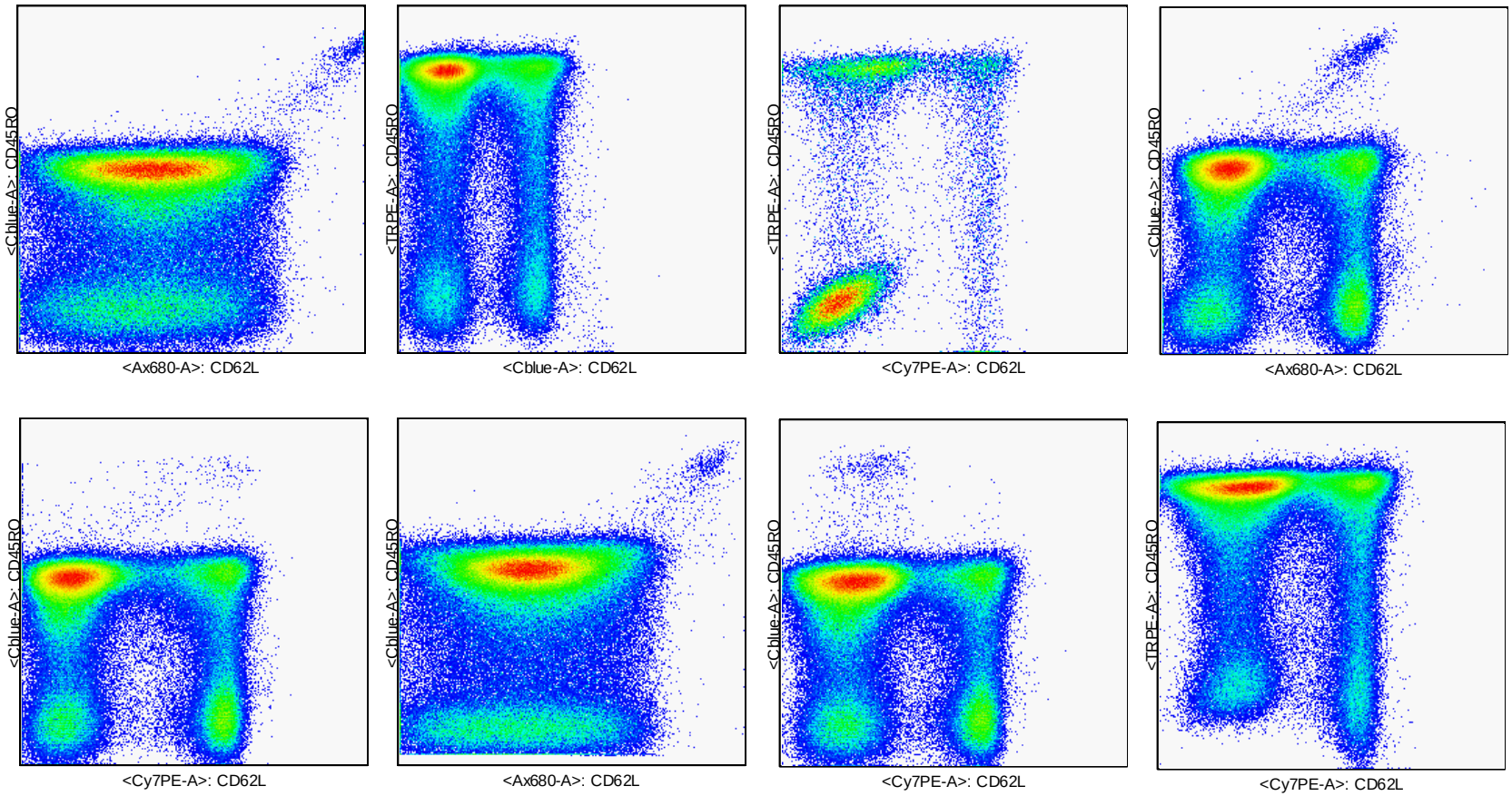
General Approach

1. Test all conjugates of Secondary reagents to determine how good they are.
2. Choose 3-4 best conjugates, and construct panels with Primary reagents “slotted” in.
3. Evaluate expression patterns to ensure appropriate identification of naïve/memory subsets.
4. Evaluate potential sensitivity of FITC and PE channels (where CXCR3 and CCR4 will be used).

First set of panels

	TRPE	Cy5PE	Cy55PE	Cy7PE	APC	Cy55APC Ax680	Cy7APC	CB	QD655
1			CD45RA	CD4	CD27	CD62L	CD11a	CD45RO	“CD3”
2	CD45RO			CD4	CD27	CD45RA	CD11a	CD62L	“CD3”
3	CD45RO		CD45RA	CD62L		CD27	CD4	CD11a	“CD3”
4			CD45RA			CD62L	CD4	CD45RO	“CD3”
5				CD62L	CD4	CD45RA		CD45RO	“CD3”
6		CD45RA		CD11a	CD27	CD62L	CD4	CD45RO	“CD3”
7	CD4	CD45RA		CD62L	CD27	CD28	CD11a	CD45RO	“CD3”
8	CD45RO	CD3		CD62L	CD28	CD11a	CD4	CD27	CD45RA

Panel Evaluation: CD45RO vs. CD62L



Cy5.5APC CD62L: Too much smearing in some panels. CD45RO: Looks good in all panels

Panel Optimization

Is a long, complicated, iterative process.

Plan to spend 5 experiments minimum.

(1): Survey range of reagents

(2): Construct 8-12 possible multicolor combinations

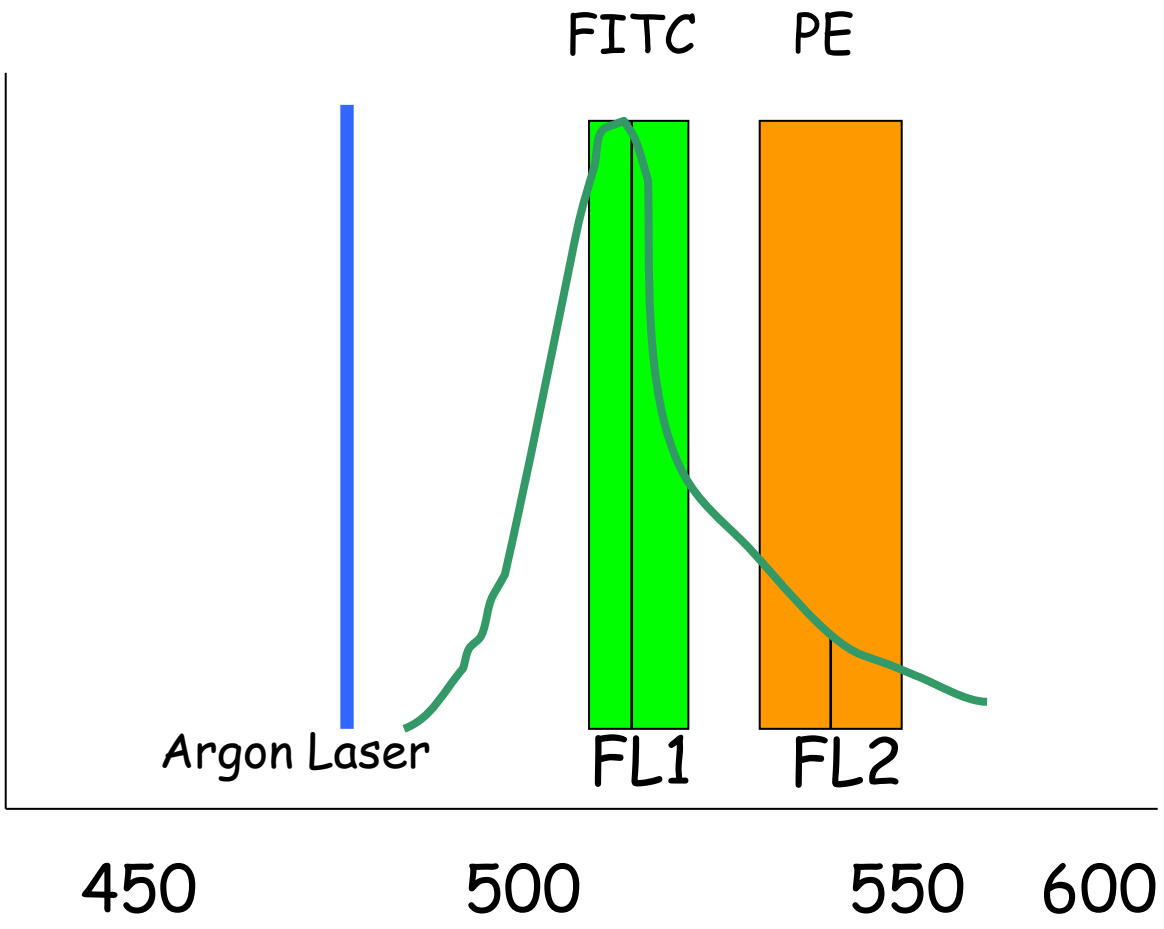
(3): Rank each combination, deriving rules about reagents and combinations. Construct 4-6 derivative combinations

(4): Repeat step 3, winnowing down the combinations.

Record the process as you go along!

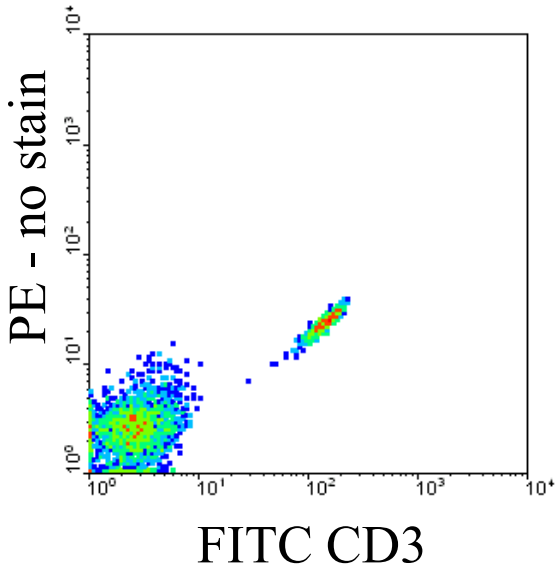
Quality Control, Standardization and Data Analysis.

FITC Single Stain Control



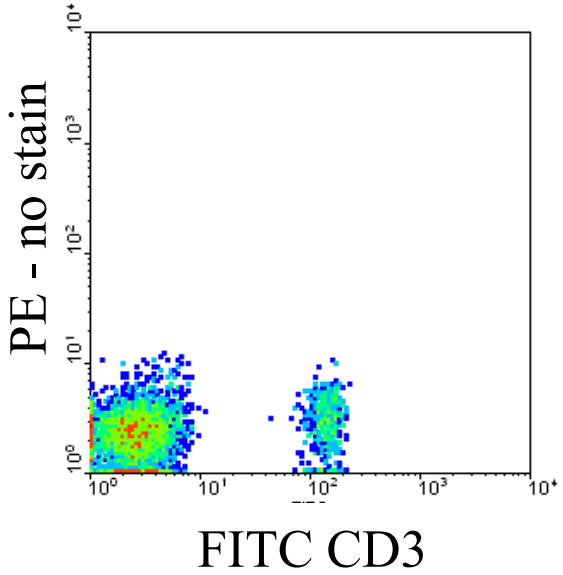
FITC Compensation Control

Uncompensated



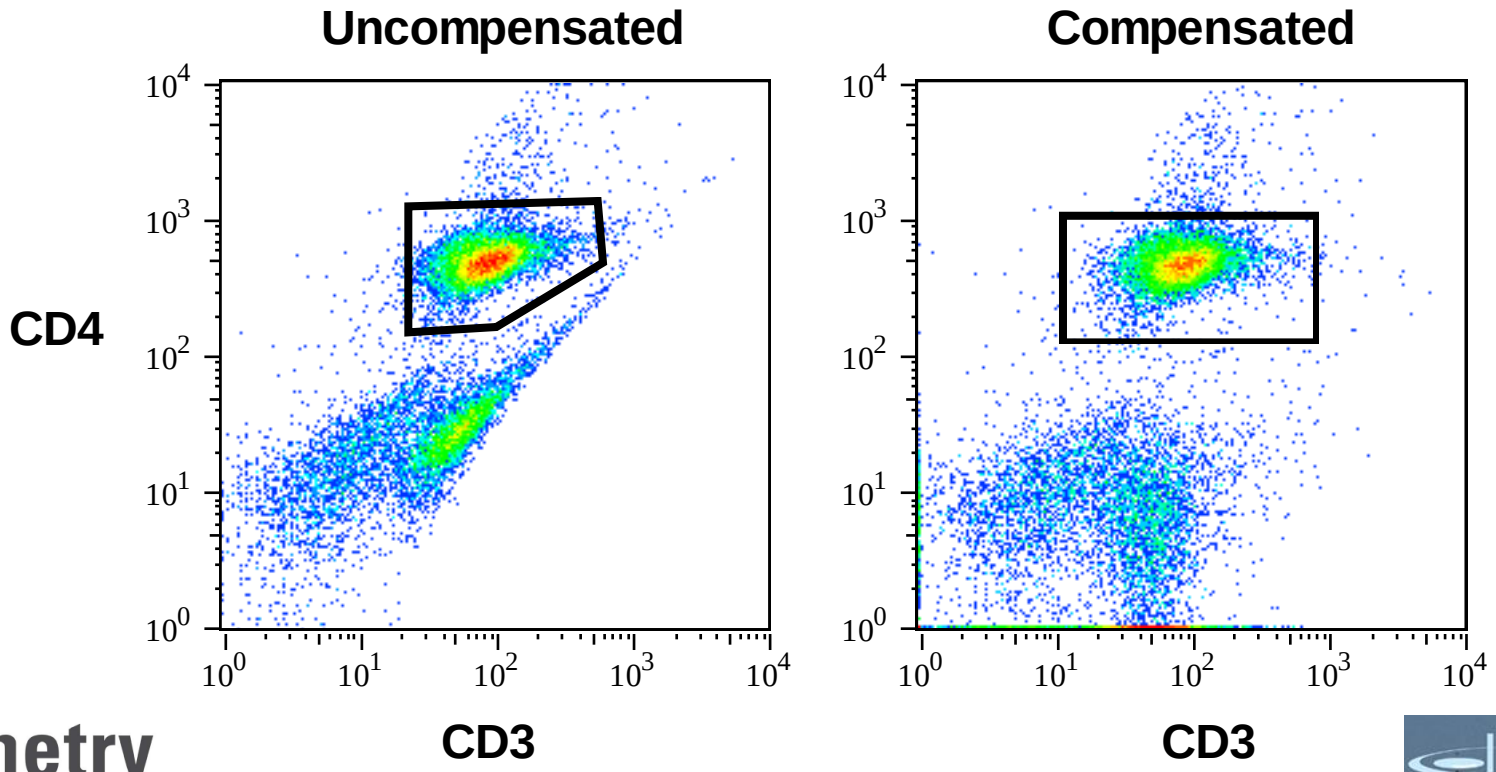
FL2-15%FL1

Compensated



Compensation in 2 colors: Mostly aesthetic

Accurate identification and enumeration of subsets is still easy in two color experiments



Compensation: Mostly aesthetic

- **Accurate discrimination** of subsets is possible with uncompensated data
- However, this is true only when the expression of all **antigens is uniform** on each subset (e.g., CD45 / CD3 / CD4 / CD8)
- Otherwise, it may not be possible to gate on subsets (with current tools)

New **automated software** is on the way for unbiased analysis (no gating).

Impact of Compensation on Visualization and Analysis of Data

- "Visualization artifacts" lead to:
  - Manual overcompensation
  - Incorrect gate settings
- Specific staining controls become essential

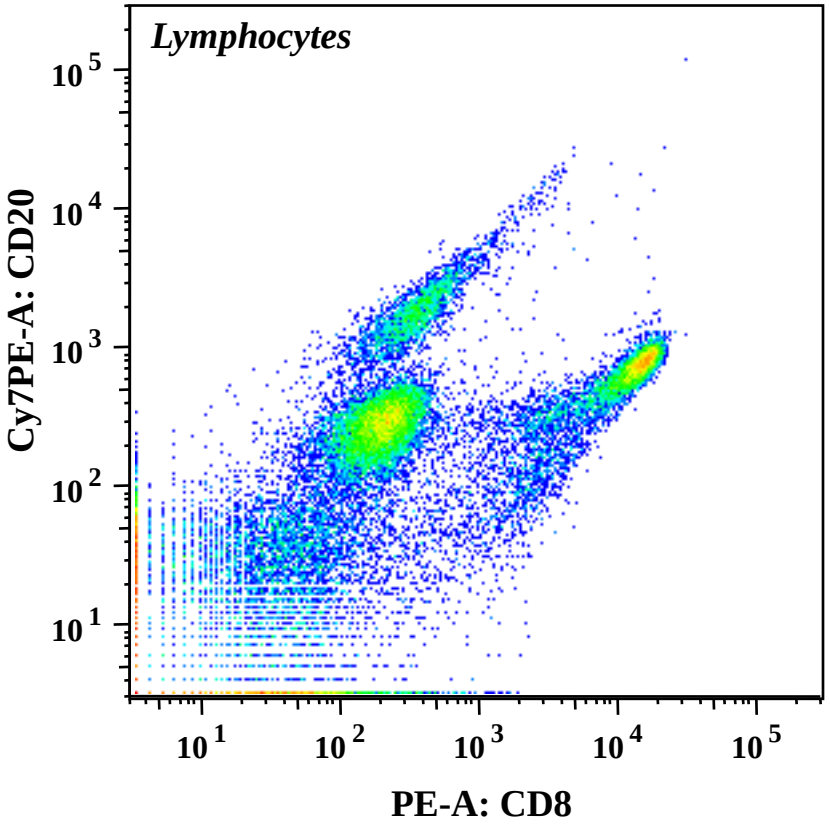
What causes this artifact?

Spreading due to Measurement Error

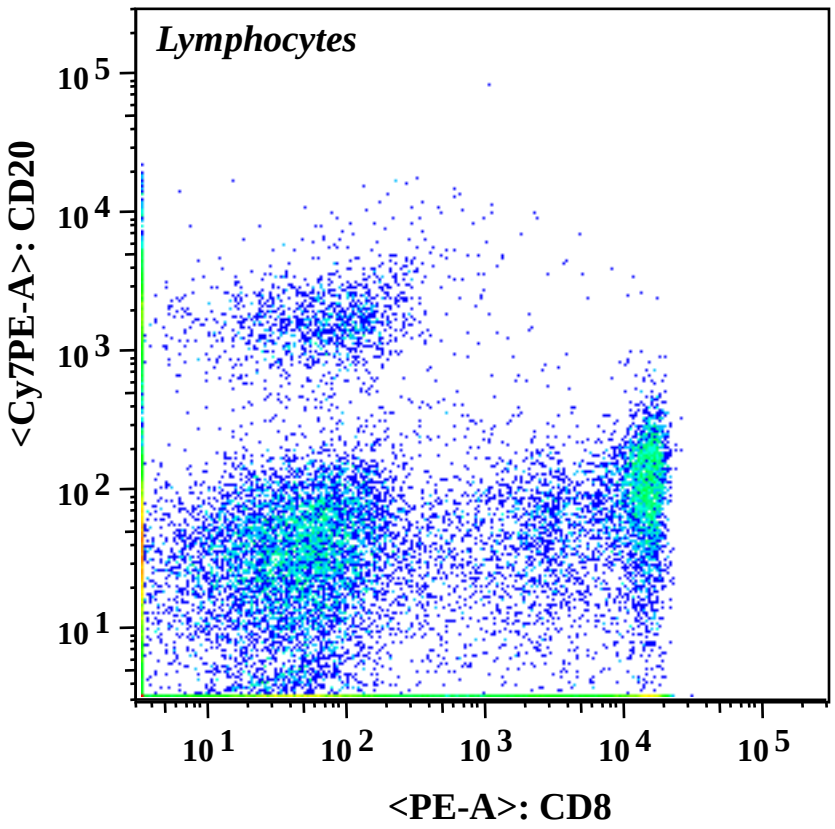
Why do these populations look funny?

Multicolor Compensation

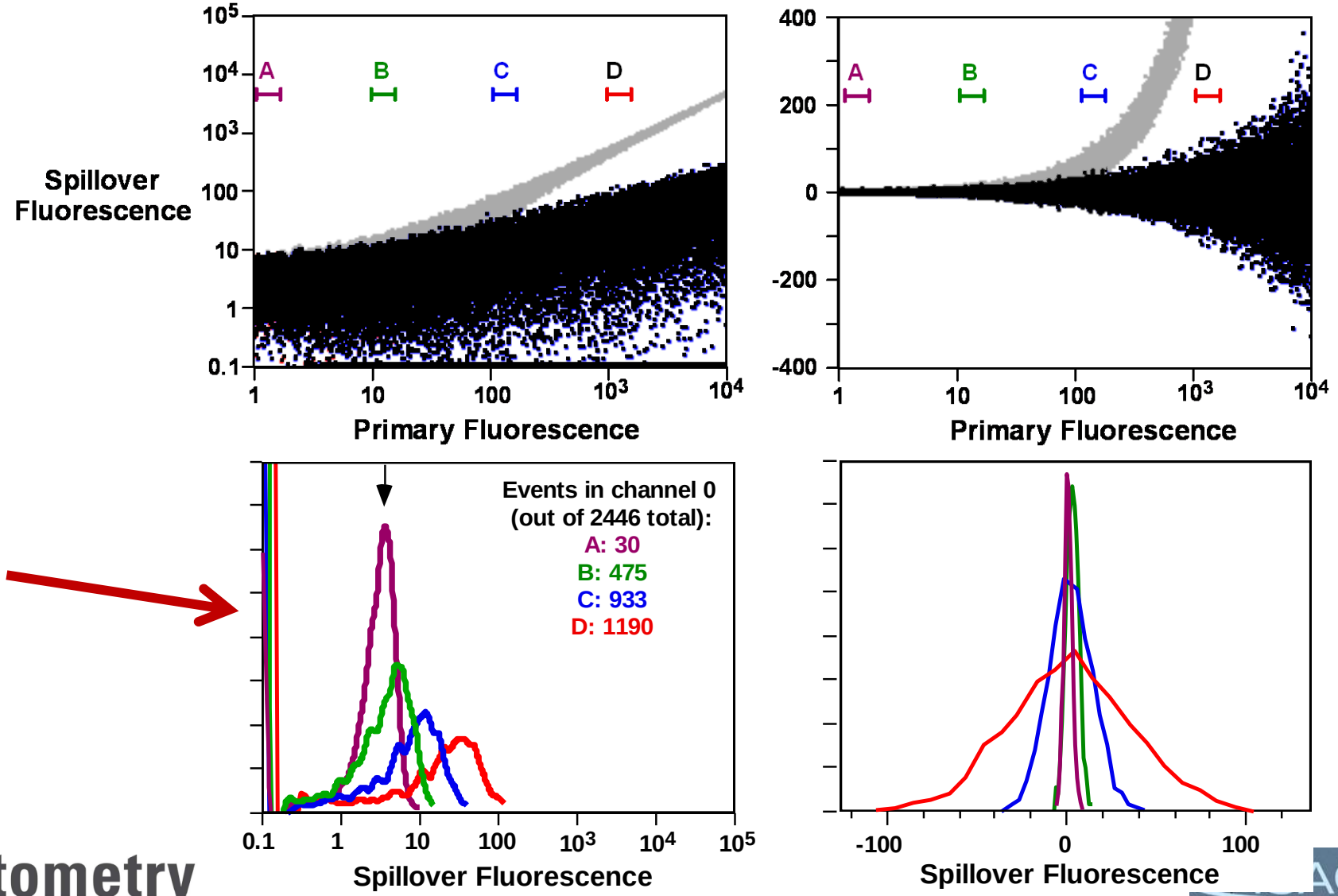
Uncompensated



Compensated



Log Transformation of Data Display Leads to Manual Overcompensation



Compensation Does NOT Introduce or Increase Error:

Compensation Only Reveals It!

Spread of Compensated Data

- Properly compensated data may not appear rectilinear ("rectangular"), because of **measurement errors**.
- This effect on compensated data is **unavoidable**, and it cannot be "corrected".
- It is important to distinguish between incorrect compensation and the effects of measurement errors.

Controls

Staining controls fall into three categories:

Instrument setup and validation (compensation, brightness)

Staining/gating controls (Viability, FMO)

Biological

Instrument Setup Controls

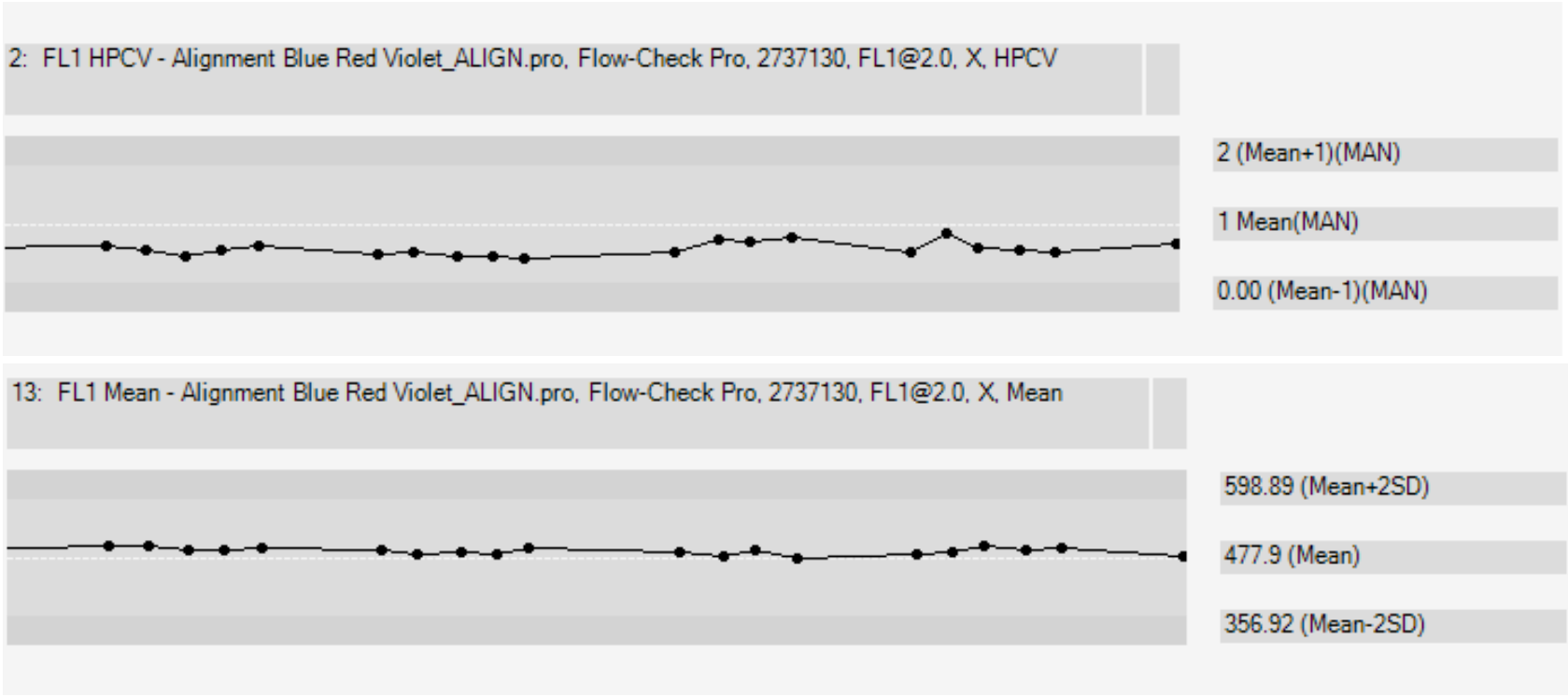
Typically, **fluorescent beads**… with a range of fluorescences from “negative” to very bright.

Use these to validate:

- Laser stability & focusing
- Filter performance
- PMT sensitivity (voltage)
- Fluidics performance
- Daily variability

Consider setting target fluorescences for alignment: this allows for greatest consistency in *analysis* (gating) between experiments.

Stability of instrumentation



Compensation Controls

Single-stained samples…must be at least as bright as the reagent you are using in the experiment!

Can use *any* "carrier", as long as the positive & negative populations have the same fluorescence when unstained:

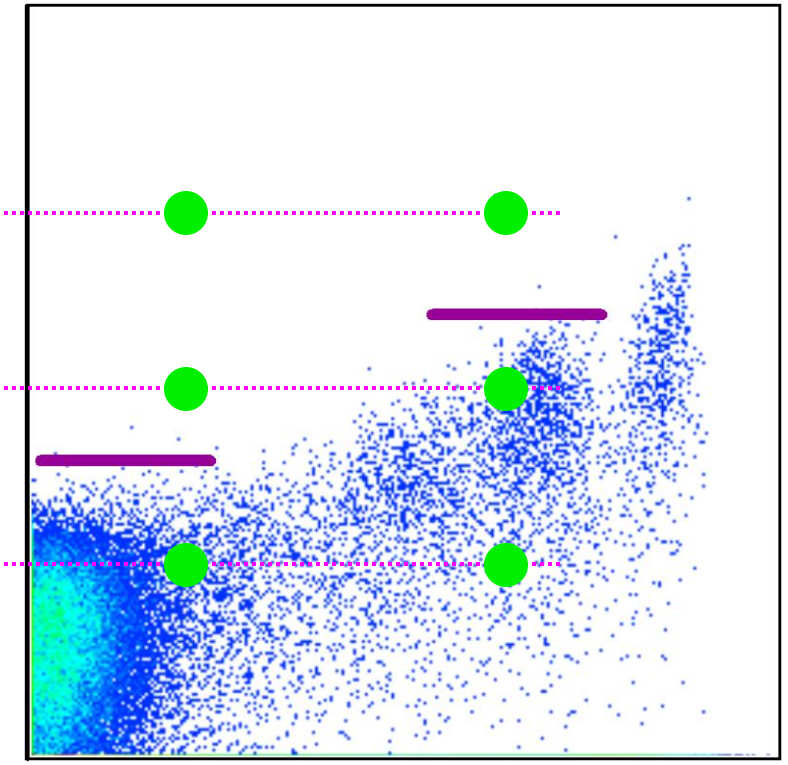
Cells (mix stained & unstained)
Subpopulations (CD8 within total T)
Beads (antibody-capture)

One compensation for every color… and one for each unique lot of a tandem (Cy5PE, Cy7PE, Cy7APC, TRPE)

Staining Controls

- Staining controls are necessary to identify cells which do or do not express a given antigen.

- The threshold for positivity may depend on the amount of fluorescence in other channels!



Staining Controls

- **Unstained cells** or complete isotype control stains are ***improper*** controls for determining positive vs. negative expression in multi-color experiments.
- The **best control** is to stain cells with all reagents ***except*** **the one** of interest.

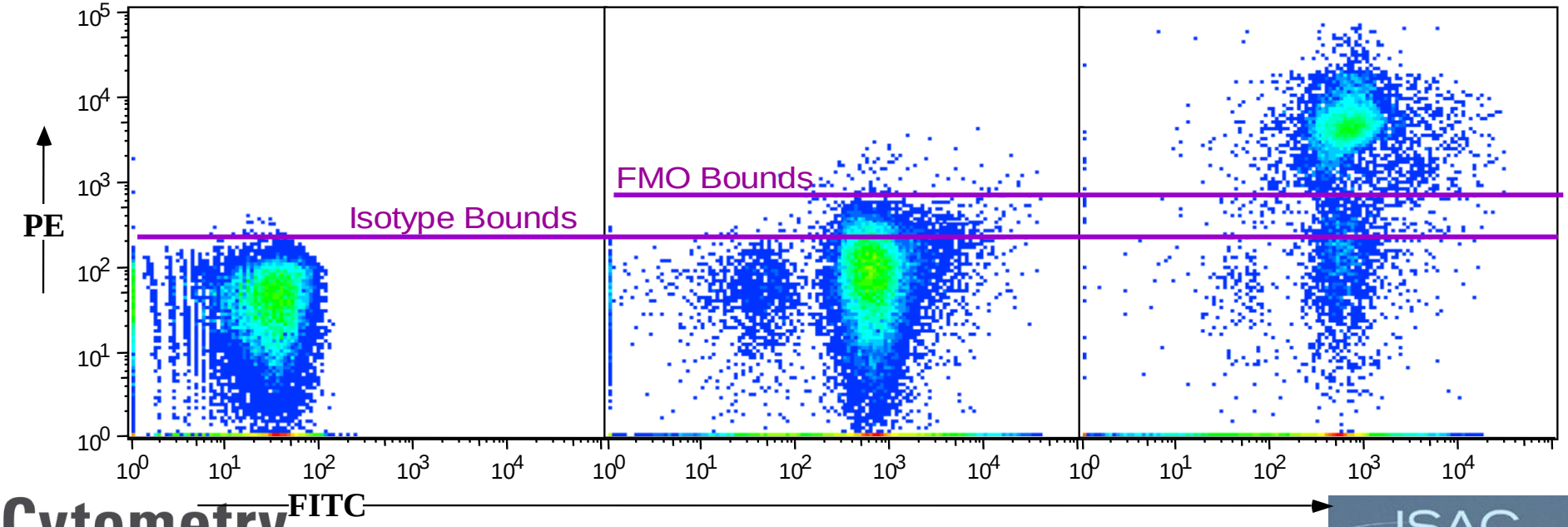
FMO Control

"Fluorescence Minus One"

Identifying CD4 cells with 4 colors

PBMC were stained as shown in a 4-color experiment. Compensation was properly set for all spillovers

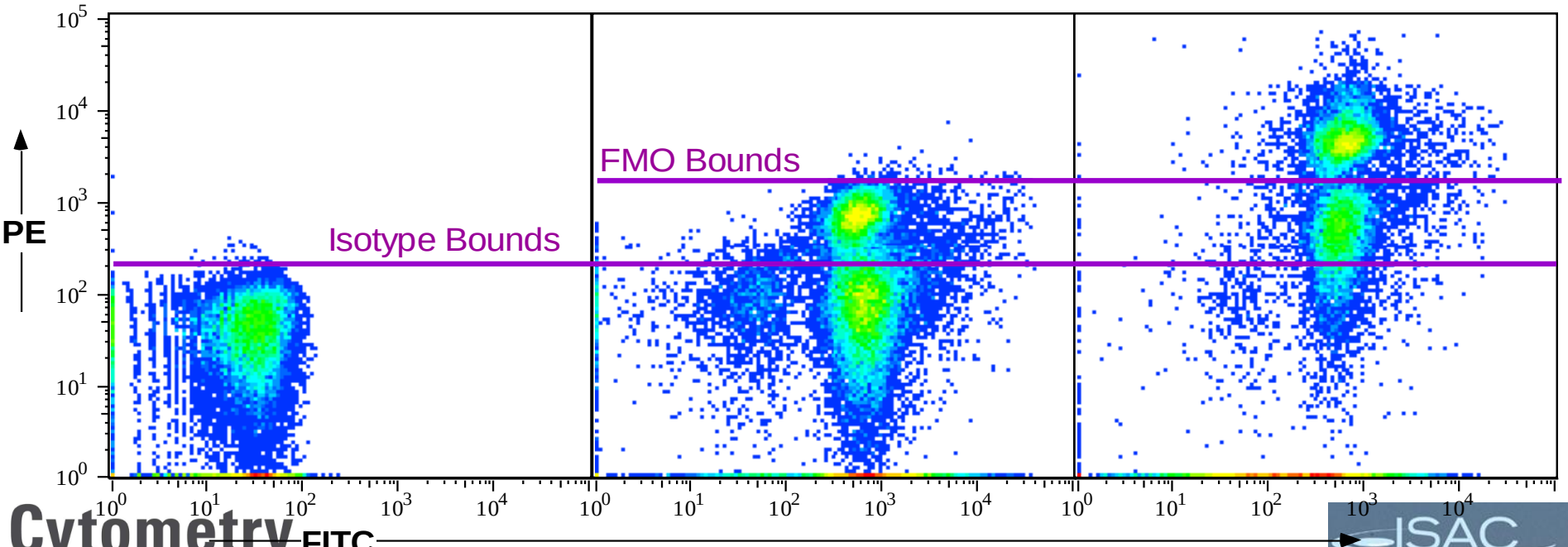
	Unstained Control	FMO Control	Fully Stained
FITC	–	CD3	CD3
PE	–	–	CD4
Cy5PE	–	CD8	CD8
Cy7PE	–	CD45RO	CD45RO



FMO controls aid even when compensation is improper

Incorrect Cy5PE into Cy7PE compensation

	Unstained Control	FMO Control	Fully Stained
FITC	Š	CD3	CD3
PE	Š	Š	CD4
Cy5PE	Š	CD8	CD8
Cy7PE	Š	CD45RO	CD45RO



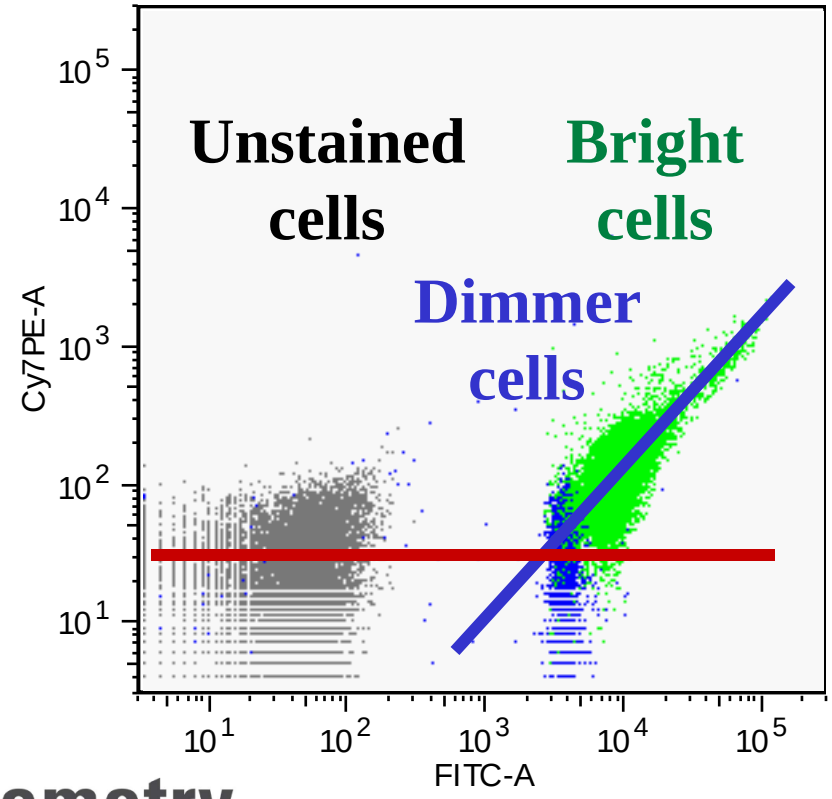
FMO Controls

- are a much better way to **identify positive vs. negative** cells
- can also help **identify problems in compensation** that are not immediately visible
- should be used whenever **accurate discrimination** is essential or when antigen expression is relatively low

Why Bright Comp Controls?

Estimating a low spillover fluorescence accurately is impossible (autofluorescence).

Therefore, compensation is generally only valid for samples that are duller than the compensation control.

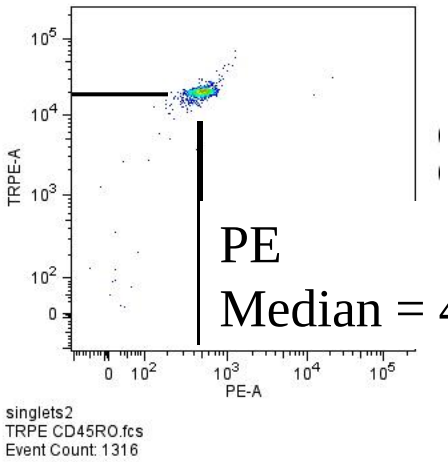


FITC spillover into Cy7PE (1%)

Autofluorescence

Different lots of tandems can require different compensation!

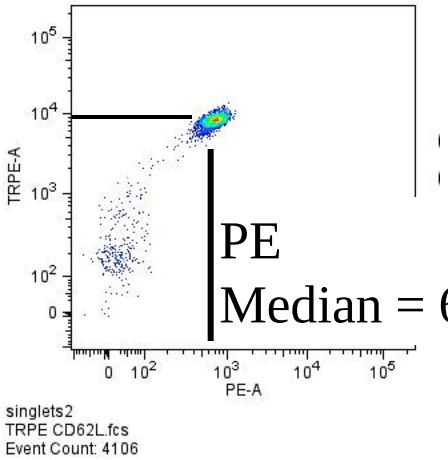
TR-PE reagent 1
Median = 21,100



Compensation Required
(ΔPE / ΔTRPE)

2.3%

TR-PE reagent 2
Median = 8,720



8.0%

Advantage of More-Than-Minimal Markers

Two extremes of gating strategy:

"**Conservative**" - drawn to be very "tight" around the visually-defined populations

- Greatest purity of subset
- Lowest sensitivity

"**Liberal**" - drawn to include much larger areas than visually appear to belong to a subset.

- Greatest sensitivity
- Greatest chance of contamination

BUT: multiple rounds of "Liberal" gating based on multiple parameters results in excellent purity and sensitivity.

Polychromatic panels

Development is time-consuming, expensive and requires substantial expertise.

Fortunately, you do not always need to reinvent the wheal because many optimized panels are already published (→ OMIPs)

OMIPs

Optimized Multicolor Immunofluorescence Panels

Mario Roederer, NIH, Bethesda

Cytometry PART A

ISAC International Society for Analytical Cytology

OMIPs

A new publication type exclusive to Cytometry A.

Proposed in 2010, with guidelines for publication:

> "Publication of optimized multicolor immunofluorescence panels," Mahnke, Chattopadhyay, and Roederer. Cytometry A. 2010;77:814

The first two OMIPs in 2010:

> **OMIP-001**: Quality and phenotype of Ag-responsive human T-cells. Mahnke, Roederer. Cytometry A 2010;77:819
>
> **OMIP-002**: Phenotypic analysis of specific human CD8+ T-cells using peptide-MHC class I multimers for any of four epitopes. Chattopadhyay, Roederer, Price. Cytometry A 2010;77:821.

A total of >**18 OMIPs** now in published and more to come

OMIPs

OMIPs have **2 parts**

A brief (2 page only!) **printed version** that summarizes information and shows an example.

An extended **online version** that has multiple required tables and information pieces.

The format and content, even of the online material, is fairly well specified and must be followed.

NATURE REVIEWS | IMMUNOLOGY VOLUME 12 | MARCH 2012 | 191

Standardizing immunophenotyping for the Human Immunology Project

Holden T. Maecker[1], J. Philip McCoy[2,3] and Robert Nussenblatt[3,4]

Abstract | The heterogeneity in the healthy human immune system, and the immunological changes that portend various diseases, have been only partially described. Their comprehensive elucidation has been termed the 'Human Immunology Project'. The accurate measurement of variations in the human immune system requires precise and standardized assays to distinguish true biological changes from technical artefacts. Thus, to be successful, the Human Immunology Project will require standardized assays for immunophenotyping humans in health and disease. A major tool in this effort is flow cytometry, which remains highly variable with regard to sample handling, reagents, instrument setup and data analysis. In this Review, we outline the current state of standardization of flow cytometry assays and summarize the steps that are required to enable the Human Immunology Project.

LIFE-Study

LIFE - Leipzig Research Center for Civilization Diseases

LIFE-study **26.500** individuals (5 % of population)

Aims: Influence of health status and life style

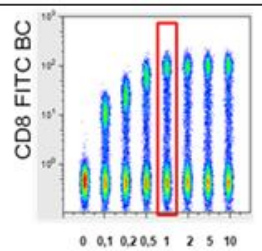
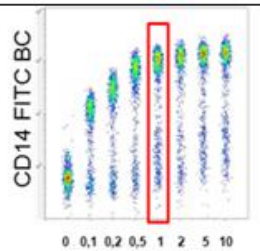
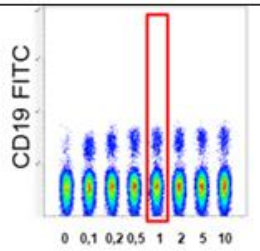
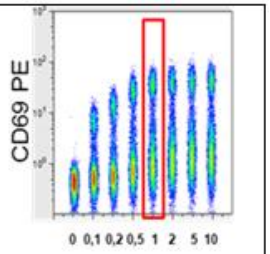
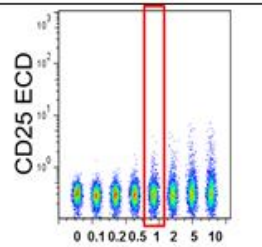
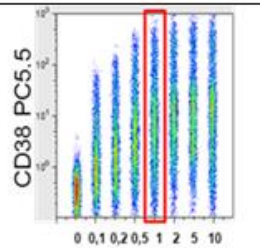
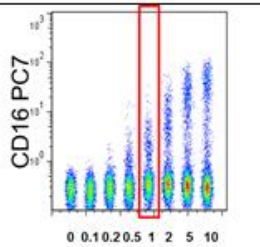
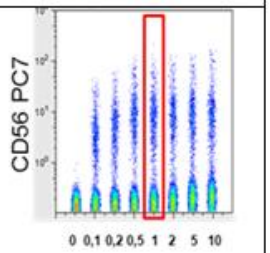
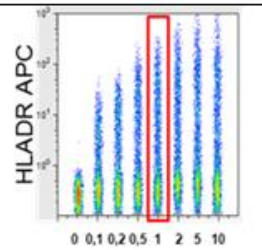
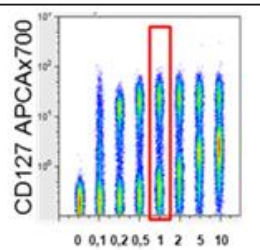
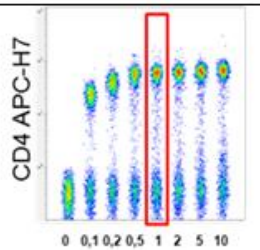
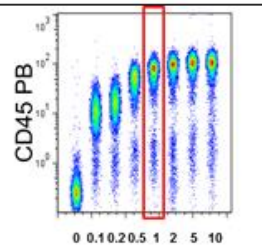
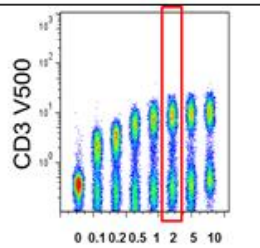
- Identification of risk factors
- Innovative ways to predict disease development and early diagnosis
-Improvement of German healthcare

Methods: Complex medical, psychological and laboratory analysis and questionnaires.
Follow up studies.

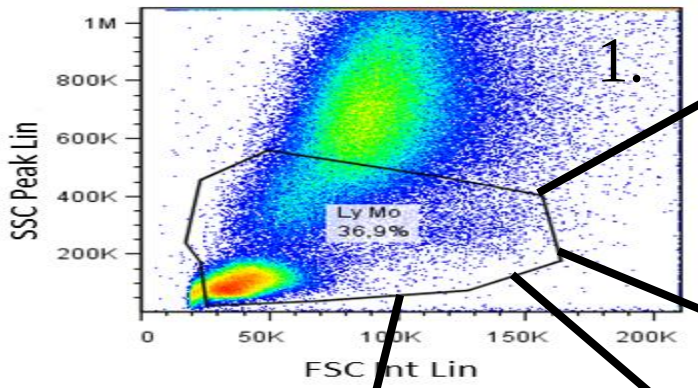
Specificity MAB	Ab Clone	Fluorochrome	Purpose	Isotype
CD8	B9.11	FITC	T-cytotoxic cells	IgG1
CD14	RMO52	FITC	LPS Rec. Monocytes	IgG2a
CD19	J3-119	FITC	B-cells	IgG1kappa
CD69	TP1.55.3	PE	Early activation	IgG2b
CD25	B1.49.9	ECD	IL-2 Receptor a	IgG2a
CD38	LS198.4.3	PC5.5	Activated T and B-cells	IgG1
CD16	3G8	PC7	Fcγ Rec III	IgG1
CD56	N901(NKH-1)	PC7	N-Cam	IgG1
HLA DR	Immu-357	APC	MHC-II	IgG1
CD127	R 34.34	APCAx700	IL-7 Receptor a	IgG1 kappa
CD4	SK3	APC-H7	T-helper cells	IgG1 kappa
CD45	J.33	Pacific Blue	PanLeukocyte antigen	IgG1 kappa
CD3	SP34-2	V500	T-cells	IgG1 lamda

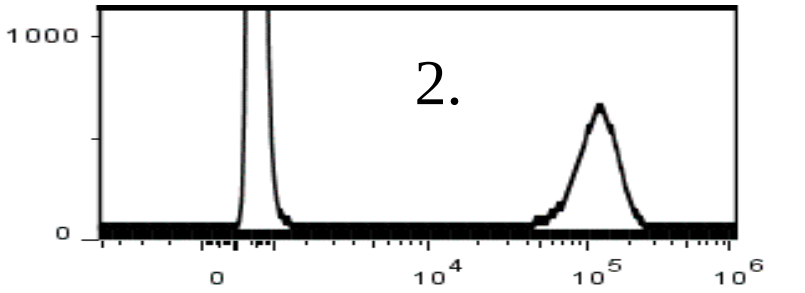
Print Table 1B: Antibodies used for OMIP-BJ-AT

- **30** defined cell phenotypes
- >> **5** functional information in one run!

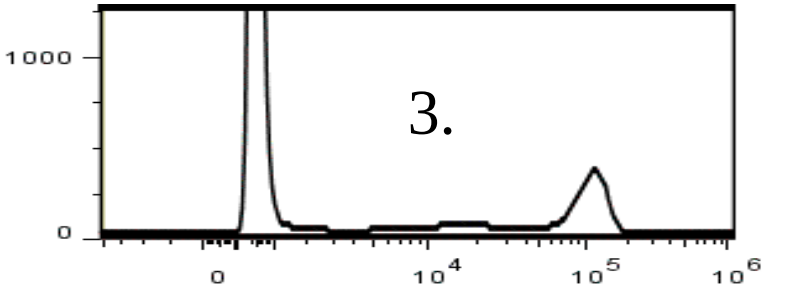
Blue laser 1 488 nm				
	CD8 FITC	CD14 FITC	CD19 FITC	CD69 PE
Blue laser 2 488 nm				
	CD25 ECD	CD38 PC5.5	CD16 PC7	CD56 PC7
Red laser 638 nm				
	HLA DR APC	CD127 APCAx700	CD4 APC-H7	
Violet laser 405 nm				
	CD45 PB	CD3 V500		

Combination of many markers on one color

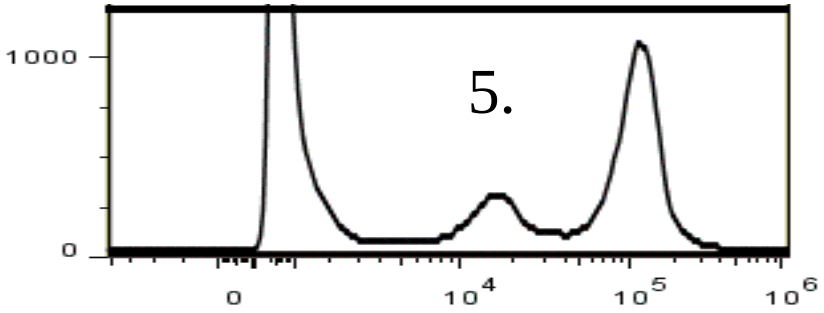




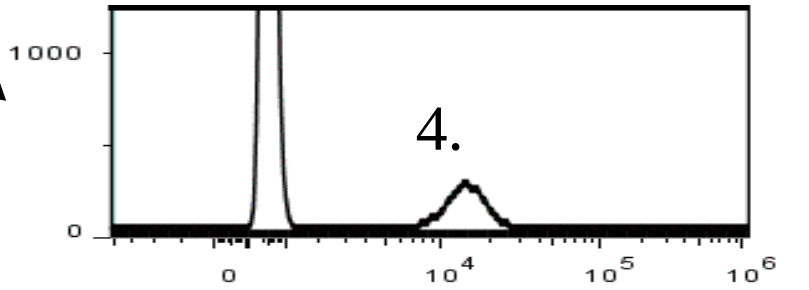
Single CD14 FITC Staining



Single CD8 FITC Staining

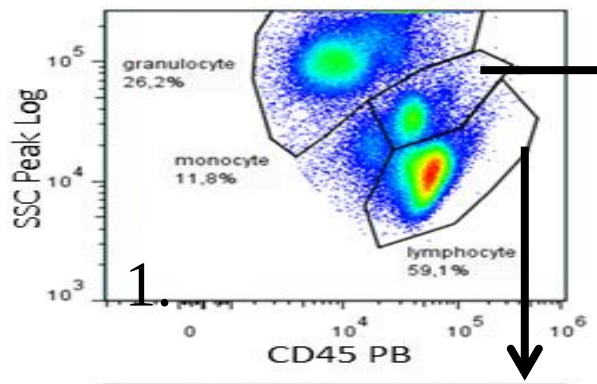


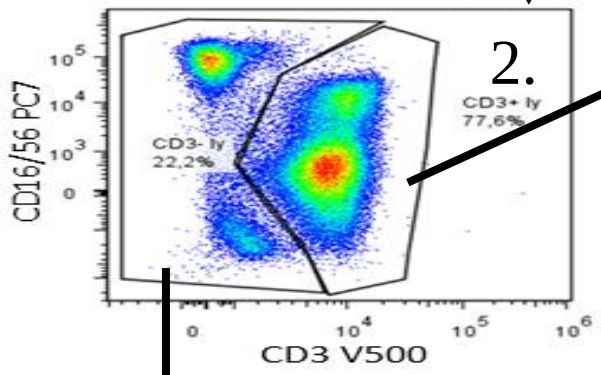
Combined Staining
CD8/14/19 FITC

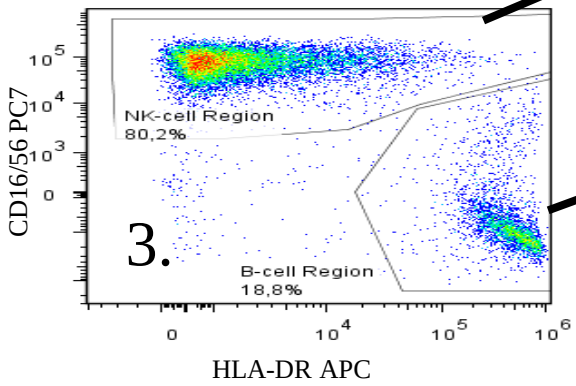


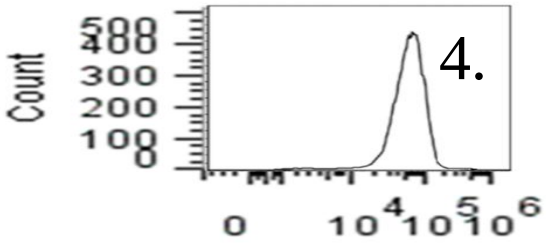
Single CD19 FITC Staining

Combination of many markers on one color

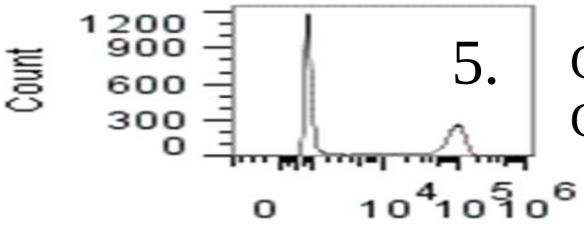




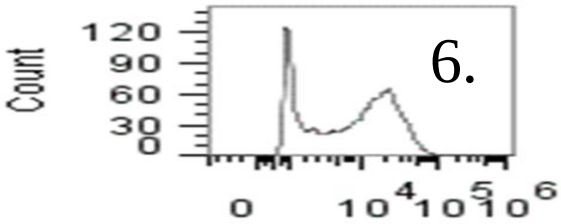




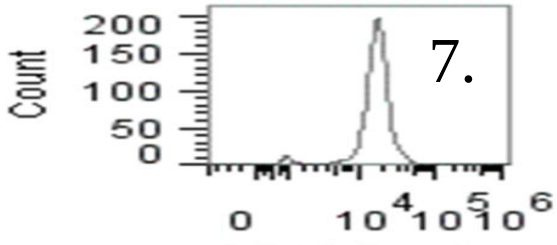
CD14 FITC
on Monocytes



CD8 FITC on
CD3+ Lymphocytes



CD8 FITC on
NK-cells
(CD3-, CD16/56+)



CD19 FITC on B-cells
(CD3-, CD16/56-, HLA DR+)

Critical assessment of automated flow cytometry data analysis techniques

Nima Aghaeepour[1], Greg Finak[2], The FlowCAP Consortium[3], The DREAM Consortium[3], Holger Hoos[4], Tim R Mosmann[5], Ryan Brinkman[1,7], Raphael Gottardo[2,7] & Richard H Scheuermann[6,7]

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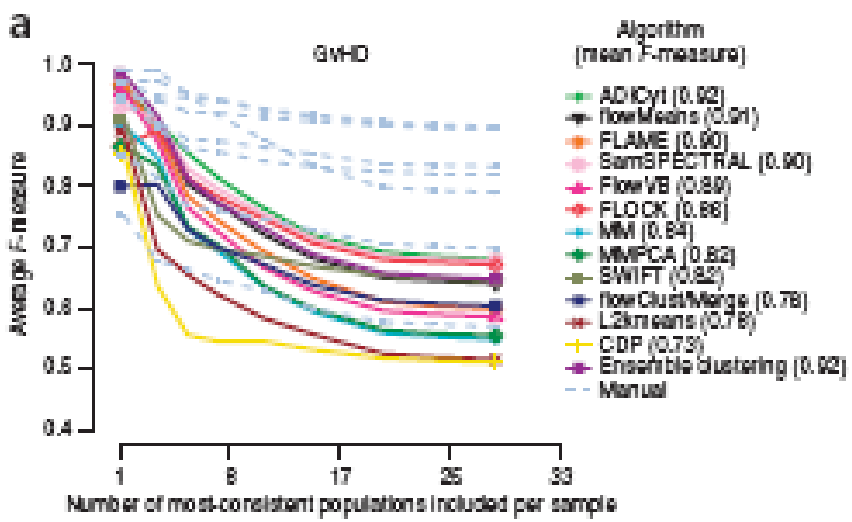
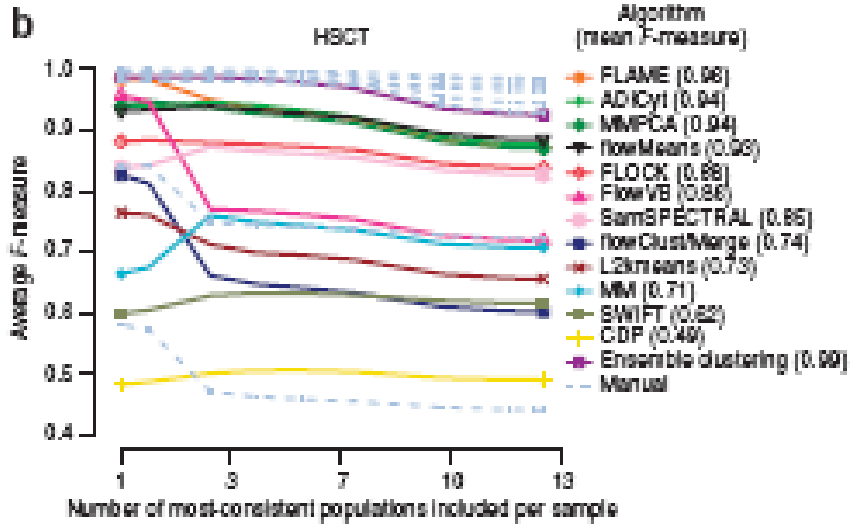
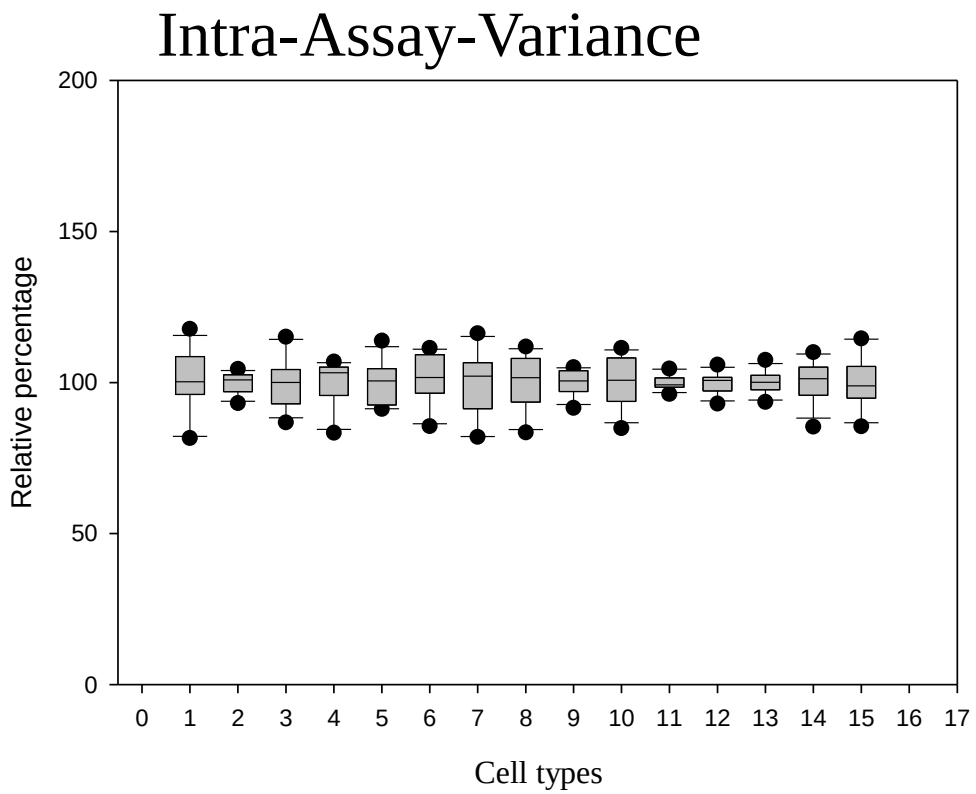


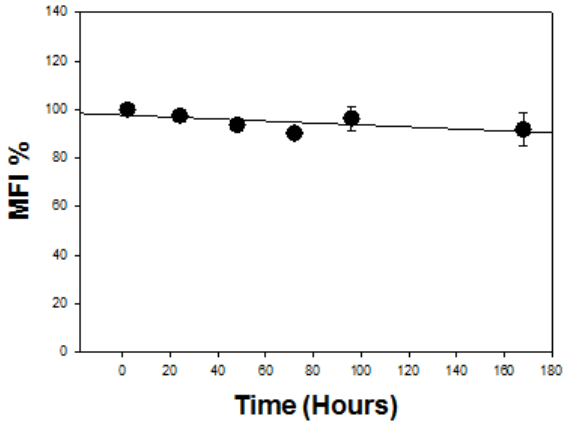


Stability of pre-analytics

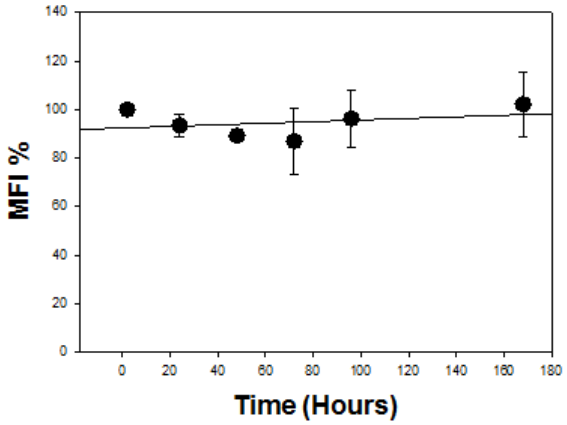


1 - Neutrophil CD16
2 – Eosinophil CD25
3 - Monocyte CD14
4 - Lymphocyte CD45
5 - T-Lymphocyte CD3
6 – NK-cells CD16/56
7 - B-Lymphocytes HLA DR
8 - Plasma Cells CD38
9 - B cells CD19
10 - T-cytotoxic cells ++ CD8
11 - T-helper cells CD4
12 - Treg cells CD25
13 – NKT cells CD16/56
14 - Monocyte Atypic CD16
15 - Monocyte Typic CD14

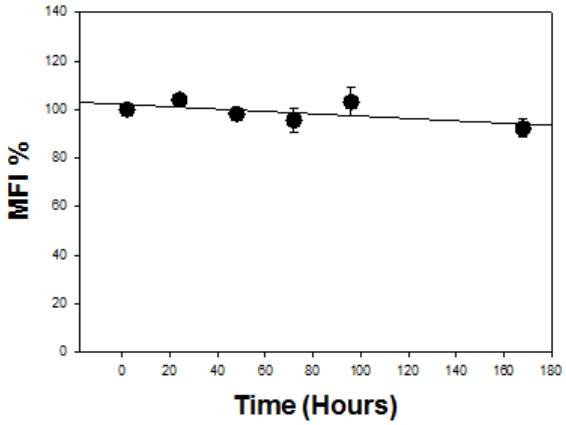
Cocktail stability



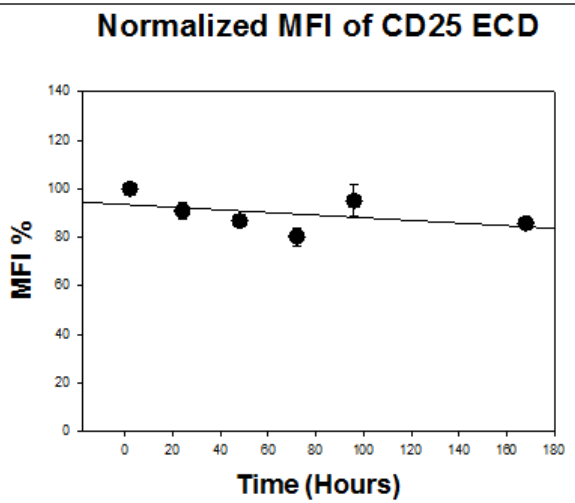
y=-0.040x+97.71; r^2=0.121



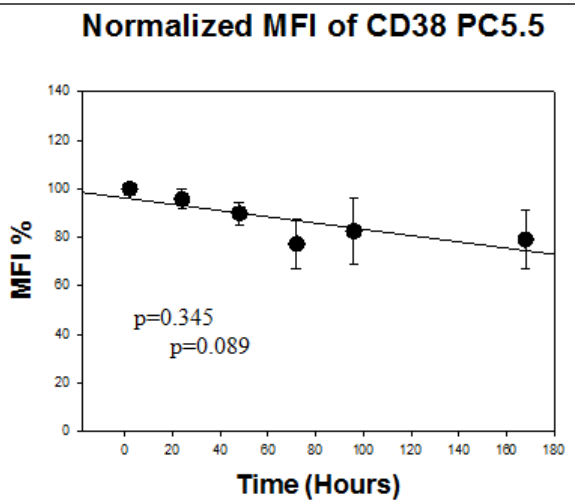
y=-0.015x+94.40; r^2=0.004



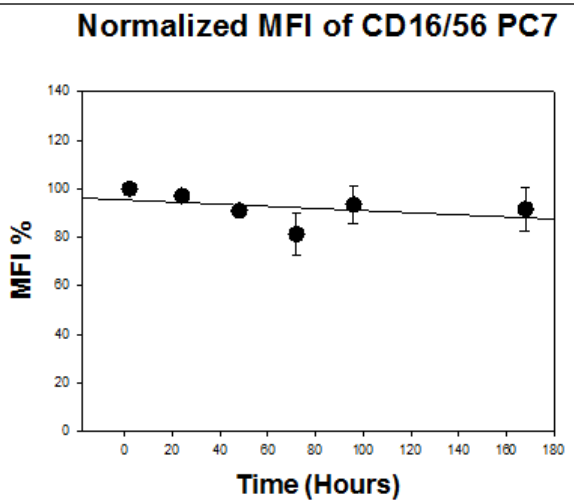
y=0.0074x+99.56; r^2=0.004



y=-0.023x+92.46; r^2=0.018

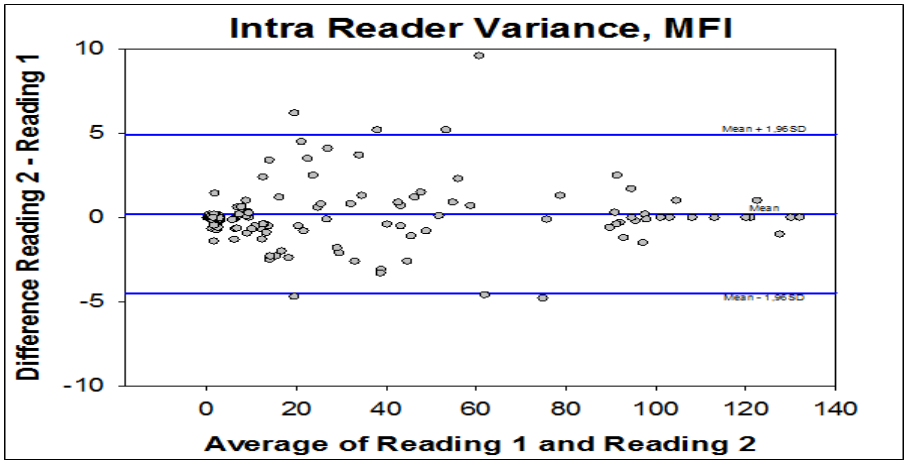


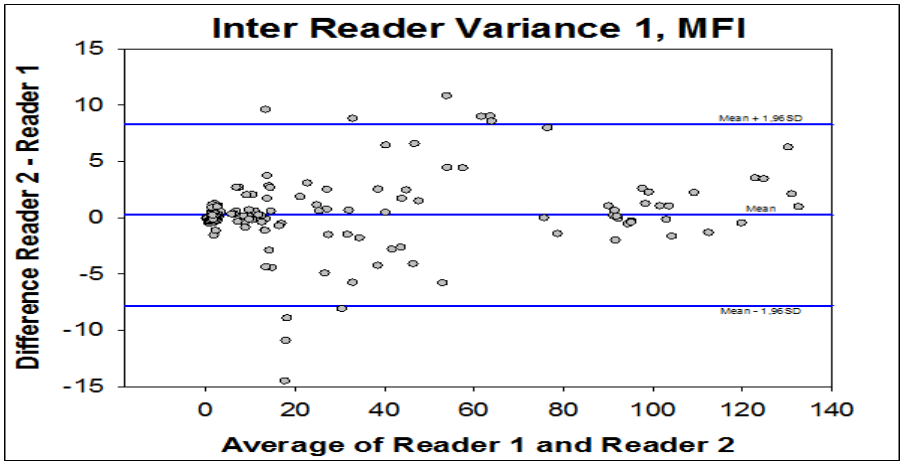
y=-0.131x+99.73; r^2=0.211

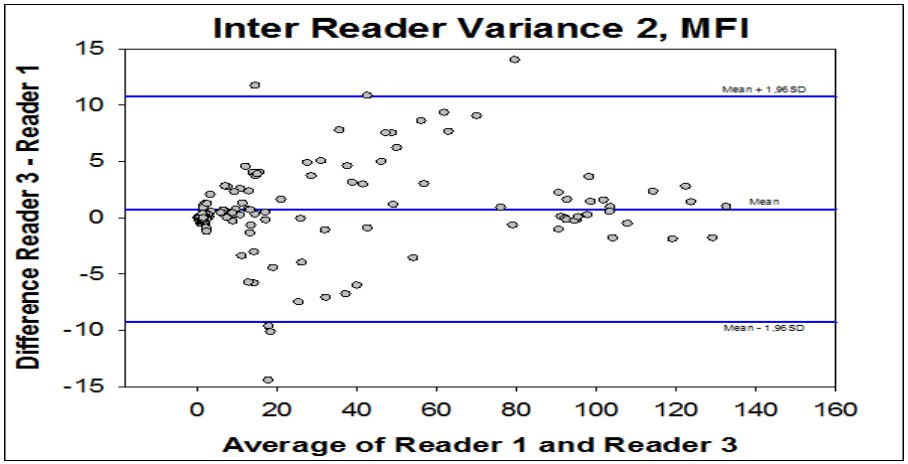


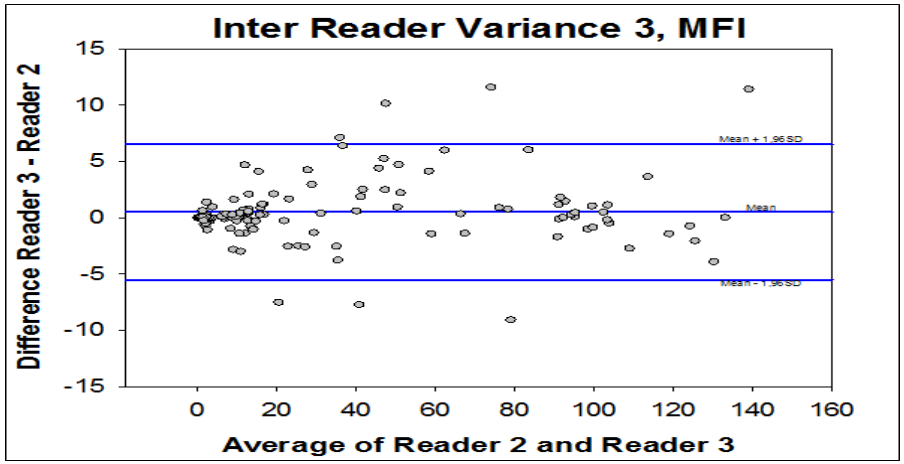
y=-0.053x+96.87; r^2=0.072

Stability of manual analysis

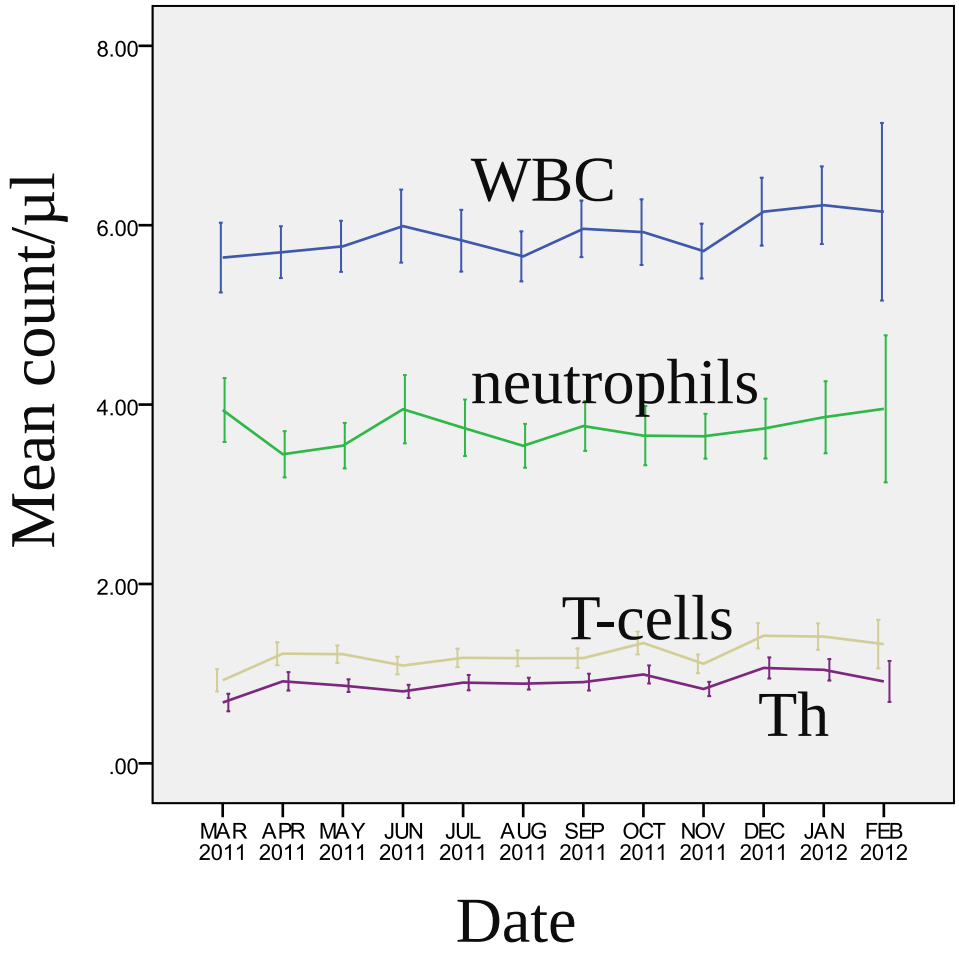








Stability of cell counts



Published OMIPS

	Organism	Cell-subtype
1	human	CD8+ T-cells
2	human	CD4+, CD8+ T-cells (HIV+)
3	human	Memory B cells
4	human	Regulatory T-cells
5	Rhesus macaque	T-cells
6	human	Regulatory T-cells
7	human	NK cells
8	human	T-cells
9	human	CD4+, CD8+ T-cells
10	human	lymphoma cells (leukemia)
11	human	circulating endothelial cells (CECs)
12	mouse	leukocytes
13	human	T-cells
14	human	T-cells
15	human	Regulatory T-cells
16	Cynomolgus macaque/human	CD4+, CD8+ T-cells
17	human	CD4+ T-helper-cells
18	human	CD4 T-cells
19	human	gd T-cells, iNKT-cells, haematopoietic precursors

BC Cancer Agency
CARE & RESEARCH
An agency of the Provincial Health Services Authority

Since Oct. 01. 2010 required for Cytometry A publications.

MIFlowCyt:
Minimum Information about a Flow Cytometry Experiment

Ryan Brinkman
Department of Medical Genetics, University of British Columbia
BC Cancer Research Center

Cytometry PART A
ISAC International Society for Analytical Cytology

FLOWRepository

Login

Did you know?

A guide to FlowRepository is available at the documentation site for Cytobank and FlowRepository.

We also have a Quick start guide.

You can contact us by filling out a support ticket.

Supporting journal

FlowRepository at CYTO 2012

» **Sunday, June 24:** State of the Art Lectures - Computational Analysis of High-Dimensional Data

» **Tuesday, June 26:** Parallel 8 - Cytometry Technology: Cytometry Software and Informatics

» **Wednesday, June 27:** Workshop 13 - Publishing MIFlowCyt Compliant Data to ISAC's FlowRepository.org for Cytometry A and Other Journals

FlowRepository

FlowRepository is a database of flow cytometry experiments where you can query and download data collected and annotated according to the MIFlowCyt standard.

Query

Enter a term to search all publicly available experiments:

Query

Show query fields

Links

Browse all public datasets
Browse community datasets
Browse most popular datasets
Quick start guide
Submit data
Funding
Referencing Flow Repository and Cytobank
FlowRepository Steering Committee & Advisory Board

Flow Repository Website

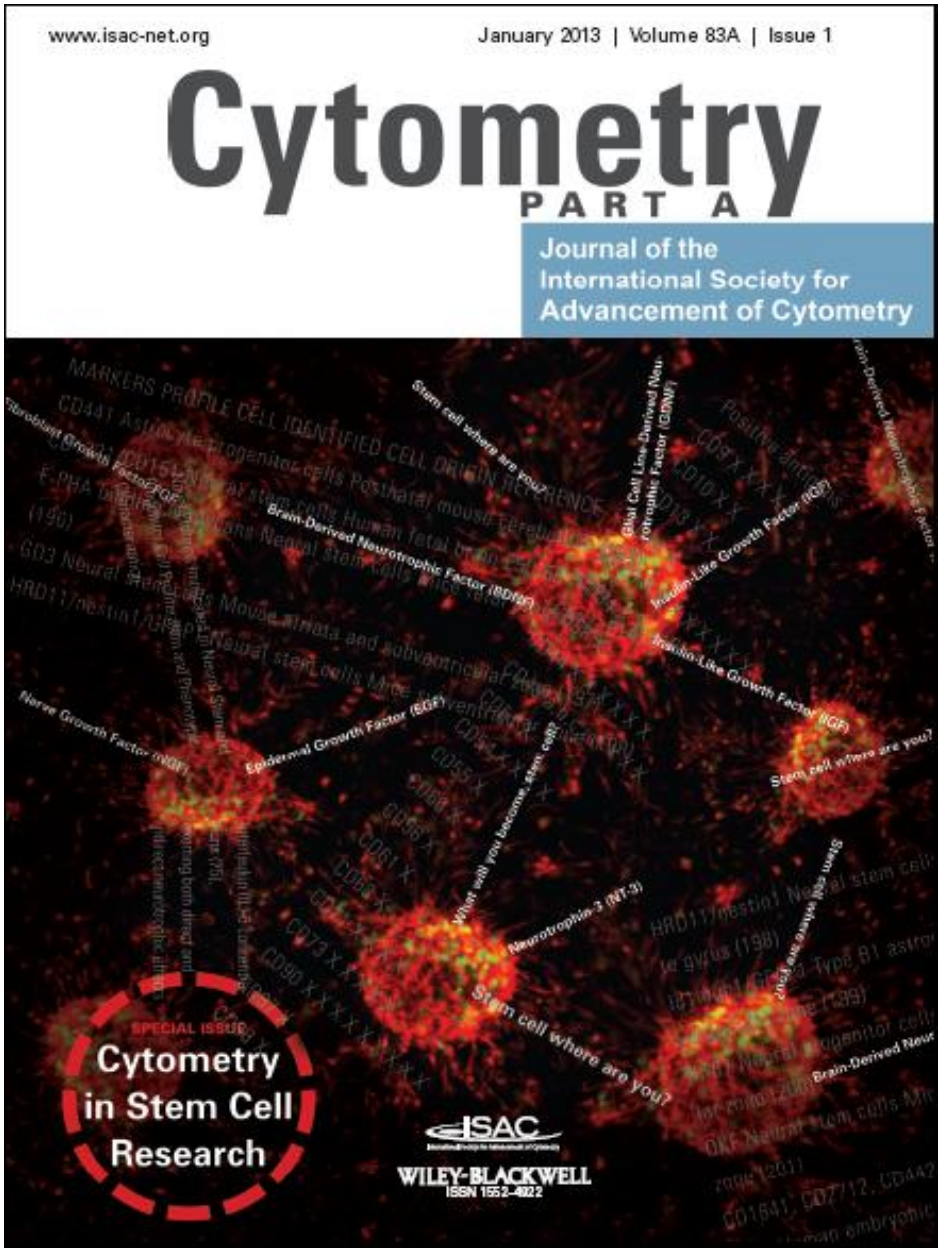
Terms of Service Privacy Policy Support Feedback

The Journal for quantitative single cell science and cell systems biology

Impact Factor 2011: 3.749 (2012 exp.: ~3.7)

Transition time
1st submission to 1st decision: < 30 days

Papers published/year
~ 100



ZYTOMIK
Leipziger Workshop

19th Leipziger Workshop
April 2-4, 2014

Workshop
Register, Login and Abstract submission
For companies only
Publications
Contact / Inquiry

I cordially invite you to the 19th Leipziger Workshop with a focus on

Cytomics and Cell Therapies

Incorporating: 12th International Workshop "Slide-Based Cytometry"
April 2-4, 2014

The goal of the Leipziger Workshop series is to transfer developments from innovative single cell based high-content analysis into applications in clinical research and diagnostics, basic biological research and drug discovery (see also **meeting history**). The Leipziger Workshop covers, among others, the fields of Cytomics, Systems Biology, Cytometry, High-Content Analysis, Modelling, new Techniques and Reagents as well as biological and clinical applications in Cardiology, Pediatric Cardiolgy, Intensive Care Medicine, Oncology and Regenerative Medicine. Different areas are highlighted by renowned key note speakers.

Who should attend? In order to combine the knowledge of different disciplines scientists and clinicians from a great variety of faculties and disciplines are invited to participate and to present their data. We in particularly encourage instrument and technology developers, basic scientists and clinical investigators to attend.

Abstracts. You are invited to submit an abstract of your presentation early. All meeting abstracts and presentations will be reviewed. Authors whose presentation was found excellent (top 10%) will be invited to submit their manuscript as "Invited Paper" that will be published following peer-review in **Cytometry Part A**.

Publication. All abstract authors are invited to submit a brief manuscript on their presentation for potential publication in **Cytometry Part A** as a fully peer-reviewed manuscript (bring a print version to the meeting or send it to us electronically).

Teaching and Practical courses are an integer and important part of the workshop. Here, theoretical and practical, hands-on courses on cutting edge cell analytical instruments and technology are held. Separate registration is mandatory.
This course is also an *excellent opportunity for companies* to introduce and teach their latest achievements and cutting edge technologies.
We hope to see your latest scientific achievements presented at the next Leipziger Workshop or to welcome you as a participant.

Prof. Attila Tárnok,
Workshop President

UNIVERSITÄT LEIPZIG
HERZZENTRUM
Organization
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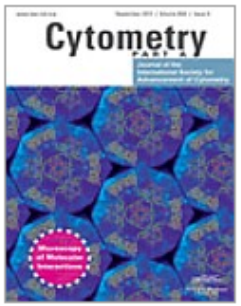
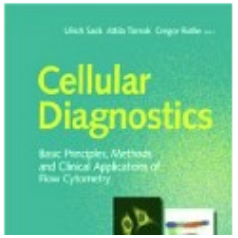
TRM Leipzig
Organization
TRM Leipzig, University of Leipzig



Editor-in-Chief
Attila Tárnok



www.leipziger-workshop.de

Cytometry PART A

ISAC
International Society for Analytical Cytology

Thank you

References and examples

- Manuscript examples are found on the Cytometry Part A – Wiley-Blackwell Website.
- **MIFlowCyt: the minimum information about a Flow Cytometry Experiment.** Lee et al. Cytometry A. 2008;73:926.

MI

- For experimental publications a minimum information (MI) has to be provided so that the experiments can be understood and repeated
- *Promoting coherent minimum reporting guidelines for biological and biomedical investigations: the MIBBI project*. Taylor CF et al. Nat Biotechnol. 2008;26:889.
- Usage of these guidelines is now obligatory for many journals. ~ 100% of FCM submissions to us claim MIFlowCyt compliance.

Advantage of More-Than-Minimal Markers

When designing your panels, try to include reagent combinations that will allow you a combination of positive and negative expression gates for every subset of interest.

Note that there is almost never a downside to including additional markers that are negative gates--the lack of this fluorescence signal on your cells of interest cannot alter the sensitivity of your measurements.

“Dump” channels and viability channels are virtually always a good thing!

Example Optimization

In this example, we wished to evaluate the expression of CXCR3 and CCR4 on naïve ($CD62L^{+}CD45RA^{+}CD45RO^{-}$) CD4 T cells.

- What fraction of naïve T cells express these molecules?
- If possible: are those cells "truly" naïve ($CD28^{+}CD11a^{dim}CD27^{+}$)?

  Requirements:

  CD4, CD3 = Primary reagents

  CD45RO/RA, CD62L = Secondary (need excellent separation)

  CXCR3, CCR4 = Tertiary reagents

  CD27, CD11a, CD28 = Luxury reagents

Selection of Marker/Color Combinations

"Primary" Well-characterized, identify broad subsets of cells, expression is usually on/off.

e.g., CD3, CD4, CD8, CD14, CD19, CD20
Typically used as "parent" gates in analysis

These reagents are usually assigned to "dimmer" colors and colors that exhibit the greatest spillover problems

e.g., Cy5.5PE, Cy7PE, Cy7APC, AmCyan

Selection of Marker/Color Combinations

"Secondary" Well-characterized, bright expression patterns

e.g., CD27, CD28, CD45RA/RO, γIFN, perforin
Expression levels can be a continuum

These are usually assigned to the next tier of colors, those that perform well with little spillover problems

e.g., FITC, TRPE, Cy5PE/PerCP, Alexa 405, Alexa 690

Selection of Marker/Color Combinations

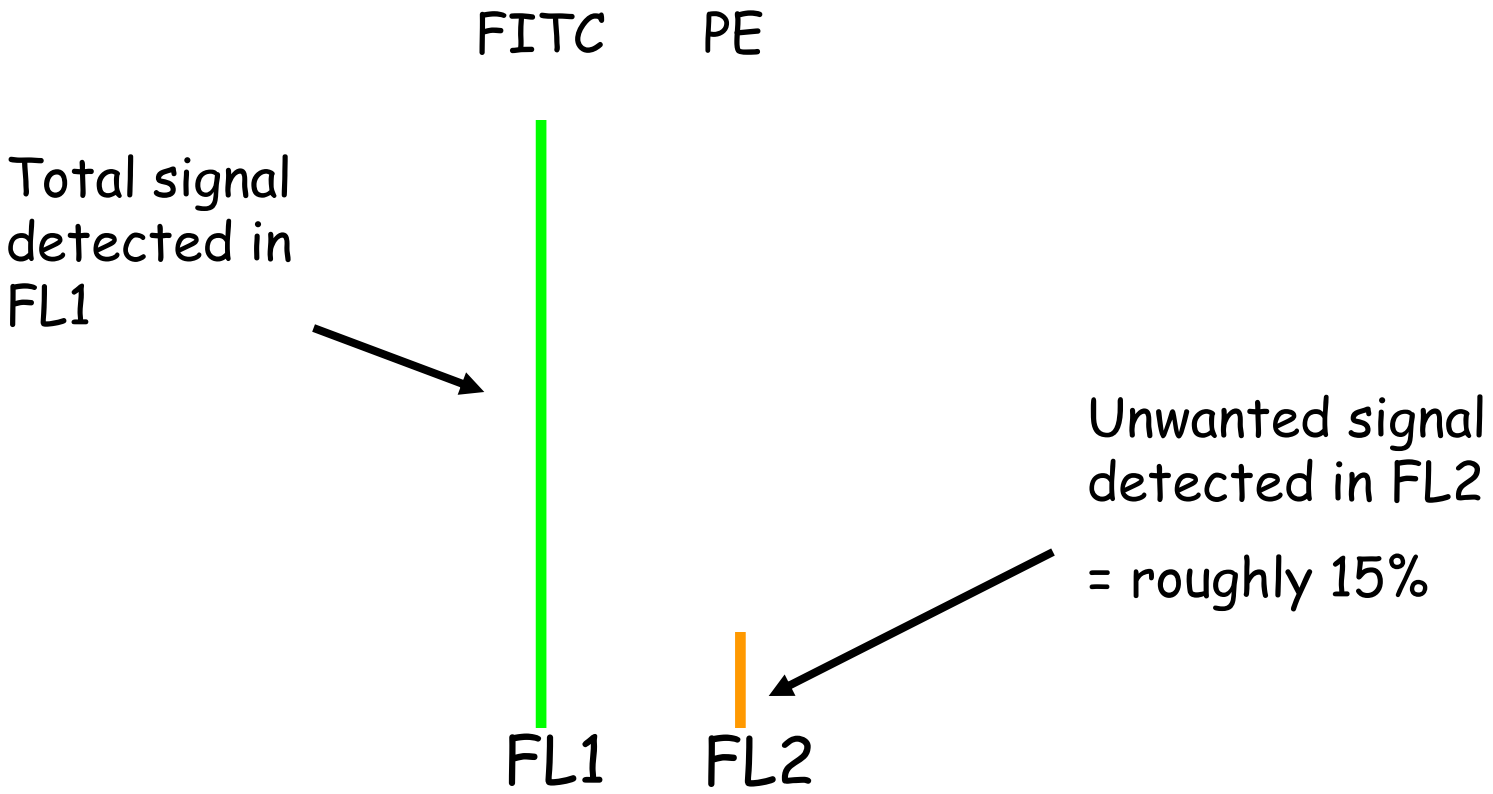
"Tertiary" Low-expression levels or uncharacterized

e.g., CD25, CCRs, "X"

These require the absolutely brightest colors, with the least spillover problems possible

e.g. PE, APC, QD655

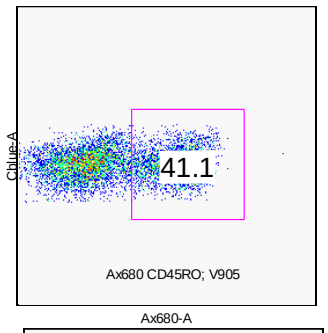
FITC Single Stain Control

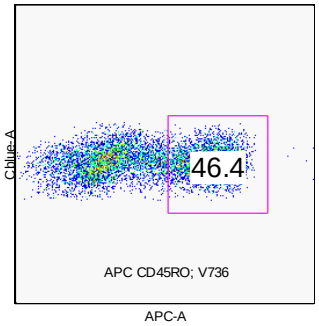


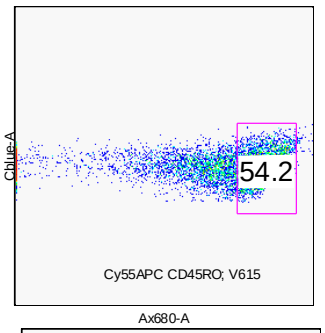
True PE = Total FL2 – 15% FL1

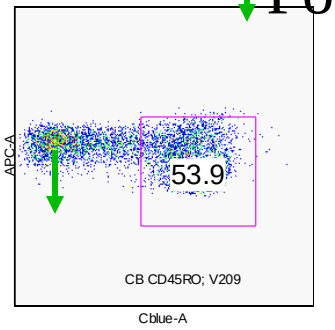
CD45RO Example Stains

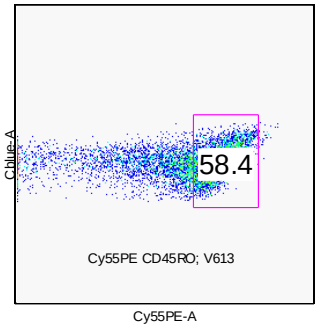
For panel

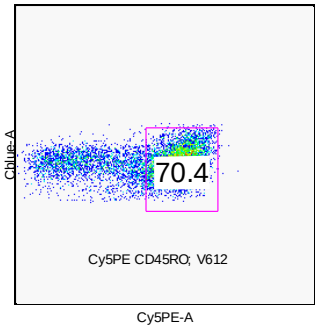


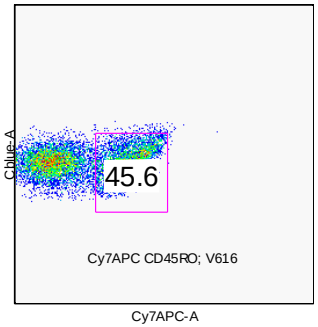


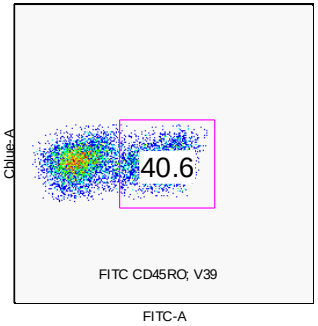


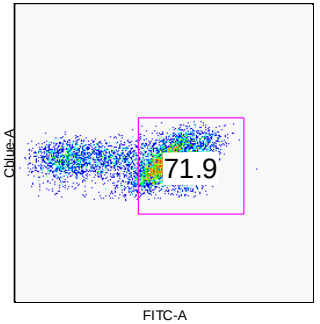


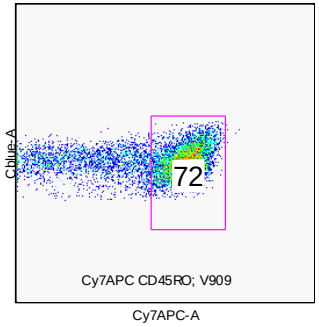


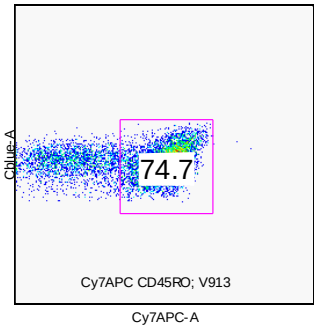


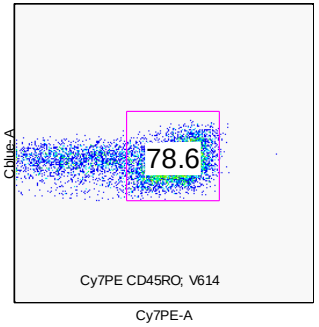


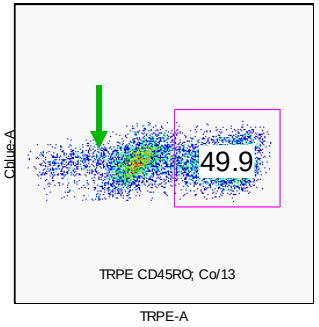






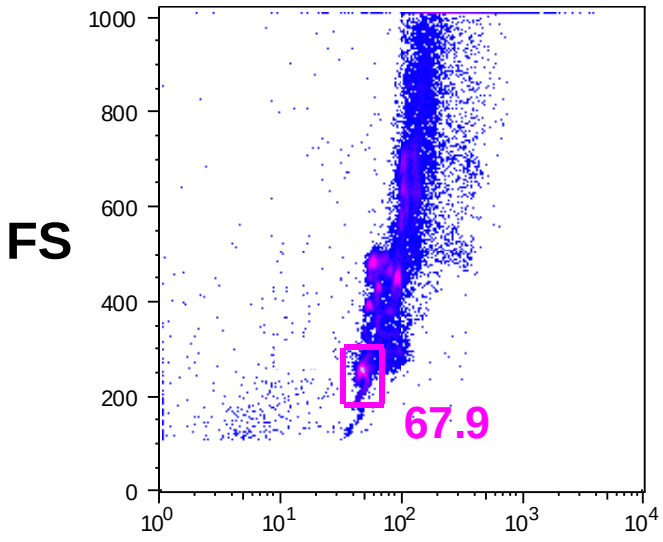


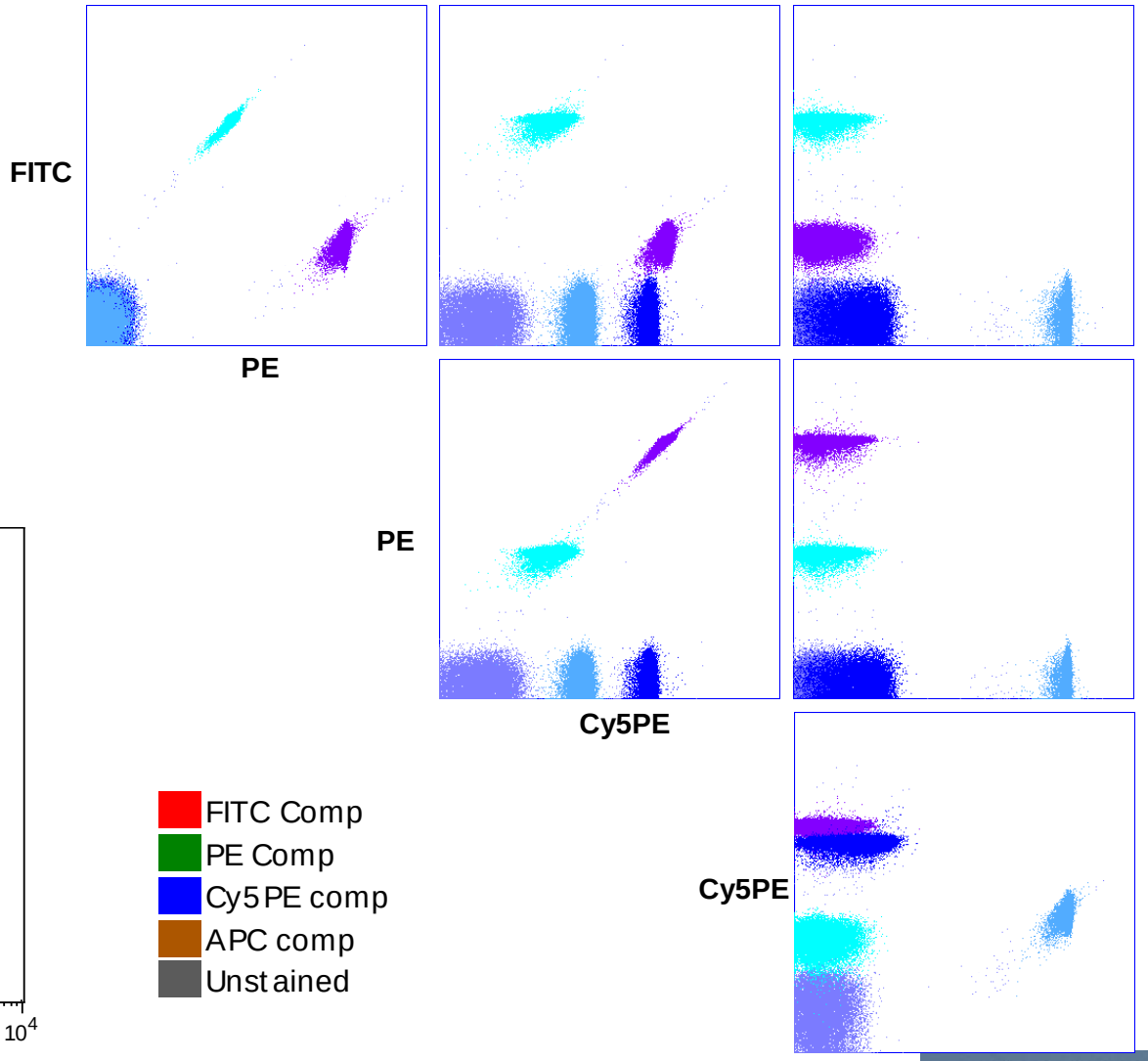




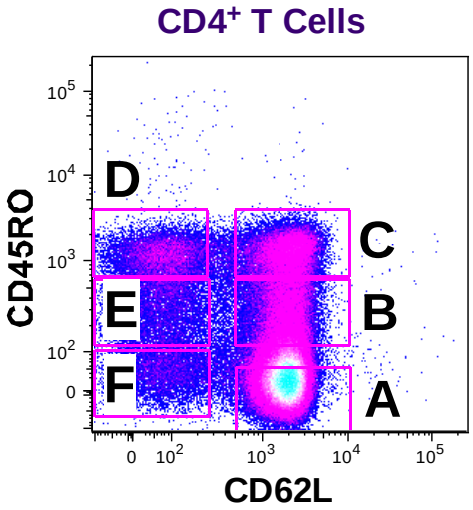
Using Beads to Compensate

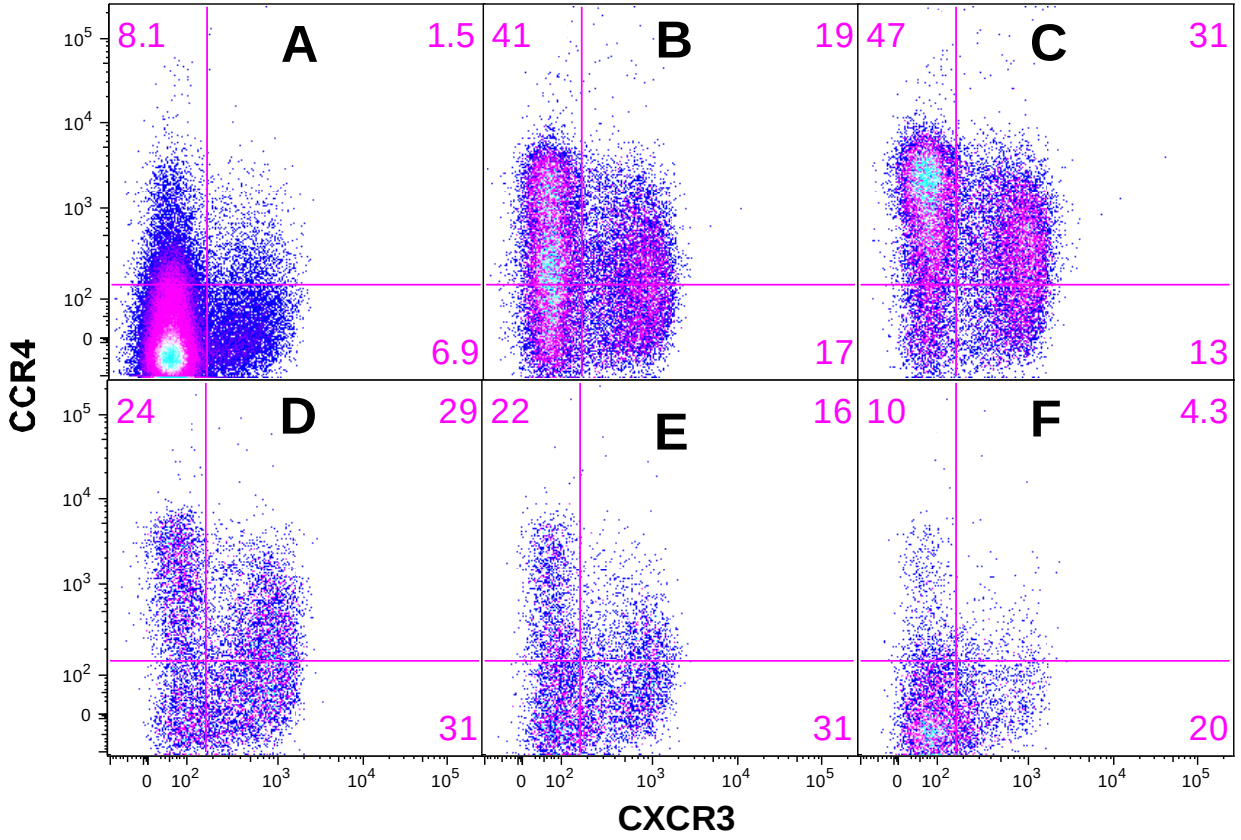
Gate on "Singlets"; then gate on single-stained beads.





Result

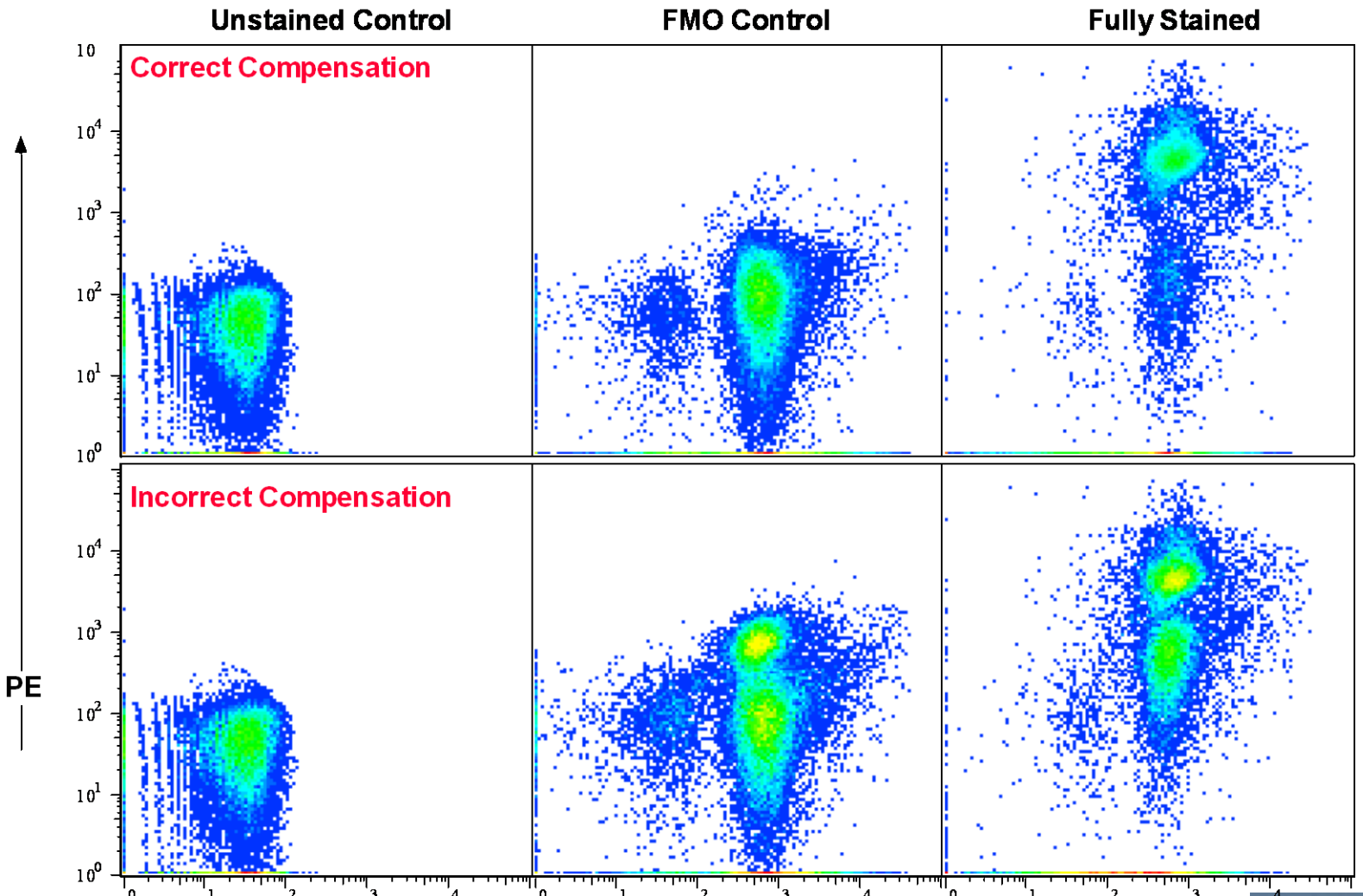




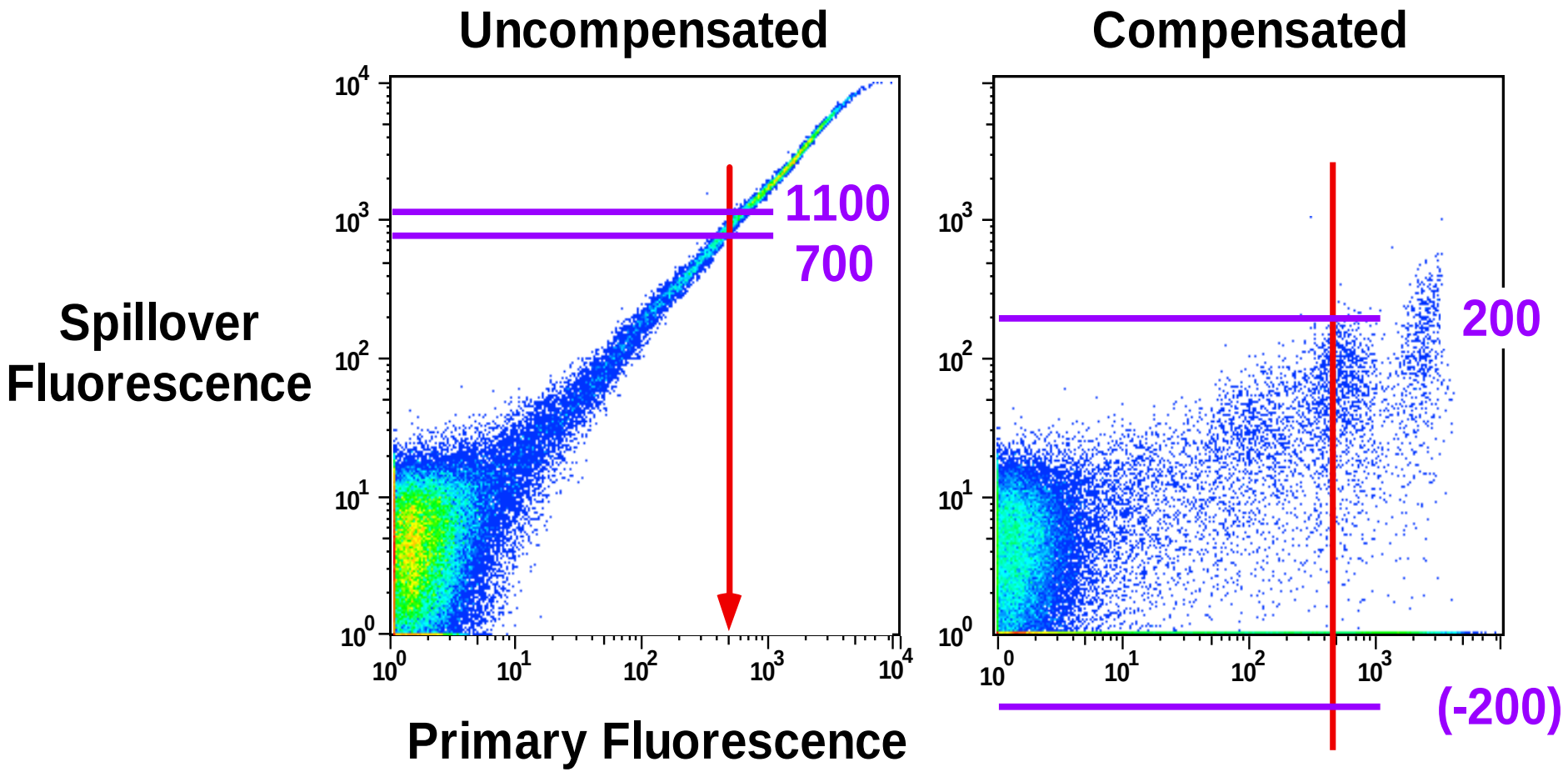
Final panel worked very well--in fact, identified expression of CCR4 not previously seen on FACSCalibur!

Complex Interactions in Compensation

The same data is shown with correct or wrong Cy5PE->Cy7PE comp setting. Note that neither of these channels is shown here!



Imperfect Measurement Leads to Apparent Spread in Compensation



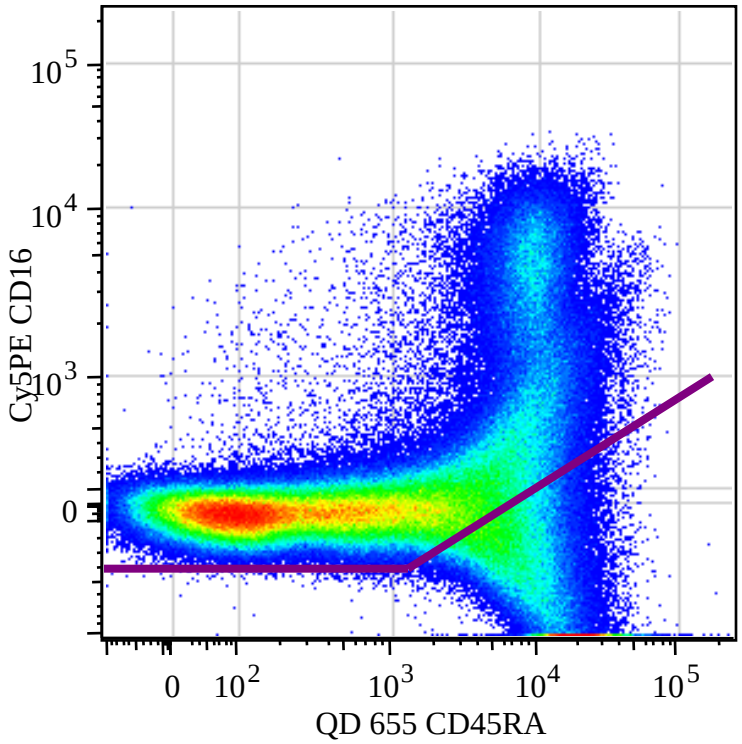
Why is there a 400-unit spread? Photon counting statistics.

Selection of Marker/Color Combinations (2)

All colors are not created equal.

The same monoclonal antibody conjugated to FITC, PE, Cy5PE, APC, Cy7APC can show apparently different distributions on multiply-stained cells.

This is due to spectral-spillover,
and the propagation of the
error in those measurements.



Selection of Marker/Color Combinations (2)

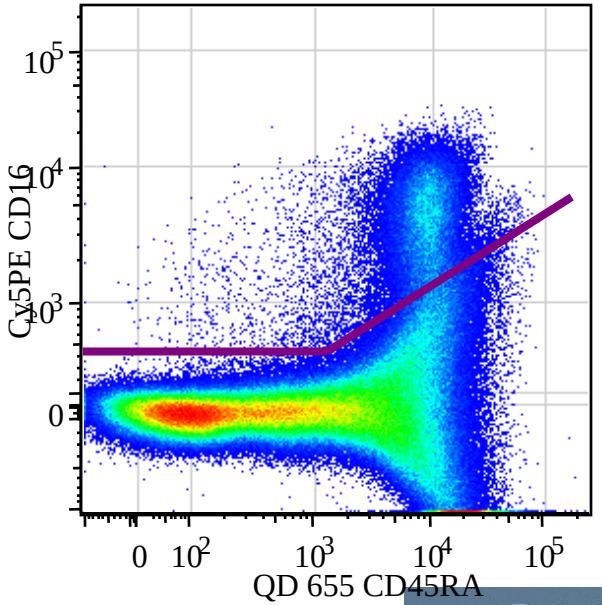
Prediction of the spillover effect is very difficult. You need to know three different aspects:

(1) The brightness of the *other* reagents in your panel

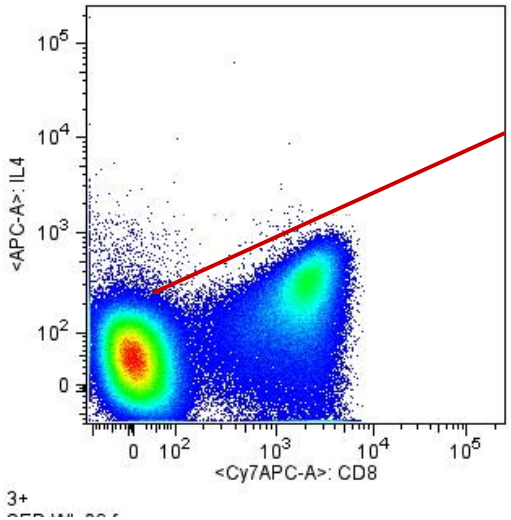
(2) The spillover of these reagents into your channel

(3) The absolute brightness of every measurement

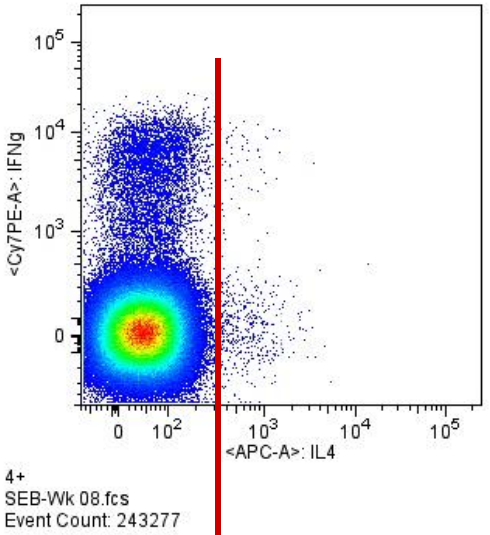
Amount of spread in your measurement channel is equal to the sum of all other reagents' brightnesses multiplied by their spillover coefficient and by the inverse square root of the absolute brightness….

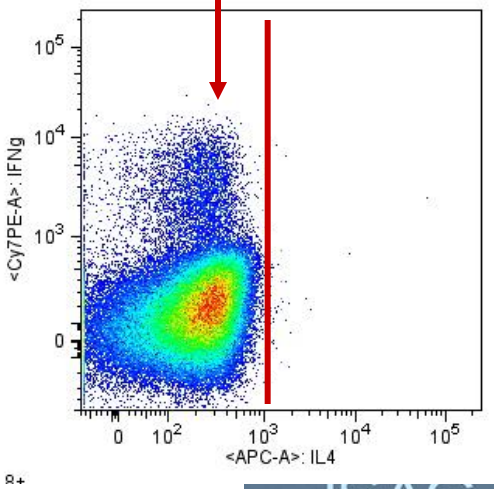


Fix/Perm Changes Cy7APC Compensation Requirement



The longer Cy7APC is in fixative, the more it "falls apart", leading to more APC compensation

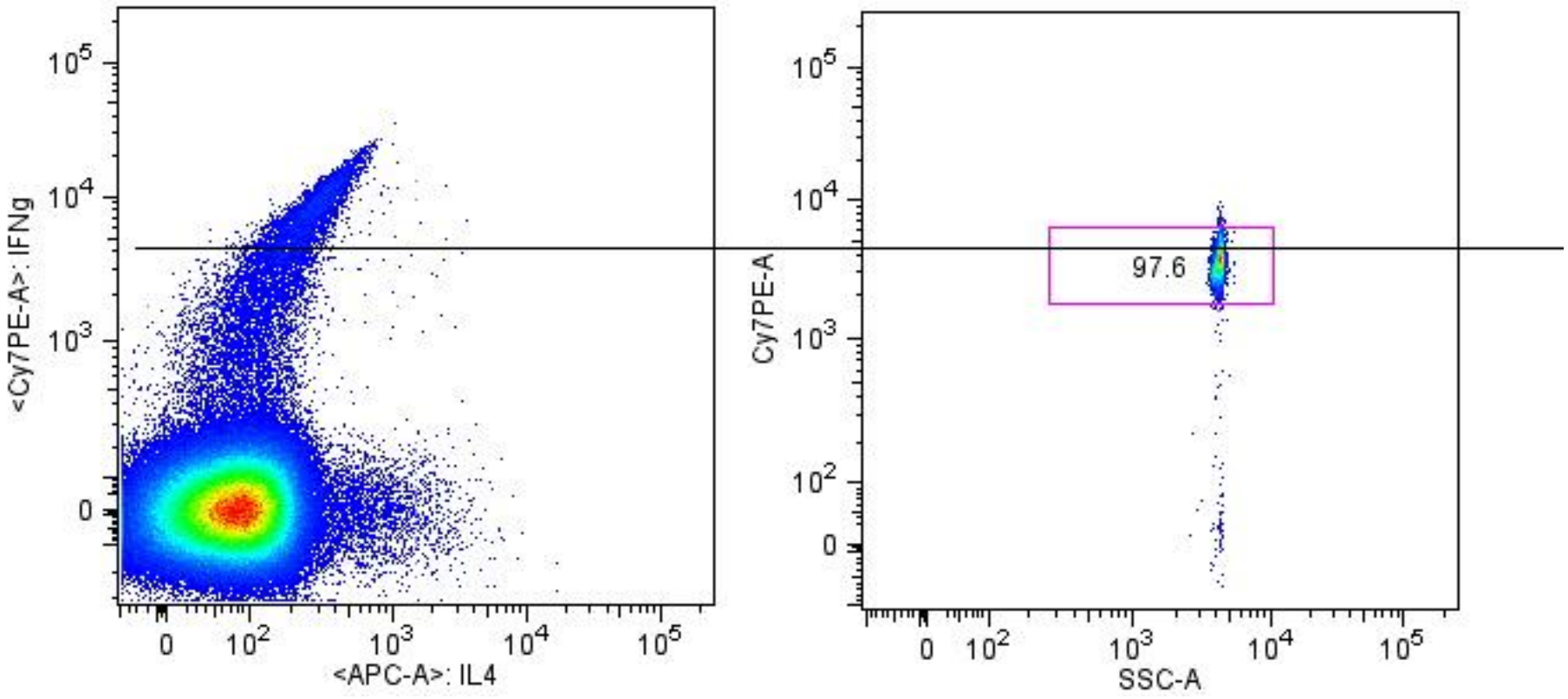




Note that this exacerbates the higher "IL4+" gate required for CD8 cells.

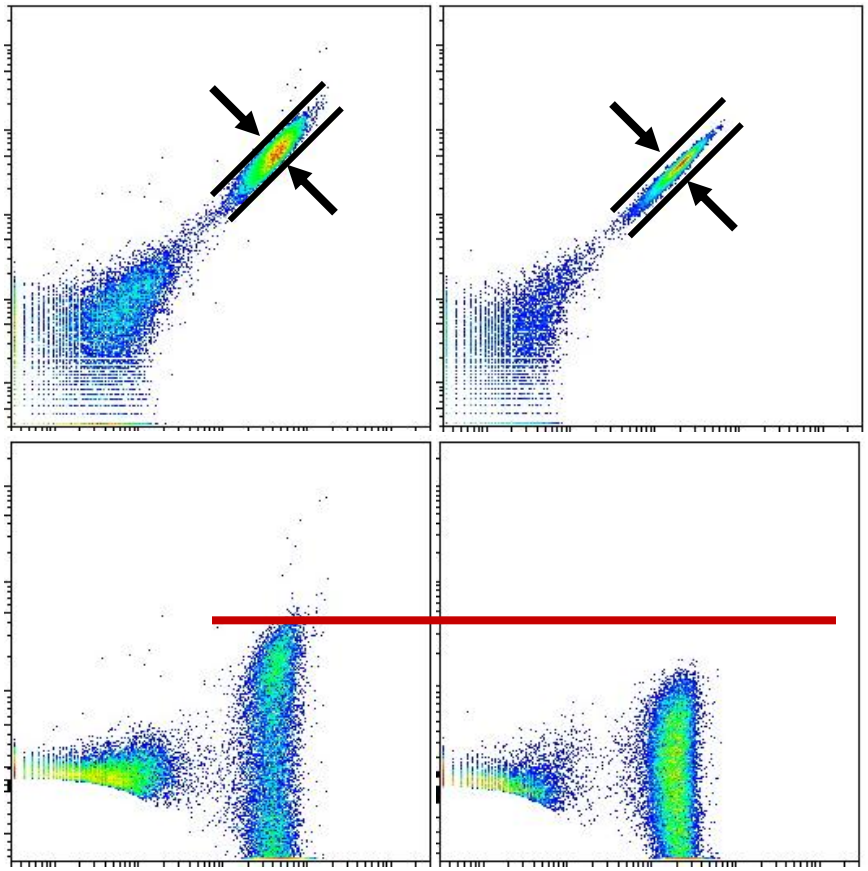
The undercompensation would not have been detected except by looking at the APC vs. Cy7APC graphic…

Insufficiently-Bright Comp Control Is …. Bad!



Note that either under- *or* over-compensation can result from using comp controls that are too dim!

Good Instrument Alignment Is Critical!



While the amount of compensation did not differ, the measurement error (correlation) decreased leading to much better visualization of the population!

Compensation for more colors: It's not just pretty pictures

- **Spillover from unviewed measurement channel can alter event positions– without obvious visual evidence (no diagnostic diagonals!)**
- **Thus, gate positions may depend on unviewed measurement channels and be different for various tubes in a panel**
- **Separation of populations may require multi-dimensional surfaces.**

Using Beads to Compensate

- Antibody-capture beads
- Use reagent in use
- Lots positive
- Small CV, bright
- Sonicate
- Some reagents won't work (IgL, non mouse, too dim, EMA/PI)--mix with regular comps

Final Panels

Based on the evaluation of the first sets of panels, certain combinations were eliminated. The good aspects of other combinations were combined and fine-tuned.

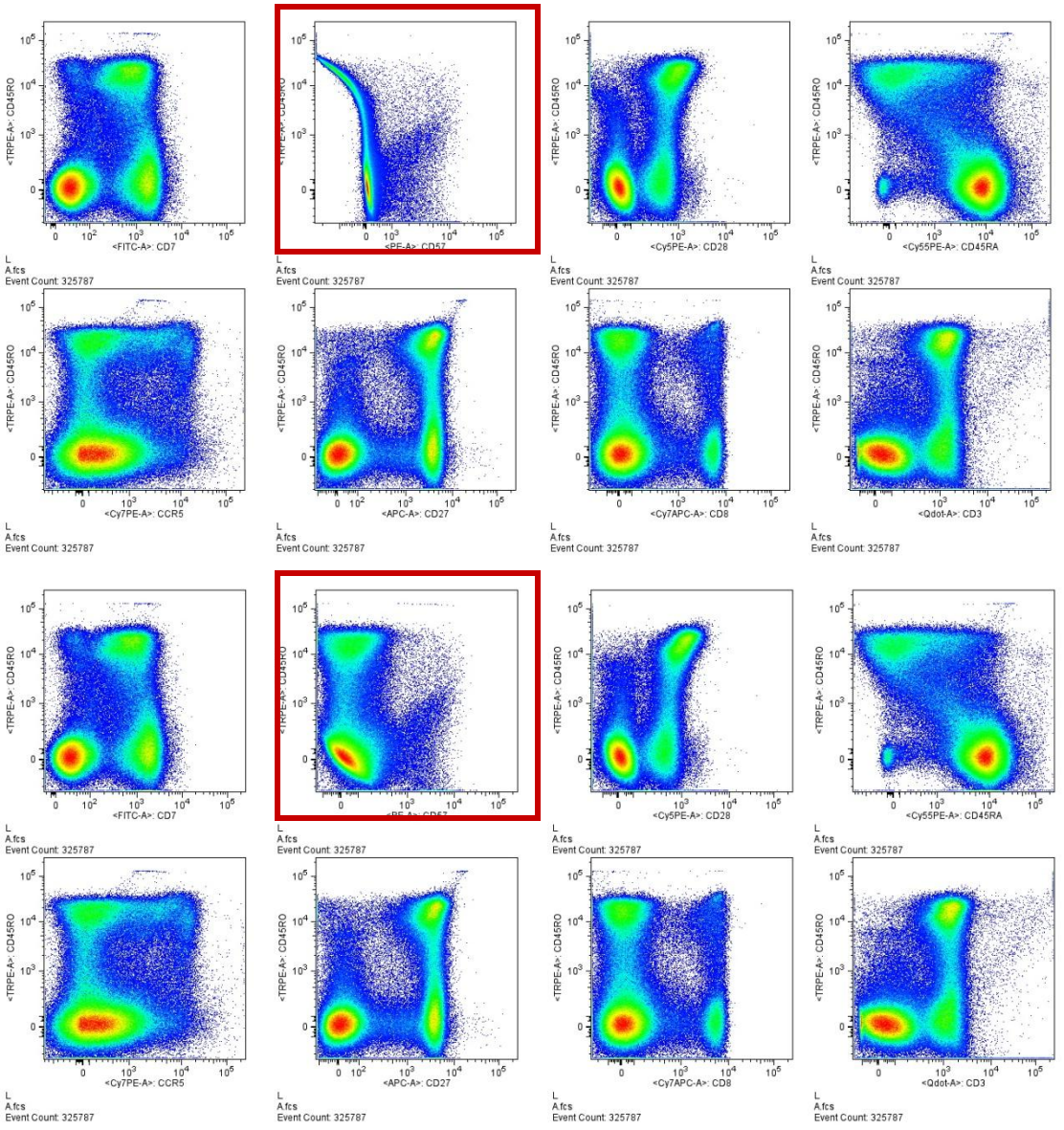
	TRPE	Cy5PE	Cy55PE	Cy7PE	APC	Cy55APC Ax680	Cy7APC	CB	QD655
1						CD62L	CD4	CD45RO	CD45RA
2	CD45RO	CD3		CD62L	CD28	CD11a	CD4	CD27	CD45RA
3	CD45RO				CD27	CD4	CD11a	CD62L	CD45RA

Note: CD3 was dropped from 1 & 3 as CD4 staining was deemed good enough to identify CD4 T cells.
Panel 2 will validate this assertion!
Panels 2 & 3 add more memory markers to verify the final phenotype of the chemokine-expressing cells.

Compensating with the wrong TRPE

Wrong TR-PE comp control

Right TR-PE comp control



Some Examples of Problems

- The following four examples illustrate some types of problems that can be occur related to compensation.
- In each case, compensation itself is not the problem: there is an underlying reagent, instrumentation, or analysis problem.
- However, the manifestation of this problem is an apparent incorrect compensation!

Design of panels

Since optimal sensitivity was desired, I tried to minimize reagents that would have spillover-spreading into FITC and PE.

Optimal separation of CD62L and CD45Rx was required.

Other memory markers were less important: therefore, some panels were designed to test minimal requirements, and others were part of the “wish list”.

Why do we need OMIPs?

Developing a multicolor panel is extremely labor-intensive.

The complexity increases geometrically with number of colors… it often takes us 4 months to develop a 12-14 color panel.

Publishing these panels accomplishes two goals:

(1) Sharing the panel for others to use, adapt, or build upon

(2) Providing a mechanism by which recognition for panel development is achieved (attribution by citation)

OMIPs: OPTIMIZED

The **key part** of OMIPs is the optimization.

Without optimization, there is no intellectual contribution, nor is their evidence that the panel should not be improved!

Optimization includes:

- Comparing as **many variations** of each reagent as possible (choosing the best – and why!)
- Comparing variations of combinations of reagents
- Showing that each reagent is optimal (titration!)

Lack of optimization demonstration is the most frequent reason for rejection!

Example OMIP (OMIP-001)

Print portion

Purpose and Appropriate Sample Types

The present panel was optimized for the evaluation of $CD4^+$ and $CD8^+$ T-cell responses to various HIV-1–derived peptide pools in peripheral blood mononuclear cells (PBMC) from HIV-1^+ individuals with differences in clinical progression. It works well with cryopreserved PBMC, and we have observed similar results with fresh specimens. Other tissue types have not been tested.

Example OMIP (OMIP-001)

Print portion

Background

The approach used for the development of this panel has been described in detail (1). Briefly, a large number of Ab-conjugates were screened for each antigen of interest, as available, to select those Ab-conjugates providing best detection. As the focus of the panel was the detection of cytokine-producing T-cells, the brightest fluorochromes were used for interleukin-2 (IL-2), interferon (IFN)-γ, and tumor necrosis factor

Example OMIP (OMIP-001)

Online material

Required tables include:

- **Instrument configuration** (lasers/optics)
- **Commercial reagents** (fluorochrome, vendor, clone, catalog number, dilution, staining conditions)
- **In-house** synthesized **reagents** (no proprietary materials)

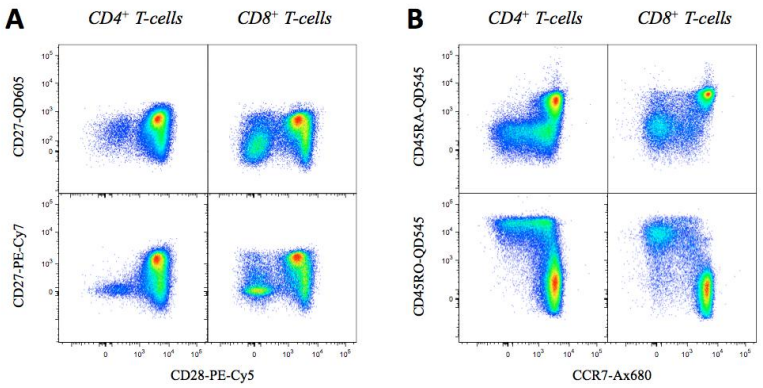
Example OMIP (OMIP-001)

Online material

Development strategy: how many (which) reagents were tested. Why was each chosen or eliminated?

Specificity	# reagents screened	# fluorochromes tested	# reagents tested in panels
IFN-γ	12	9	2
IL-2	10	6	4
TNF-α	6	6	3
CD3	29	15	5
CD4	37	21	5
CD8	63	31	2
CCR7	17	11	5
CD27	14	12	6
CD28	12	8	1
CD45RO	1	1	1
CD57	5	5	4
CD127	8	7	4
PD-1	6	5	2
CD14	1	1	1
CD19	1	1	1
Dead cells	3	3	2
Biotin	8	8	2

Priority rating	Category	Rationale for priority level	Reagents concerned
1	Cytokines	Reason for development of panel	IFN-γ, IL-2, TNF-α
2	Low density molecules	Not many good reagents available	CCR7, PD-1
3	Dump channel	Select 3 reagents for one detector	CD14, CD19, ViViD
4	T-cell subset markers	Identify cells of interest for analysis of cytokine production	CD3, CD4, CD8
5	T-cell activation	Determine activation state of cytokine producing cells	CD27, CD28, CD45RO
6	Luxury markers	Interesting to include if good reagents are available for the remaining detectors	CD57, CD127



Online Fig.2 Sample illustrations for choice of reagents. **A** CD27-PE-Cy7 was chosen as it provided better separation of $CD27^{neg}$, $CD27^{int}$ and $CD27^{hi}$ cells. **B** CD45RO gave a more defined separation of naïve and memory T-cells than CD45RA. Dot plots show CD3-gated live cells from healthy donor PBMC. All samples were stained with 14-colour panels, including reagents for cytokine-detection.

Example OMIP (OMIP-001)

Online material

Detailed staining protocol

STAINING PROTOCOL

Commercial materials:

BD Cytofix/Cytoperm Fixation/Permeabilisation Kit (BD Biosciences, San Diego, CA)
Brefeldin A (GolgiPlug; BD Biosciences)
FCS (Gibo, Carlsbad, CA)
Monensin (Golgistop; BD Biosciences)
Penicillin (Sigma, St. Louis, MO)
RPMI 1640 with phenol red (Gibco)
RPMI 1640 without phenol red (Gibco)
Sodium azide (Sigma)
Streptomycin (Sigma)

In-house media:

Culture medium	RPMI 1640 with phenol red 10 % FCS 100 IU/ml penicillin 100 µg/ml streptomycin
Staining medium	RPMI 1640 without phenol red 4% FCS 0.02% sodium azide

Note: cells are pelleted in a desk-top centrifuge with a 25 cm diameter rotor, while reagent

The Future of OMIPs

The collection of published OMIPs will provide a valuable resource for development of new panels.

Using an existing OMIP gives you an assurance that the panel is likely to work well on your instrument.

OMIPs are “living” – as new reagents come about, we expect to update the online portion of OMIPs so as to always have the most recent optimized version available.

OMIPs provide a mechanism to credit the huge amount of work that goes into a panel.

Advantage of More-Than-Minimal Markers

Two extremes of gating strategy:

"**Conservative**" - drawn to be very "tight" around the visually-defined populations

- Greatest purity of subset
- Lowest sensitivity

"**Liberal**" - drawn to include much larger areas than visually appear to belong to a subset.

- Greatest sensitivity
- Greatest chance of contamination

BUT: multiple rounds of "Liberal" gating based on multiple parameters results in excellent purity and sensitivity.

LIFE-Study

LIFE - Leipzig Research Center for Civilization Diseases

Aims: - to explain the causes of widespread common diseases (metabolic & cardiovascular diseases, heart attack, diabetes, depression, dementia, head- and neck cancer, allergies …)
- Identification of risk factors
- Establishment of effective forms of prevention and early diagnosis
-Improvement of German healthcare

Methods: Complex medical analysis and questionnaires
Leipzig population 531.800 (12/2011) with a density of 1,787/km^2

LIFE-study 26.500 (5 % of population)

Cytomics for LIFE

cytometric analysis of 1200 EDTA-anticoagulated fresh blood samples over a 3 year period

complex antibody panel with 13 fluorescent antibodies on 10 colours

immunophenotyping: differentiation of over 30 leukocyte subpopulations and activity

Calculation of reference intervals for leukocyte subpopulations for adults (20-80 years)

Correlation of biological variability with lifestyle and diseases