Multiparameter Flow Cytometry Immunophenotyping Course

Holden Maecker, Maria Jaimes, Caroline Duault

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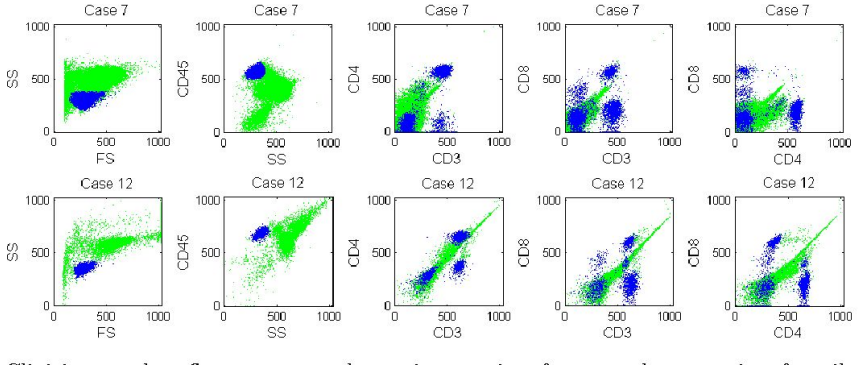
Federation of Clinical Immunology Societies



What's so great about flow cytometry?

"Cells are the quanta of biology"

-Mark Davis

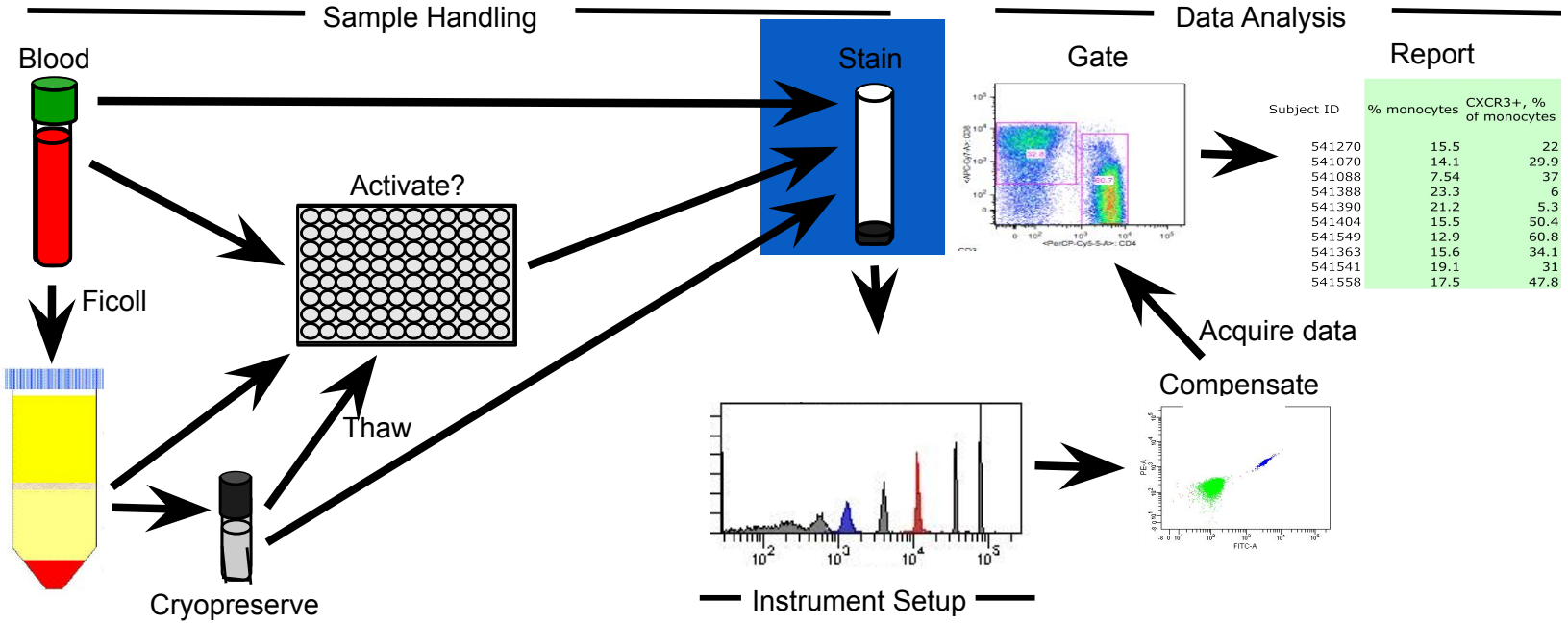
What's so great about flow cytometry?

- The analysis of multiple parameters on many individual cells
  - 40+ colors in fluorescence flow cytometry or 50+ parameters in mass cytometry (CyTOF)
  - Up to 10^6 cells or more per sample

Allows detailed phenotypic and functional analysis of rare cell populations

Set voltage gains & acquisition parameters

Where are the sources of variability?



Maecker et al., 2010

Course Outline

1. **Pre-analytical variables/sample processing (1:00-1:40)**
   a. Sample collection and transport
   b. PBMC isolation or other sample processing
   c. Stimulation/functional assays
2. **Experimental design (1:40-2:45)**
   a. The instrument: lasers, filters, conventional vs. spectral
   b. Panel building and testing: fluorochromes, spillover
   c. Batching
   d. Controls

   Break (2:45-3:00)
3. **Instrument setup and acquisition (3:00-3:30)**
   a. Standardizing instrument setup, inter-instrument qualification
   b. Acquisition variables: # of events, speed, collection criteria
4. **Data analysis (3:30-4:30)**
   a. Quality control steps
   b. Manual and automated analysis

Holden Maecker

Maria Jaimes

Caroline Duault

Course Outline

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   a. Sample collection and transport
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2. **Experimental design (1:40-2:45)** — Maria Jaimes
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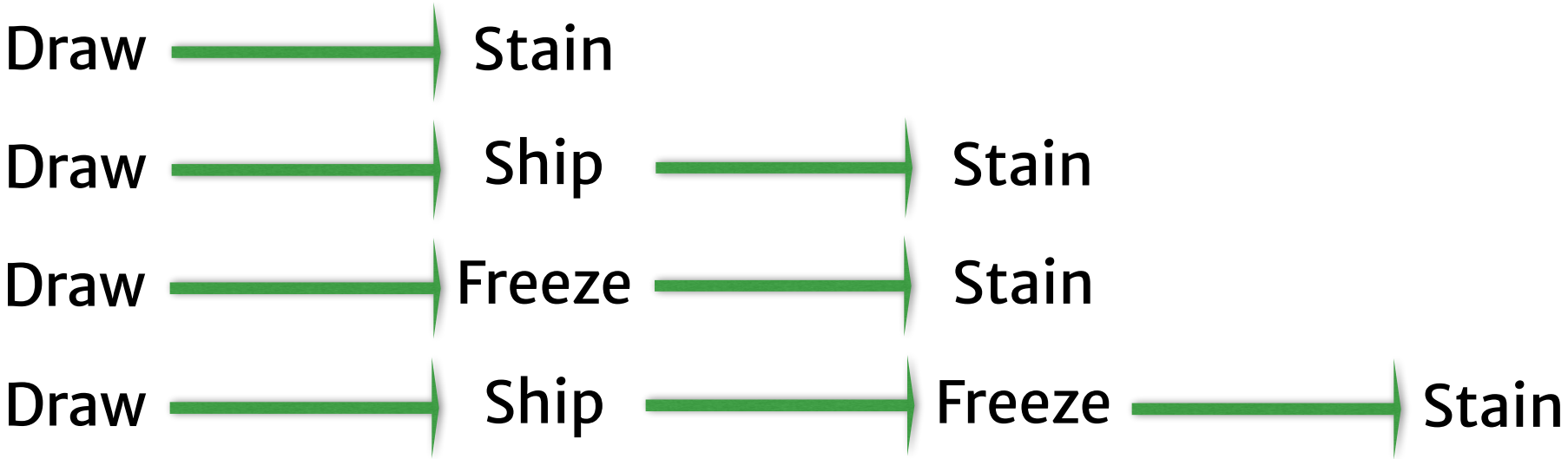
3. **Instrument setup and acquisition (3:00-3:30)** — Caroline Duault
   a. Standardizing instrument setup, inter-instrument qualification
   b. Acquisition variables: # of events, speed, collection criteria
4. **Data analysis (3:30-4:30)**
   a. Quality control steps
   b. Manual and automated analysis

Sample handling

- How long can blood wait to be processed?
- Cryopreservation versus fresh staining



Sample handling

- Type of assay
  - Phenotype versus function (functional assays more sensitive)
  - APC function required (processing of protein antigens is very sensitive to cryopreservation)

- Markers to be assessed
  - CD62L: lost upon storage or cryopreservation
  - Activation markers: may be induced with storage (CD69)

PBMC Isolation and Cryopreservation

- CPT vs. SepMate vs. traditional Ficoll
- Centrifugation time and speed
- Cell recovery and counting
- Freezing medium
- Method of controlled rate freezing
  - How long before transfer to LN2
- Cycles of LN2 -> dry ice -> LN2
- Thawing protocol

Fixing Samples Prior to Staining

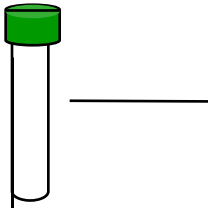
- CytoCHEX
- PROT1 (Smart Tube, Inc.)
- BD FACS Lysing Solution
- Whole Blood Cell Stabiliser (Cytodelics)

No more functional assays…
Need to know stability of epitopes targeted…

Phospho-Flow Assays

- Measure phosphorylation events in very short-term stimulated whole blood, PBMC, etc.
- Can measure multiple cell-surface and intracellular markers in combination, using multiparameter flow cytometry
- Can detect signaling events through T cell receptors, surface Ig, cytokines, etc.
- May detect signaling defects in aging or immune-mediated diseases

Principle of Phospho-Flow



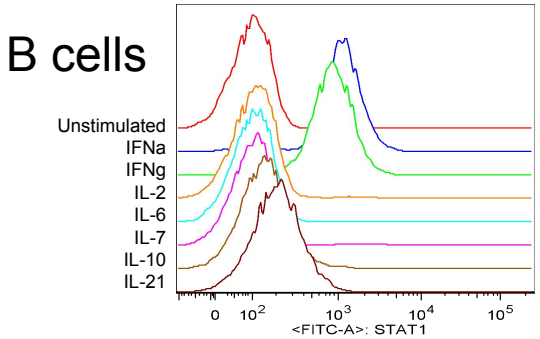
PBMC or whole blood

stimulate

Incubate 2-30 min

- Fix cells
- Permeabilize
- Stain

Gate on cells of interest

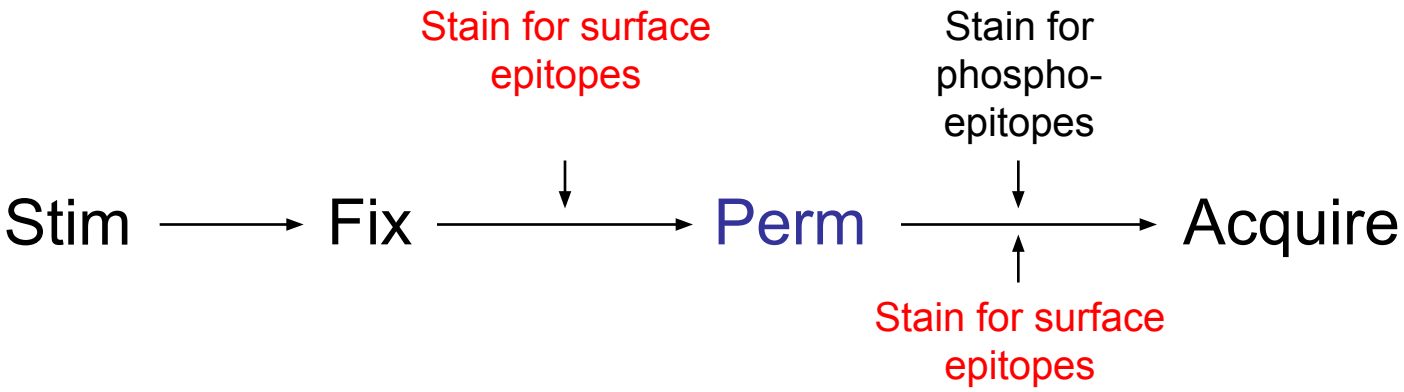


Phospho-flow: key variables

- **Stimulation**
  - Whole blood versus fresh or cryopreserved PBMC
  - Resting before activation
  - Stimulation time
- **Processing and staining**
  - Method of fixation/permeabilization*
  - Staining pre- or post-permeabilization*
  - Choice of fluorochromes*
  - Staining time, temperature, and agitation
  - Number of washes
- **Acquisition and analysis**
  - Reporting of results (percentile, fold-change, normalization)

How to perm, when to stain?

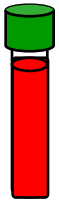
- Methanol destroys most cell-surface epitopes, as well as organic dyes (PE, APC, PerCP, etc.)
- Intra-nuclear staining (e.g., pSTAT proteins) is inefficient without harsh permeabilization



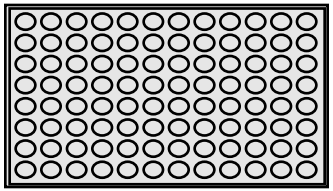
ICS Assays

- Measure production of cytokines in short-term stimulated whole blood, PBMC, etc.
- Can measure multiple cell-surface and intracellular markers in combination, using multiparameter flow cytometry
- Can detect rare events such as antigen-specific T cells
- Useful in vaccine monitoring, transplantation, etc.

ICS Assay Protocol

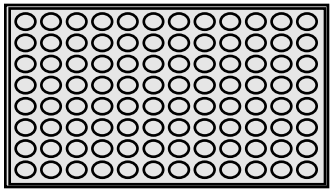


PBMC or whole blood



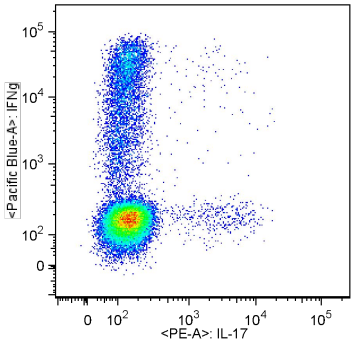
Antigenic stimulus + brefeldin A

Incubate 6-24 h



- Fix cells
- Permeabilize
- Stain

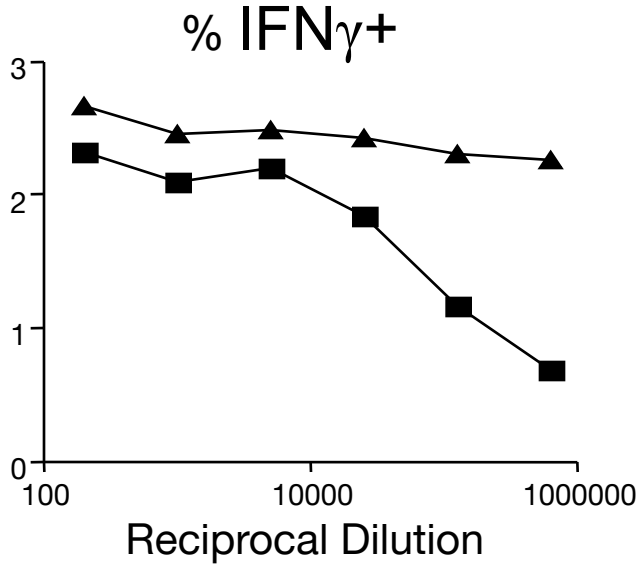
Gate on cells of interest

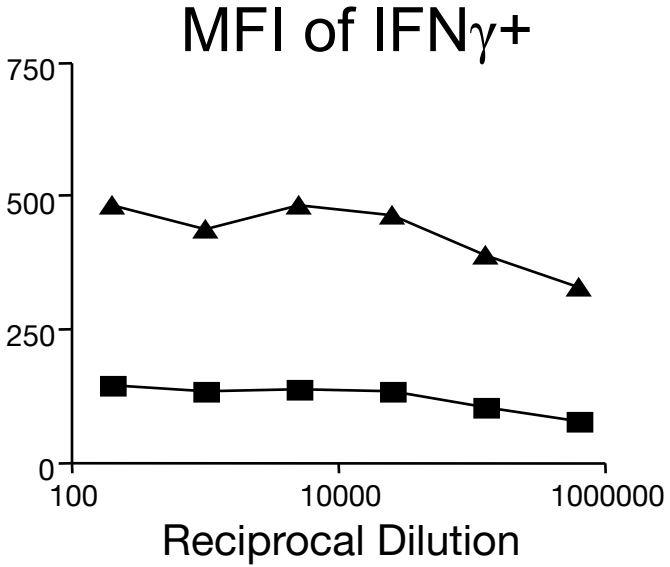


ICS assays: key variables

- **Stimulation**
  - Whole blood vs. fresh or cryopreserved PBMC
  - Resting PBMC before activation*
  - Costimulatory Abs (CD28+CD49d)
  - Stimulation time*
  - Brefeldin A and/or monensin*
- **Processing and staining**
  - Method of fixation/permeabilization
  - Surface vs. intracellular staining for CD3, CD4, CD8, etc.*
  - Staining time, temperature, and agitation*
  - Number of washes (post-perm and post-stain)
- **Acquisition and analysis**
  - Number of events to collect*
  - Gating*
  - Determining a positive response and/or significant differences

Effect of overnight rest on cryo PBMC

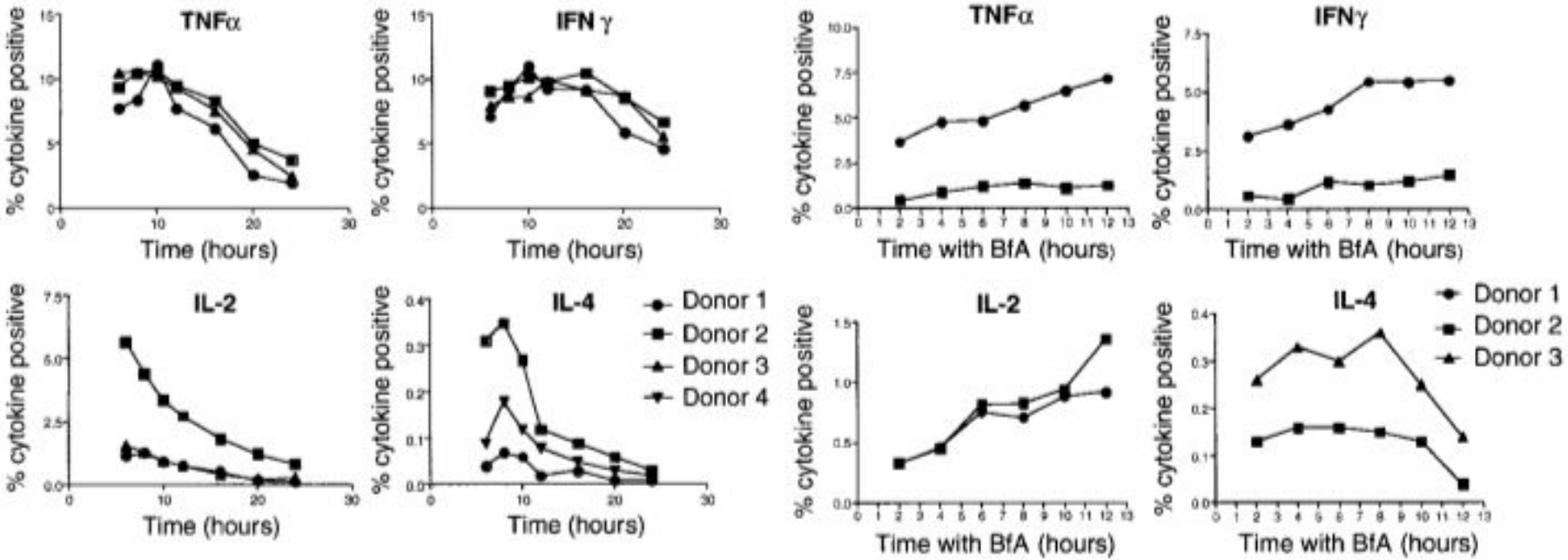




No rest, 6 h peptide

Overnight rest, 6 h peptide stimulation

Kinetics of cytokine production



Nomura et al., 2000, Cytometry 40: 60

Monensin vs. Brefeldin Summary

- Brefeldin A preferred (10 μg/mL):
  - TNFα, IFNγ, IL-2, IL-4, IL-12
- When combining any of the above with CD107, IL-17, TGFβ, or IL-10:
  - 5 μg/mL monensin + 5 μg/mL brefeldin A

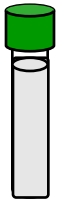
Number of events to acquire

		# events to collect	
% bkgd	lowest % positive	90% power, p<0.05	99% power, p<0.005
0.01	0.02	260,000	720,000
0.01	0.05	32,000	90,000
0.01	0.1	12,000	32,000
0.02	0.05	67,000	190,000
0.02	0.1	16,000	45,000
0.03	0.05	170,000	480,000
0.03	0.1	23,000	63,000
0.04	0.1	33,000	93,000
0.05	0.1	52,000	140,000
0.06	0.1	86,000	240,000
0.07	0.1	160,000	450,000
0.08	0.2	17,000	46,000
0.1	0.2	26,000	72,000

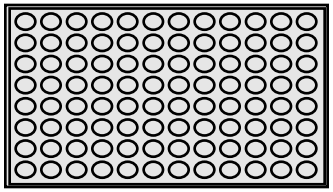
Proliferation Assays

- Measure proliferation in longer-term stimulated PBMC or other cells.
- Can measure multiple cell-surface and intracellular markers in combination, using multiparameter flow cytometry
- Can detect defects in T cell responsiveness that may not be apparent in short-term assays.
- Useful for vaccine monitoring, transplantation, etc.

Proliferation Assays: CFSE vs BrdU

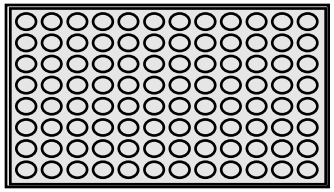


Label cells with CFSE

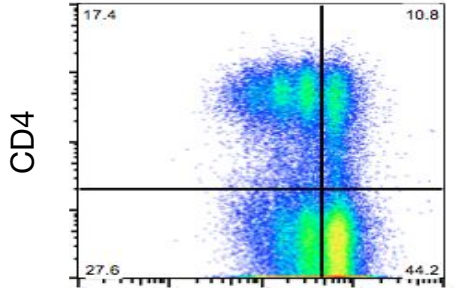


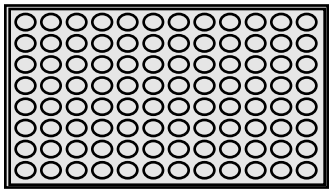
Add stimulus

Incubate 3-7 days



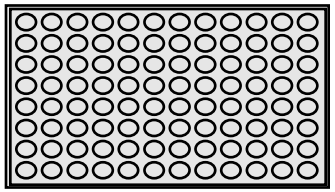
Stain for cell surface markers



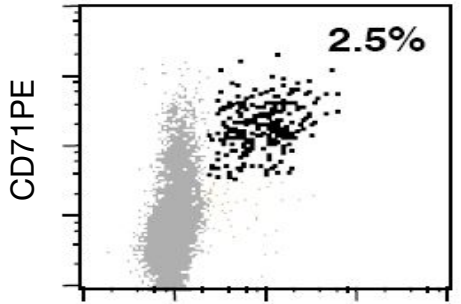


Add stimulus + BrdU

Incubate 3-7 days



- Fix cells
- Permeabilize
- Stain+DNAse



Proliferation assays: key variables

- **Stimulation**
  - Type of activation plate or tube
  - Media additives (FCS or defined media)
  - Initial cell density
  - Days of stimulation
- **Processing and staining**
  - Cell labeling technique (for CFSE)
  - Permeabilization and DNAse treatment (for BrdU)
  - Time of BrdU addition
- **Acquisition and analysis**
  - Use of a live cell dye
  - Gating strategy
  - Determining a positive response and/or significant differences

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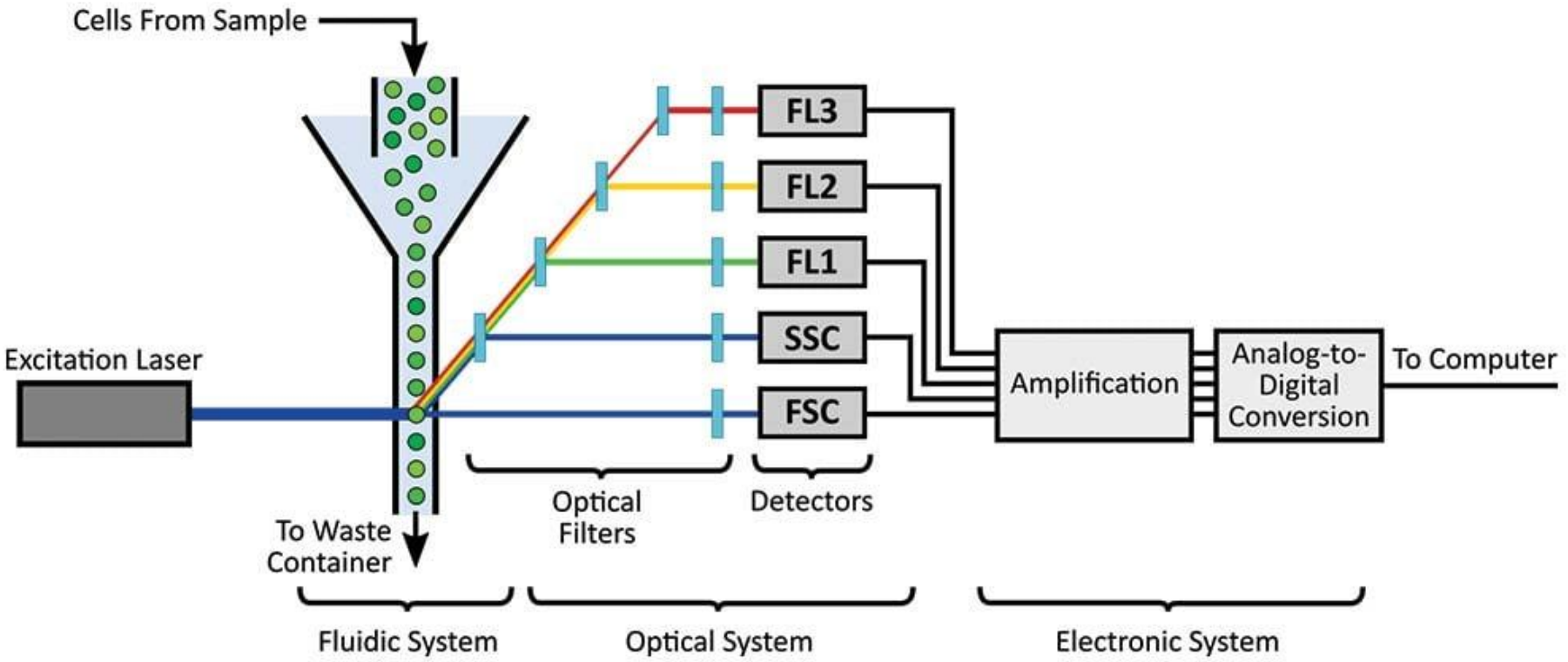


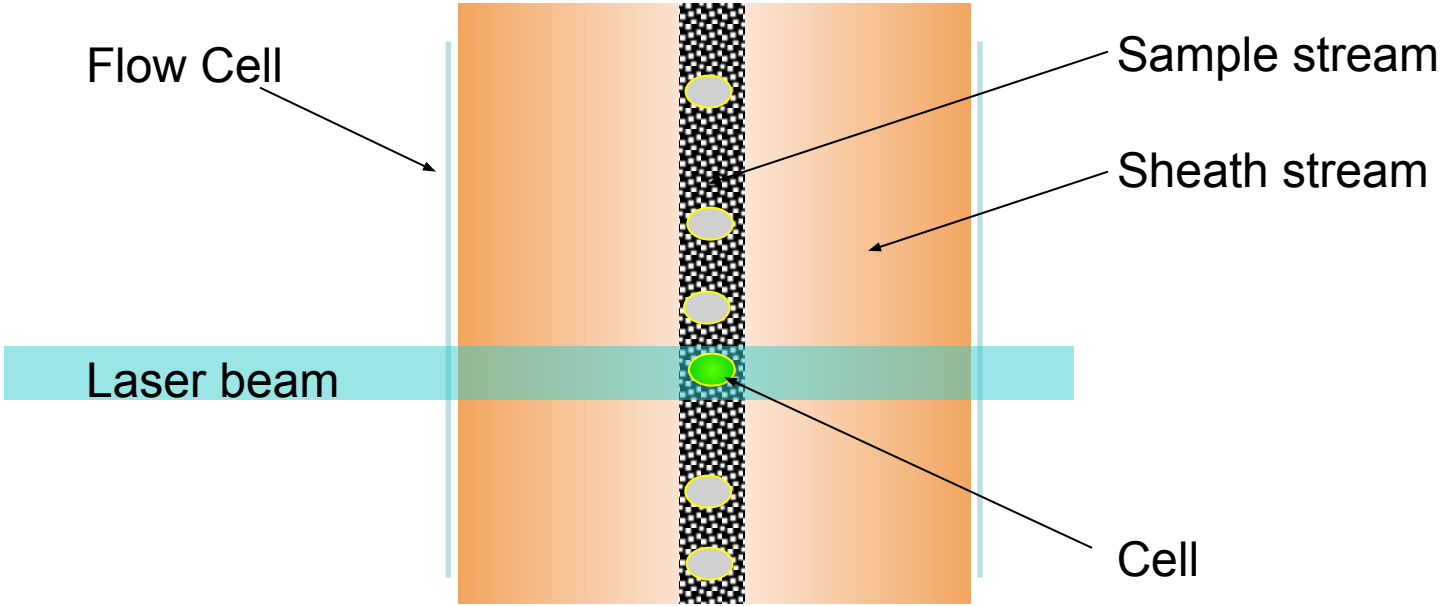
https://microbenotes.com/flow-cytometry/

Major components of a flow cytometer

- Fluidics
  - Sample Intake Port (SIP)
  - Flow cell
  - Sheath and waste reservoirs
- Optics
  - Laser(s)
  - Optical filters
- Electronics
  - Photomultiplier tubes (PMTs) or photodiodes
  - Signal processor

Fluidics

Cytometer fluidics create laminar flow

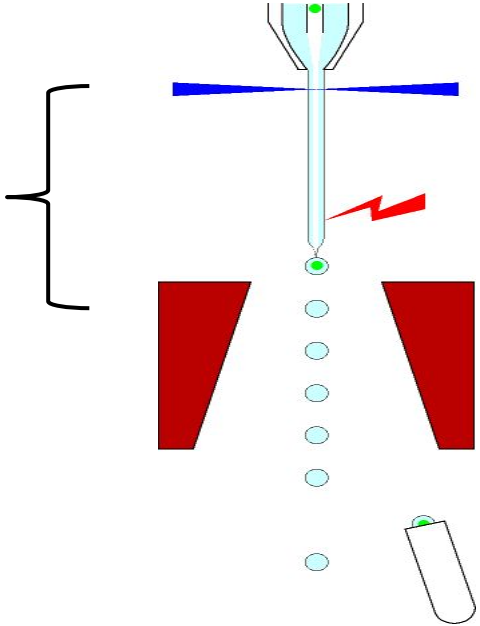


Special cases: Acoustic focusing cytometers, Imaging cytometers, Cell sorters

Cell sorting

Drop delay refers to the time between laser intercept and deflection of the drop containing the identified cell event.

It must be set accurately to sort the correct cells.



Events falling within specified fluorescence and/or scatter gates are sorted via electromagnetic deflection into a receiving vessel (tube or plate).

Instruments may support 2-way, 4-way, or 6-way sorting

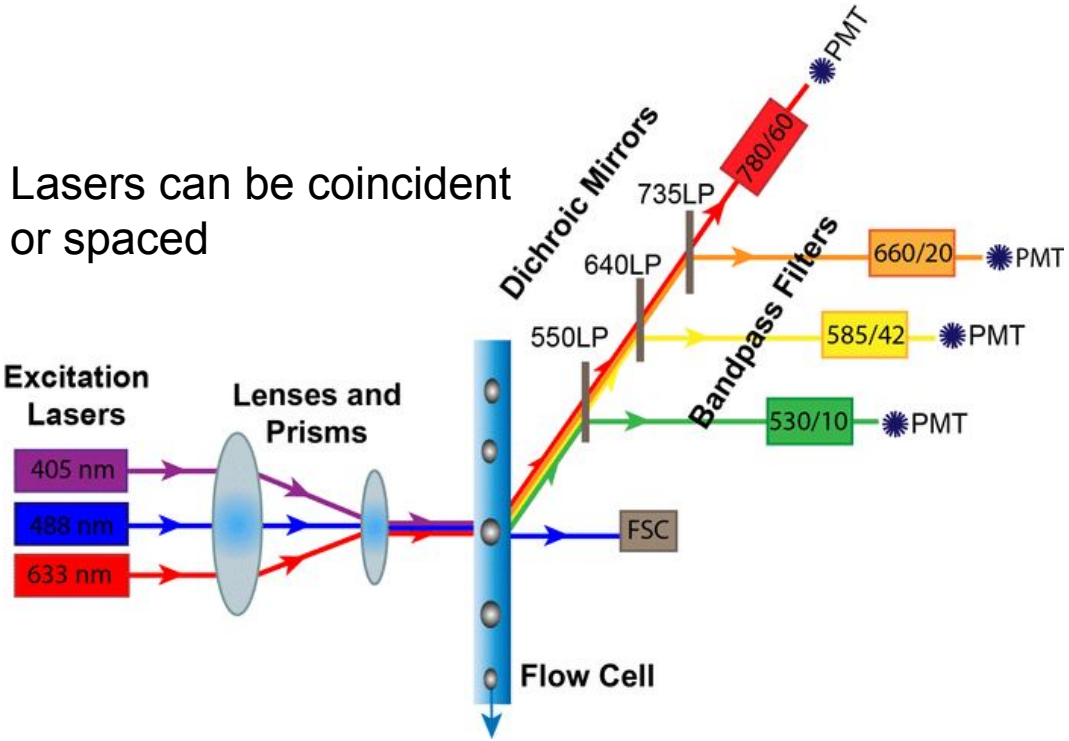
Effect of flow rate

- Higher flow rates mean a broader sample stream (=less precise focusing)
- Less precise focusing means less accurate fluorescence measurement of dim populations (=population spreading)
- Higher flow rates also increase the frequency of coincident events (can be gated out based on FSC area vs. height)
- While flow rates up to 8000-12,000 events per second are possible, this is application-dependent, and rates of 2000-3000 are more common

Optics

Multicolor cytometer configuration

Lasers can be coincident or spaced



Filter Nomenclature Conventions

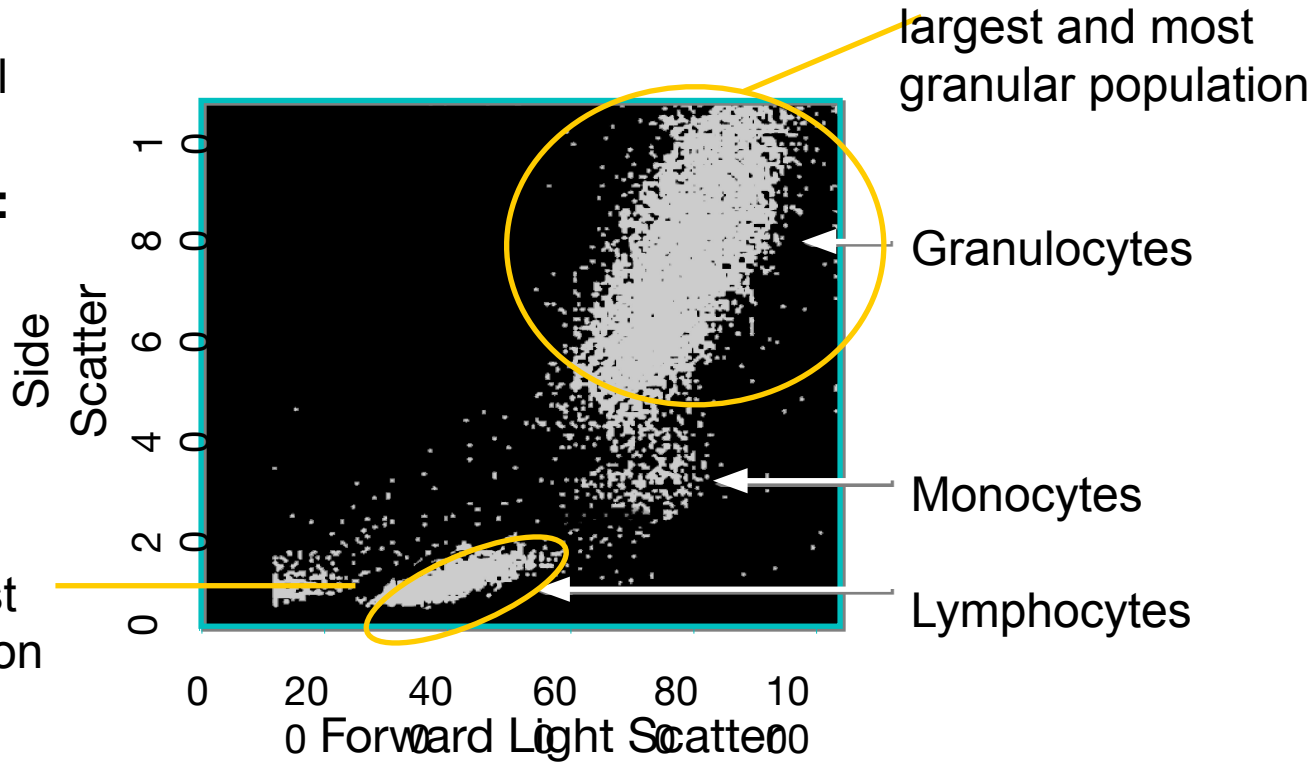
- Longpass filter: allows light above a certain wavelength to pass, reflects shorter wavelengths
  - Example: 505LP = 505 nm longpass
- Bandpass filter: allows light within a certain range of wavelengths to pass (above and below a specified midpoint)
  - Example: 530/30BP = 515-545 nm bandpass

Some common lasers and fluorochromes

Laser	Fluorescent Channel	CytoFLEX Channel Names	Commonly used Fluorescent Dyes
405 nm	450/45 BP	PB450	Pacific Blue™ dye, V450, eFluor™ 450, BV421
	525/40 BP	KO525	Krome Orange, AmCyan, V500, BV510
	610/20 BP	Violet610	BV605, Qdot® 605
	660/10 BP	Violet660	BV650, Qdot® 655
488 nm	525/40 BP	FITC	FITC, Alexa Fluor™ 488, CFSE, Fluo-3
	690/50 BP	PC5.5	PC5.5, PC5, PerCP, PerCP-Cy5.5, PI, DRAQ7™
561 nm	610/20 BP	ECD/mCherry	ECD, mCherry, PE-CF594
	585/42 BP	PE	PE, DsRed
	690/50 BP	PC5.5	PC5.5, PC5, PI, DRAQ7™
	780/60 BP	PC7	PC7, DRAQ7™
638 nm	660/10 BP	APC	APC, Alexa Fluor™ 647, eFluor™ 660, Cy5
	712/25 BP	APC-A700	APC-A700, Alexa Fluor™ 700, Cy5.5, DRAQ7™
	780/60 BP	APC-A750	APC-A750, APC-Cy7, APC-H7, APC- eFluor™ 780, DRAQ7™

Forward and Side Light Scatter

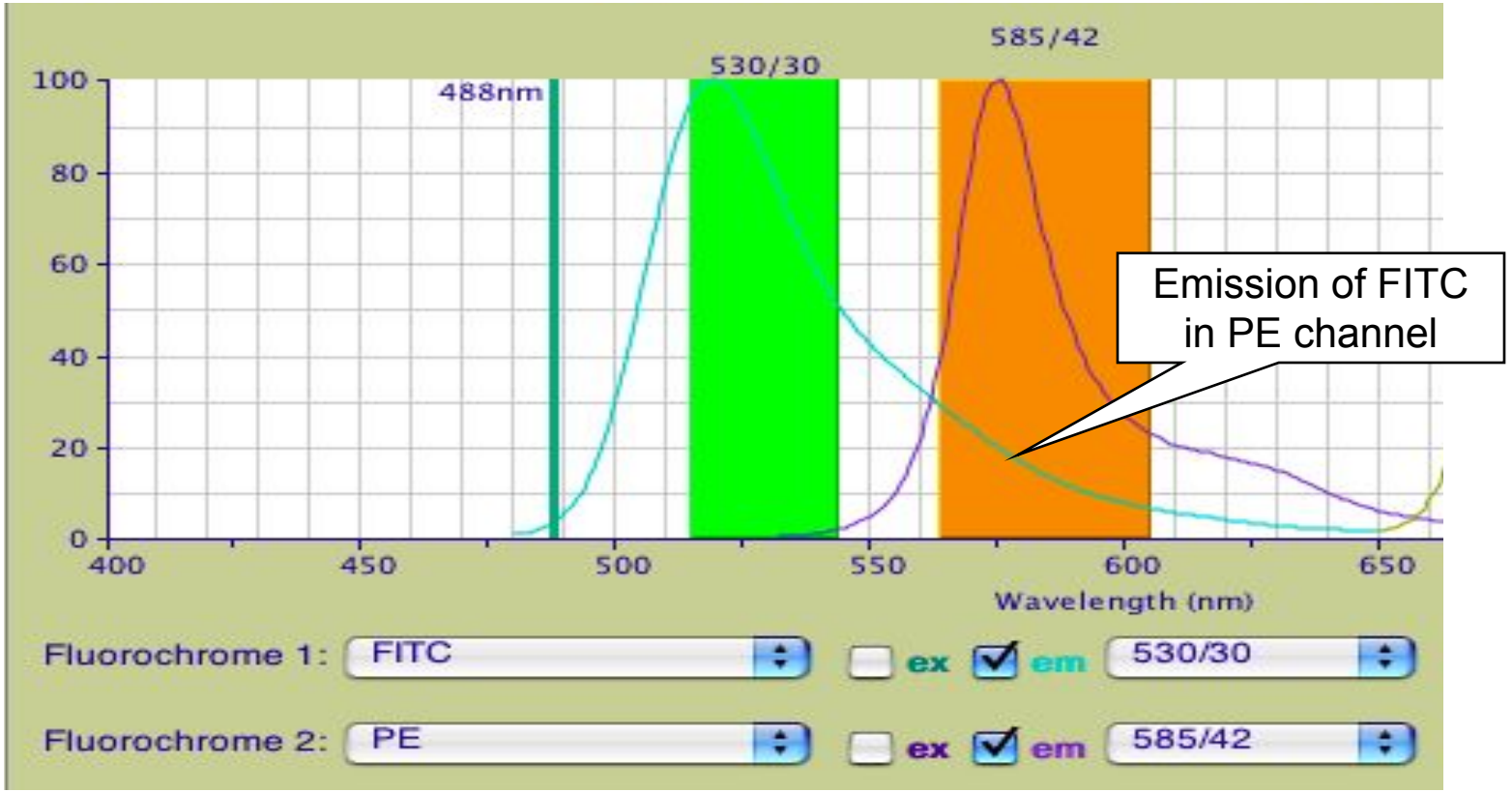
- **Forward scatter (FSC):** Proportional to cell size
- **Side scatter (SSC):** Proportional to cell granularity



Background and autofluorescence

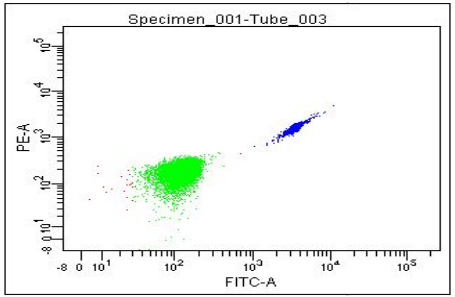
- All cells have a certain level of background fluorescence, due to:
  - Autofluroescence: from pigments and fluorescent moieties on cellular proteins
  - Non-specifically bound antibodies, and free antibody in the sample stream
- The level of autofluorescence varies with the wavelength of excitation and collection:
  - Highest in FITC, PE detectors; lowest in far red (APC, Cy7) detectors

Fluorescence spillover

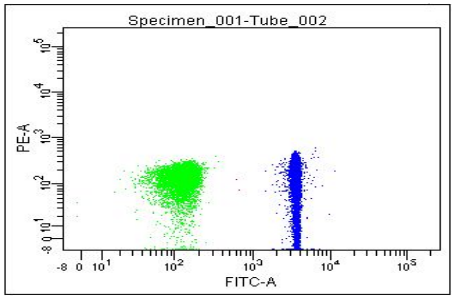


Compensating for spillover

uncompensated



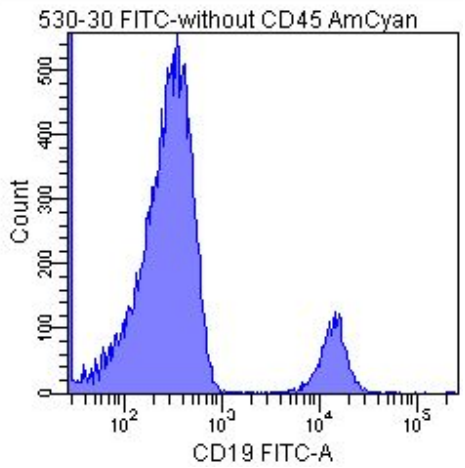
compensated

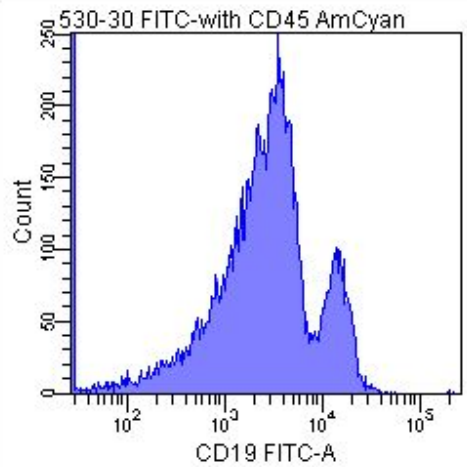


	FITC mean fluorescence		PE mean fluorescence	
	negative	positive	negative	positive
uncompensated	125	3540	185	1650
compensated	125	3560	135	135

$$\% \text{ Spillover} = \frac{1650 - 185}{3540 - 125} \times 100$$

Spillover affects resolution senstivity

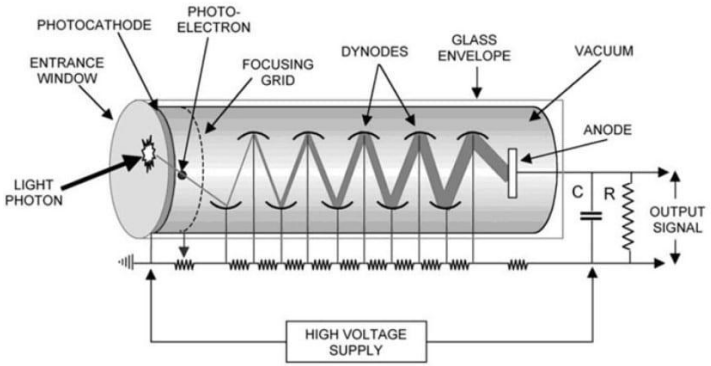




Electronics

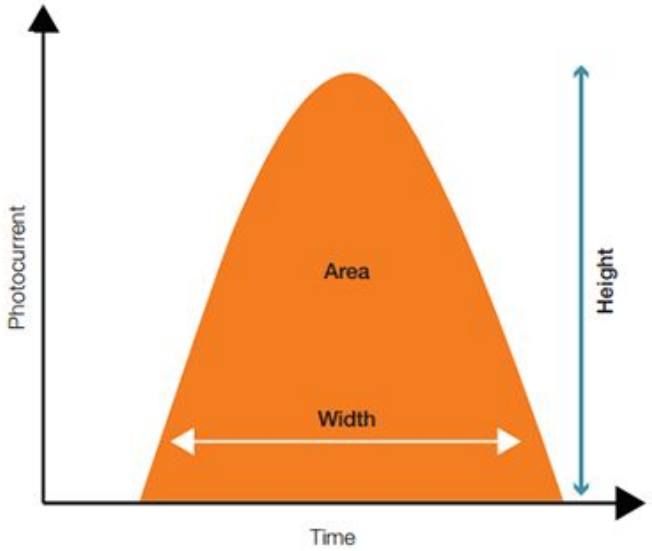
Photomultiplier Tubes (PMTs) vs. Photodiodes (PDs)

- PMTs:
  - High sensitivity due to signal amplification
  - Less sensitive in far red end of spectrum



- Photodiodes:
  - Cheaper
  - No amplification
  - Better performance in far red

Pulse Processing



Height: The maximum amount of current output by the PMT.

Area: The integral of the pulse.

Width: The time interval during which the pulse occurs.

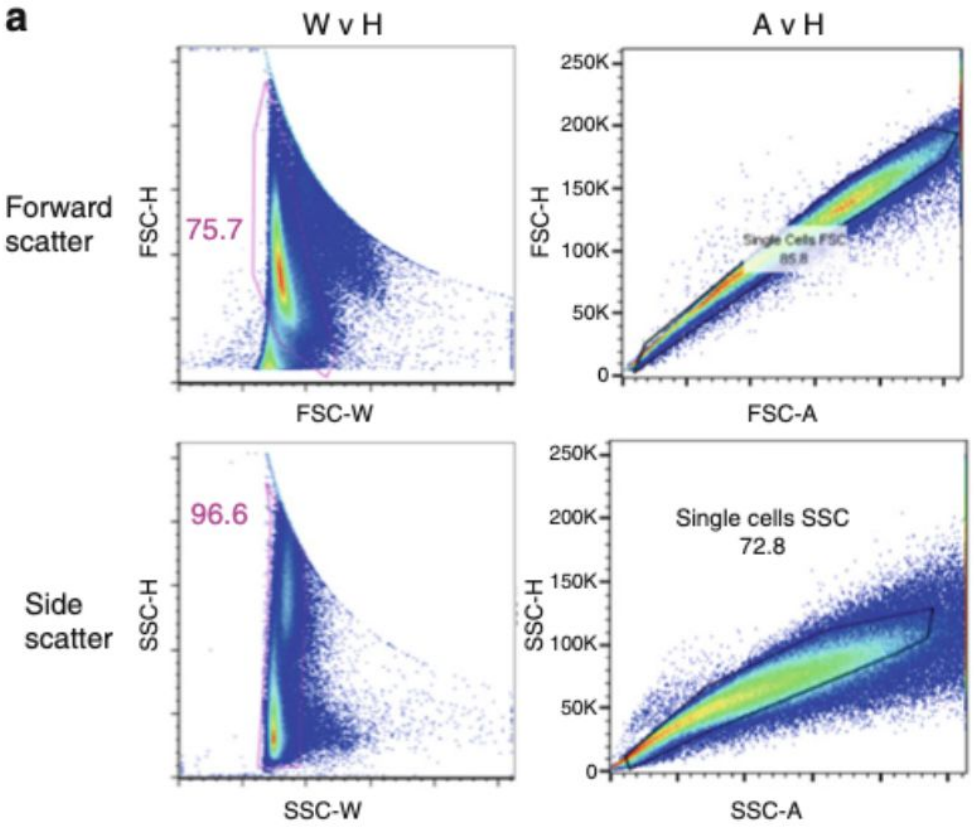
Signal intensity can be measured by either height or area.

The width parameter measures the **time that the cell spends in the laser.**

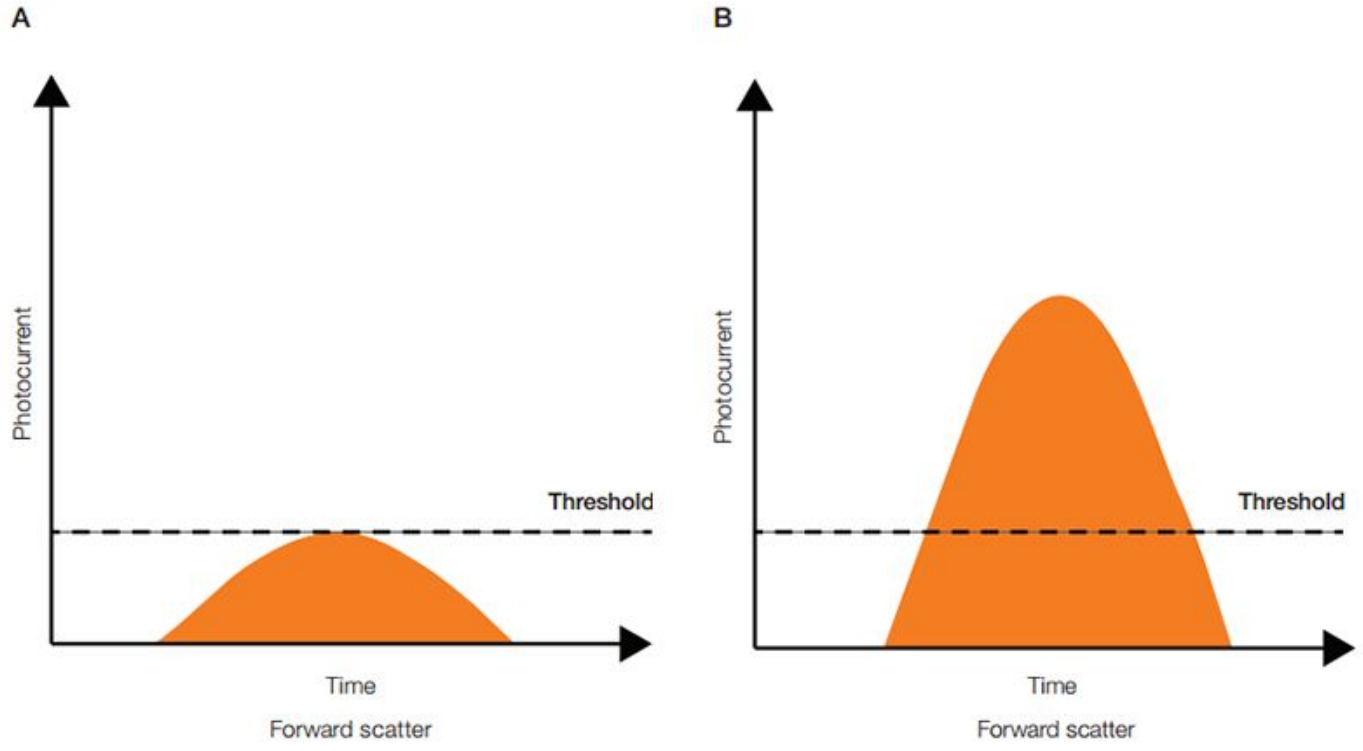
- Single cell events can be selected by FSC and/or SSC height vs. width or height vs. area
- Need to turn these parameters on in order to collect them!

https://www.bio-rad-antibodies.com/flow-cytometry-signal-processing.html

Singlet gating



Threshold Setting



Flow Cytometry Standard (FCS) Files

=

Numeric Matrix

ftr01	ftr02	ftr03	ftr04
296.7	945.4	444.9	995.8
45.9	553.8	694.2	71.4
779.9	164.8	570.9	736.6
16.9	443.4	693.3	274.1
216.7	145.3	603.4	185.2
994.0	406.5	433.7	933.7
469.1	971.8	563.7	562.8
182.4	403.8	804.3	334.6
164.3	370.4	20.2	124.5
752.7	197.3	625.8	284.8

+

Metadata

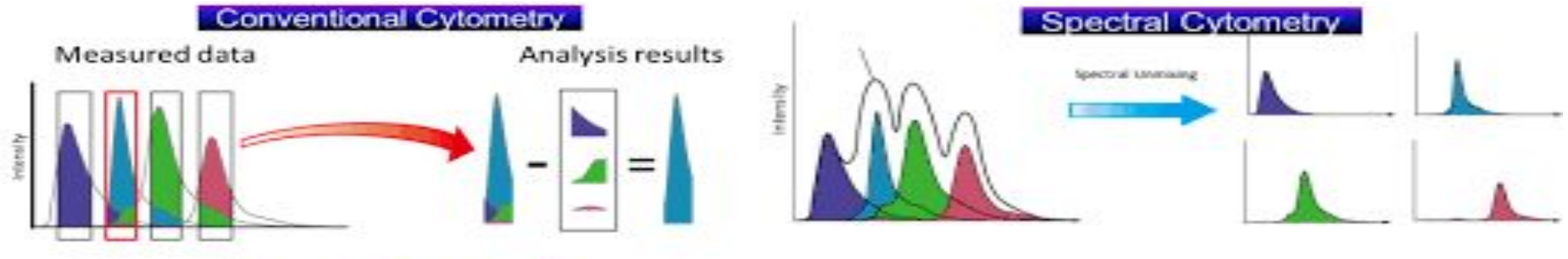
$BEGINANALYSIS	0
$BEGINDATA	1952
$BEGINSTEXT	0
$BTIM	17:16:32
$BYTEORD	4,3,2,1
$CYT	LSRII
$DATATYPE	F
$ENDANALYSIS	0
$ENDDATA	1558495
$ENDSTEXT	0
$ETIM	17:17:17
$MODE	L
$NEXTDATA	0
$P1B	32
$P1E	0,0
$P1G	1.0
$P1N	FSC-A

Read by analysis software (FlowJo, Cytobank, FCS Express, etc.)

Spectral vs. Conventional Flow Cytometry

- Conventional flow cytometry uses one detector channel per fluorochrome; spillover from other fluorochromes in that channel is subtracted out.
- Spectral cytometry measures fluorescence across the spectrum (all detectors for all fluorochromes), then deconvolute signals based on single-color spectra
- Allows for simultaneous use of fluorochromes with similar emission peaks but different shapes of emission spectra

Spectral vs. Conventional Flow Cytometry



› Cytometry A. 2020 Oct;97(10):1044-1051. doi: 10.1002/cyto.a.24213. Epub 2020 Aug 31.

OMIP-069: Forty-Color Full Spectrum Flow Cytometry Panel for Deep Immunophenotyping of Major Cell Subsets in Human Peripheral Blood

Lily M Park [1], Joanne Lannigan [2], Maria C Jaimes [1]

First words on troubleshooting the systems

- Fluidics are most commonly to blame!
  - No events, few events, parameter distortion: clog or partial clog [prime and wash]
  - Flow cytometers and air don't mix! Bubbles in the system will cause a clog [see above]
- Optics are stable, but lasers and laser alignment *can* degrade
  - Loss of signal for channels associated with one laser line only [check alignment, laser power]
- Electronics problems often relate to threshold
  - Mostly seeing debris [increase threshold]

Course Outline

1. **Pre-analytical variables/sample processing (1:00-1:40)** — Holden Maecker
   a. Sample collection and transport
   b. PBMC isolation or other sample processing
   c. Stimulation/functional assays
2. **Experimental design (1:40-2:45)**
   a. The instrument: lasers, filters, conventional vs. spectral
   b. Panel building and testing: fluorochromes, spillover — Maria Jaimes
   c. Batching
   d. Controls

Break (2:45-3:00)

3. **Instrument setup and acquisition (3:00-3:30)** — Caroline Duault
   a. Standardizing instrument setup, inter-instrument qualification
   b. Acquisition variables: # of events, speed, collection criteria
4. **Data analysis (3:30-4:30)**
   a. Quality control steps
   b. Manual and automated analysis

Spread and Panel Design

Maria Jaimes

City Sky

Suburbia

Bright Suburban

Suburban

Suburban Rural

Rural

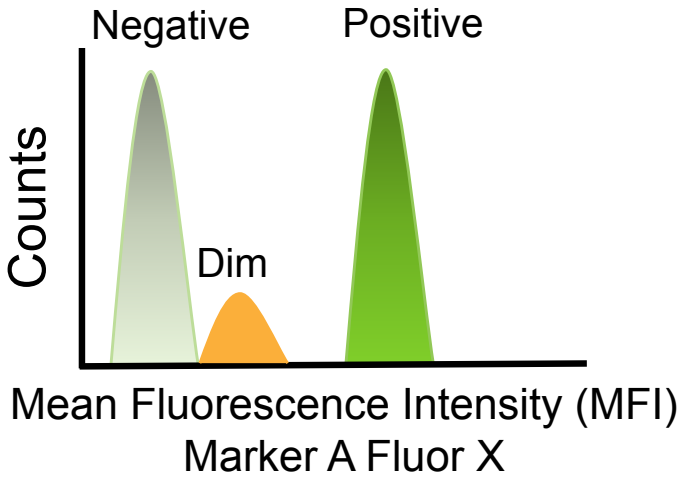
Dark Sky

Excellent Dark Sky

Population Resolution

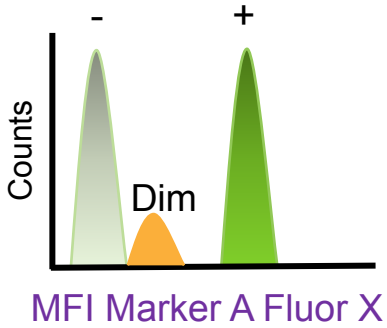
What Is What We Want To See In Our Flow Data?

- The signals from the markers we are interested at!
- Some signals are very bright, very easy to detect… but some are dim
- Our goal is to identify accurately ALL the markers present in an assay
- RESOLUTION = the ability to identify dim signals



What Factors Impact Resolution?

- First, let's focus on one marker



Negative population

Instrument

- Optical performance
- Instrument Setup

Reagents

- Non-specific binding

Biology

- Autofluorescence

Positive Population

Instrument

- Instrument Setup

Reagents

- Fluorochrome Brightness
- Antibody Performance/clone
- Antibody Titer

Biology

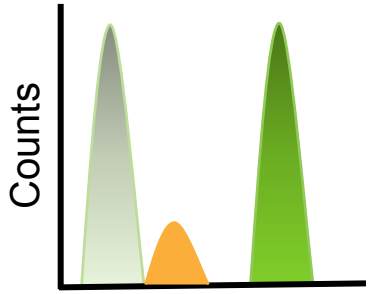
- Antigen Level of Expression

What Happens When We Combine Markers/Fluorophores?

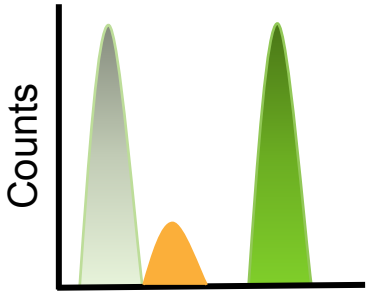
What We Want

What Might Happen…

One Marker
(Single Stained)



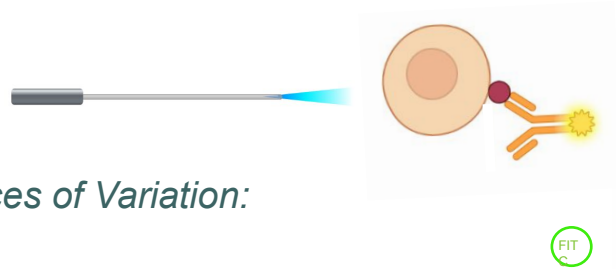
All Markers
Together
(Multicolor)

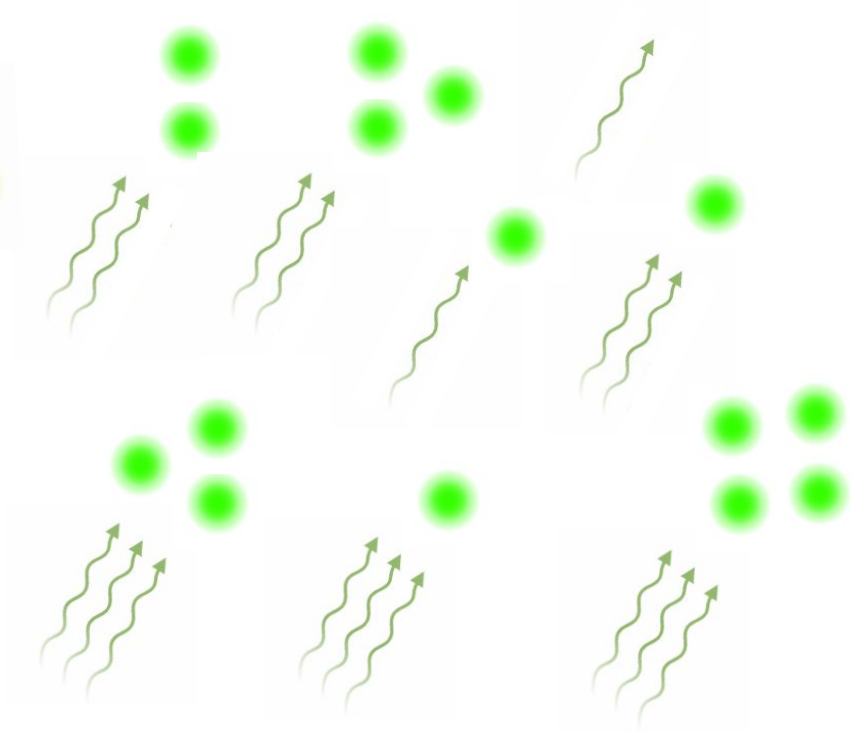


MFI Marker A
Fluor X

Photon Counting Error

Any counting process is subject to counting error
The photon counting error becomes evident as spreading of the data



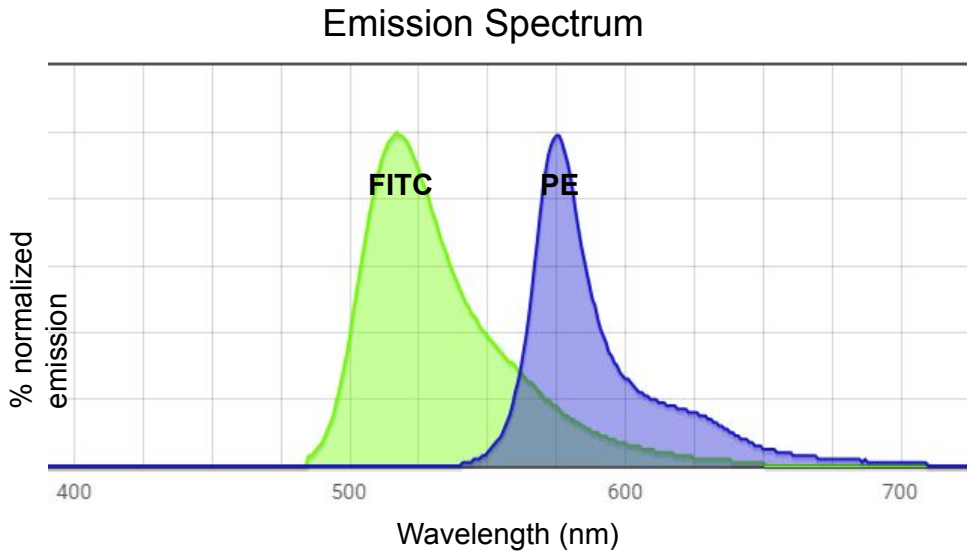


Sources of Variation:

1. Wavelength of emission
2. Photons emitted
3. Photons reaching the detector
4. Photoelectrons emitted
5. Photoelectrons amplified
6. Photoelectrons collected
7. Counted pulses

Spillover Spreading Error

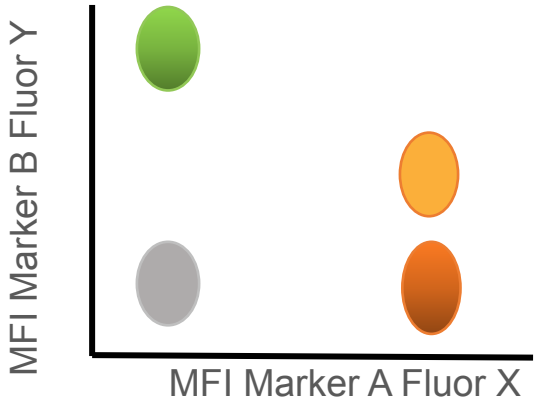
- Measurement error arises from fluorochromes spilling over into others
- Higher spillover causes higher spread
- How do we know if there is spillover between 2 dyes?



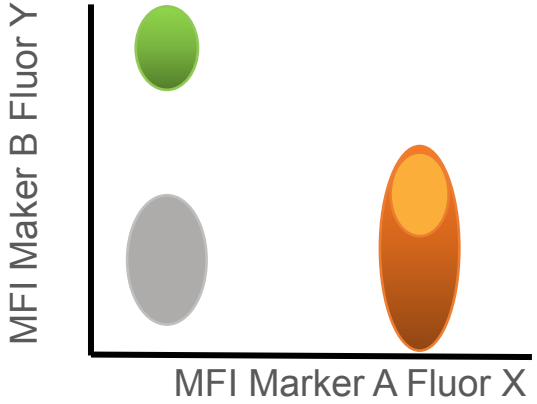
For more details, read:
Nguyen, R. et al. Cytometry A, 2013, 83(3): 306-31
Roederer, M. et al. Cytometry A, 2001, 45(3): 194-205

Spread Is the Mayor Cause Of Loss Of Resolution In Multicolor Assays

No Spread Between Fluors X and Y



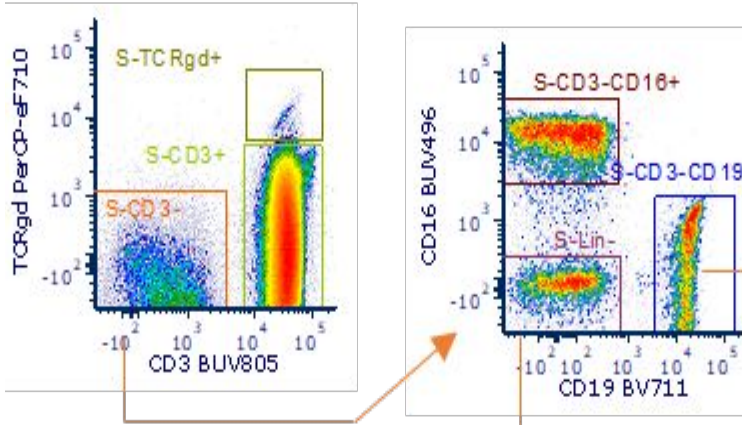
Fluor X Introduces Spread Into Y



Spread is an issue if Makers A and B are CO-EXPRESSED

Spread Visualization In Multicolor Data

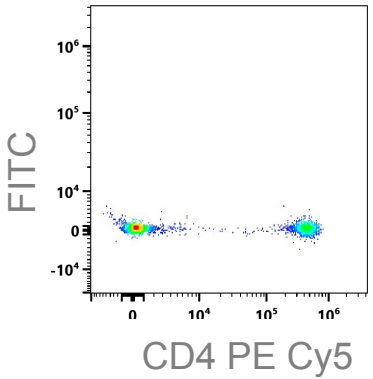
Data With High Spread/ Low resolution

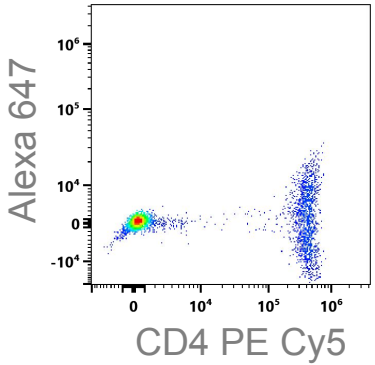


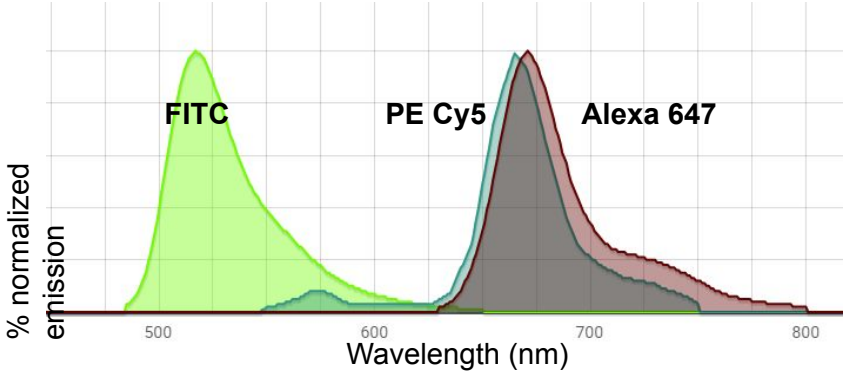
Data With Low Spread/ High resolution

Spread Visualization – Single Stained Cells

Single stained PBMCs gated on lymphocytes

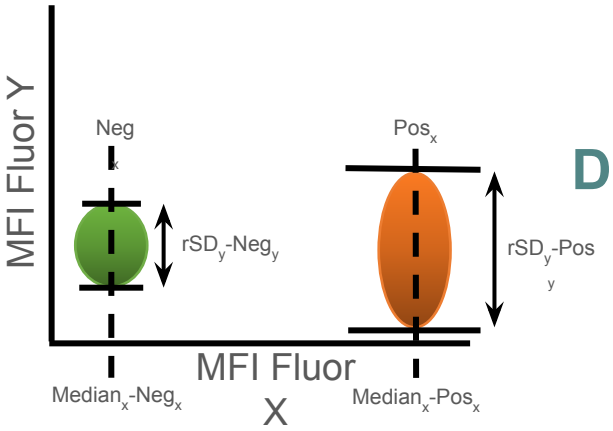






Spillover Spread Quantification

Spillover Spread Value (SSV) represents the spread increase of the fluor X into fluor Y, when compared to negatives in the same channel



Fluor X DOES SPREAD into Fluor Y

$$Spillover\ Spreading\ {}^{Primary\ (X)}_{Secondary\ (Y)} = \frac{\sqrt{\left(rSD_Y^{Pos_X}\right)^2 - \left(rSD_Y^{Neg_X}\right)^2}}{\sqrt{\left(Median_X^{Pos_X}\right) - \left(Median_X^{Neg_X}\right)}}$$

For more details, read:
Nguyen, R. et al. Cytometry A, 2013, 83(3): 306-31

Spillover Spread Matrix

Spread Receiver (Detector)

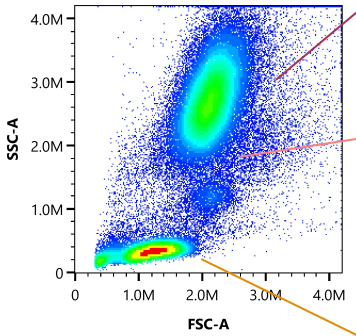
Spread Donor (Fluorochrome)	AF700	APC	APC-Cy7	BUV395	BUV615	BV421	BV510	BV786	FITC	PE	PE-CF594	PE-Cy5	PE-Cy7	PerCP-Cy5.5
Alexa Fluor 700		0,5	3,1	0,0	0,0	0,0	0,0	1,3	0,1	0,2	0,0	0,1	0,1	0,4
APC	4,1		1,8	0,0	0,0	0,1	0,0	0,7	0,1	0,0	0,0	0,4	0,0	0,4
APC-Cy7	2,6	1,8		0,0	0,1	0,1	0,0	3,1	0,0	0,0	0,1	0,1	0,2	0,1
BUV395	0,0	0,2	0,0		0,3	0,0	0,0	0,2	0,0	0,0	0,0	0,0	0,0	0,0
BUV615	0,5	0,7	0,2	0,4		0,0	0,0	0,5	0,0	0,2	0,3	0,4	0,1	0,5
BV421	0,1	0,1	0,1	8,2	0,2		1,0	0,1	0,1	0,0	0,0	0,0	0,0	0,0
BV510	0,1	0,0	0,0	1,3	2,3	0,3		0,8	0,2	0,2	0,1	0,0	0,0	0,1
BV786	0,5	0,2	1,1	0,0	0,0	0,5	0,0		0,0	0,0	0,0	0,0	0,3	0,2
FITC	0,3	0,4	0,0	0,0	0,2	0,0	1,1	0,0		0,9	0,5	0,4	0,0	0,5
PE	0,7	1,5	0,2	0,0	1,6	0,0	0,0	0,5	0,3		1,8	1,4	0,2	1,7
PE-CF594	2,2	3,9	0,4	0,0	4,2	0,1	0,0	1,4	0,2	1,7		3,5	0,7	4,8
PE-Cy5	6,4	11,9	2,7	0,0	0,2	0,1	0,2	2,6	0,1	0,5	0,3		1,1	8,2
PE-Cy7	1,5	0,4	4,1	0,0	0,2	0,0	0,0	7,8	0,3	0,6	0,3	0,3		0,6
PerCP-Cy5.5	5,7	4,1	2,8	0,0	0,0	0,0	0,4	3,9	0,0	0,0	0,1	3,4	1,3	

Oslo University Hospital https://www.ous-research.no/flow/Services/20917

For more details, read:
Nguyen, R. et al. Cytometry A, 2013, 83(3): 306-31

Antigen Density Impacts Spread

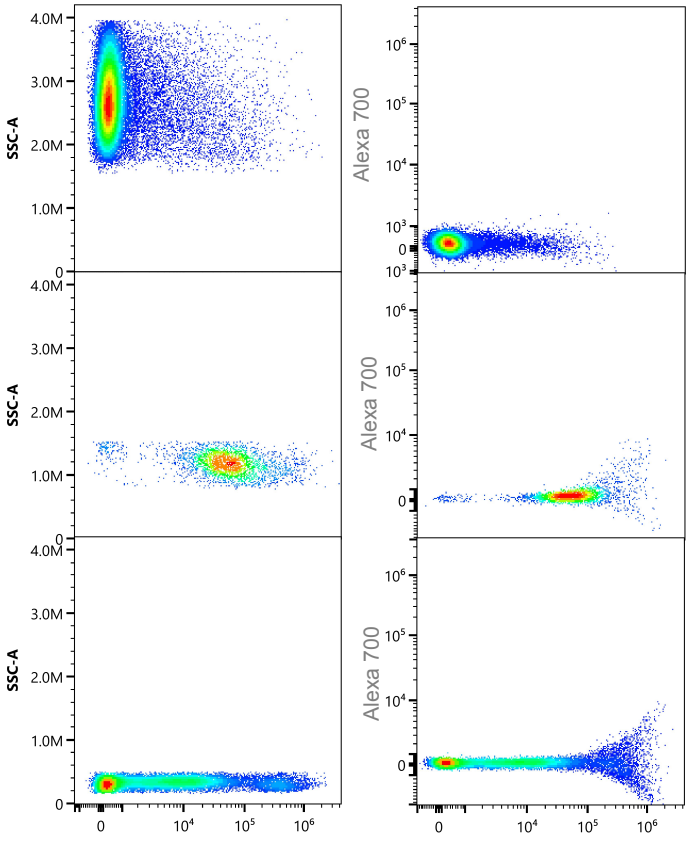
Marker density is the number of target molecules expressed on a specific cell subset



Granulocytes

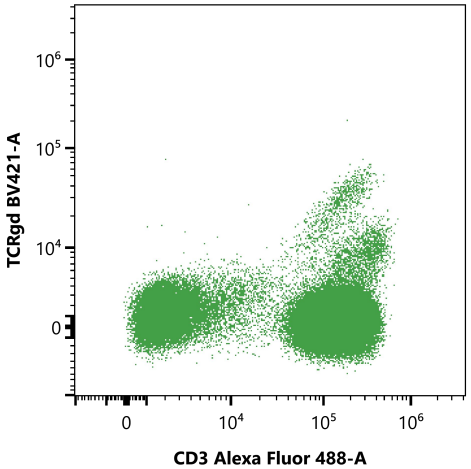
Monocytes

Lymphocytes



Let’s Review Spread And Co -Expression

No spread



CD3 AF488
TCRγδ BV421
CD19 BUV395
CD8 AF700

Gated on lymphocytes

Spread
Bright dye
No coexpression

Spread
Dim dye - no coexpression
Bright dye - coexpression

What Can We Do About Spread?

- We need to learn to work around it!
- Careful panel design minimizes the impact of spread
- A lot of available information.. Use it!
- When analyzing data, look for it and determine if you need to make changes to your assay

Panel Design: Step By Step Approach

1. Step 1: The Biology
   - Marker Level of Expression and Co-Expression
   - Clone Selection
2. Step 2: Fluorochrome Selection
3. Step 3: Reagent Availability
4. Step 4: Panel Design
5. Step 5: QC Panel Design

Step 1: The Biology

Scientific Question

What is the goal of the assay?

Experimental Considerations

- Sample type
- Sample size
- Number of cells to record
- Need for stimulation or other treatment
- Special handling conditions

Antigen Information

- Antigen expression: High or low?
- Antigen expression across samples/donors
- Co-expression

Where To Find Information About The Biology?

Where to access information about your marker of interest?

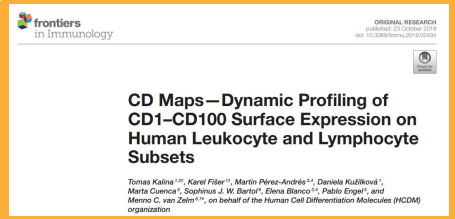
Cytometry Part A – Wiley online library


Cytometry
PART A
Journal of the International Society for Advancement of Cytometry

Human NK Cell Receptors/Markers: A Tool to Analyze NK Cell Development, Subsets and Function

Elisa Montaldo,[1] Genny Del Zotto,[2] Mariella Della Chiesa,[1] Maria Cristina Mingari,[1,3] Alessandro Moretta,[1] Andrea De Maria,[3,4,5] Lorenzo Moretta[2]*

CD Maps


frontiers in Immunology

ORIGINAL RESEARCH
published: 22 October 2019
doi: 10.3389/fimmu.2019.02434

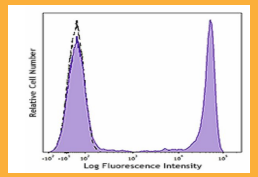
CD Maps—Dynamic Profiling of CD1–CD100 Surface Expression on Human Leukocyte and Lymphocyte Subsets

Tomas Kalina[1,2†], Karel Fišer[2†], Martin Pérez-Andrés[3,4], Daniela Kudlíková[1], Marta Cuenca[3], Sophinus J. W. Bartol[3], Elena Blanco[3,4], Pablo Engel[3], and Menno C. van Zelm[5,6], on behalf of the Human Cell Differentiation Molecules (HCDM) organization*

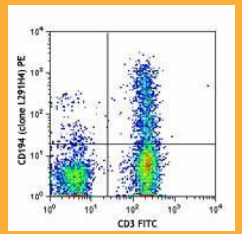
Frontiers in Immunology 2019 Vol 10 Article 2434

Reagent Vendors Website

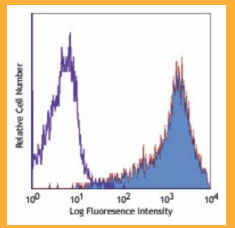
CD8 PE



CCR4 PE



ICOS PE



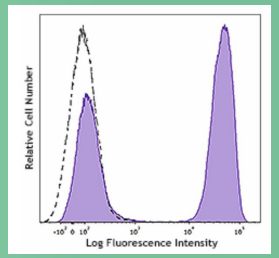
A Practical Approach: Classifying Antigens in 3 Groups

Primary

High density
on or off expression

CD45
CD3
CD19

CD3

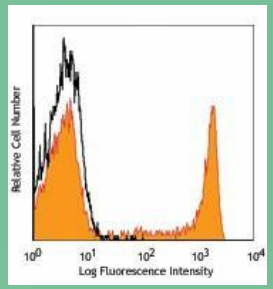


Secondary

Intermediate density
continuous expression

CD8

CD8

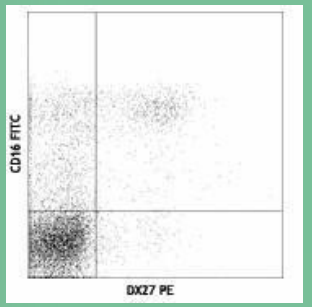


Tertiary

Critical marker in the panel
Low experession antigens

PD-1
CXCR5
CD158b

CD158b



Fluorochrome Brightness

Level of Antigen Expression

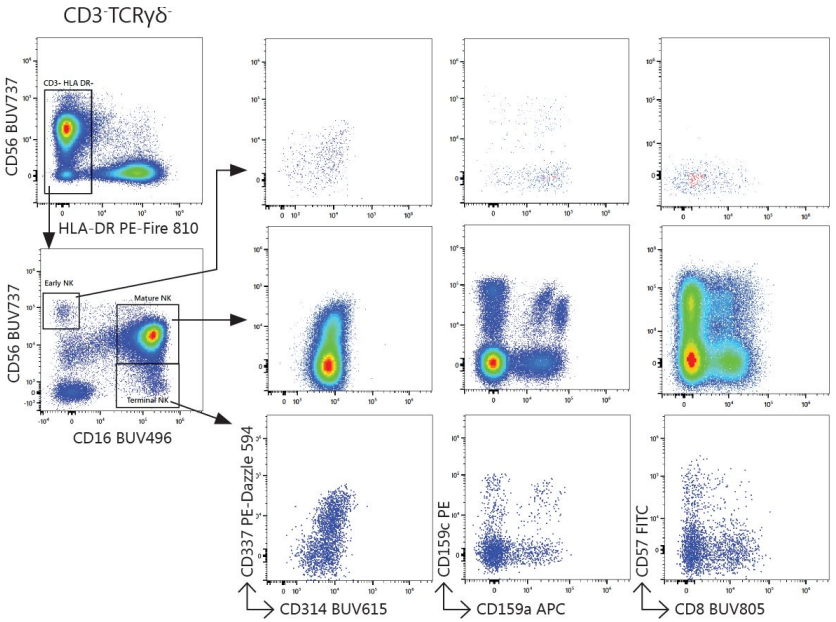
Mahnke, Y. and Roederer, M. Clin Lab Med. 2007 September ; 27(3): 469

Learn How To Extract Key Information From Publications

Cytometry A. 2020 Oct;97(10):1044-1051. doi: 10.1002/cyto.a.24213. Epub 2020 Aug 31.

OMIP-069: Forty-Color Full Spectrum Flow Cytometry Panel for Deep Immunophenotyping of Major Cell Subsets in Human Peripheral Blood

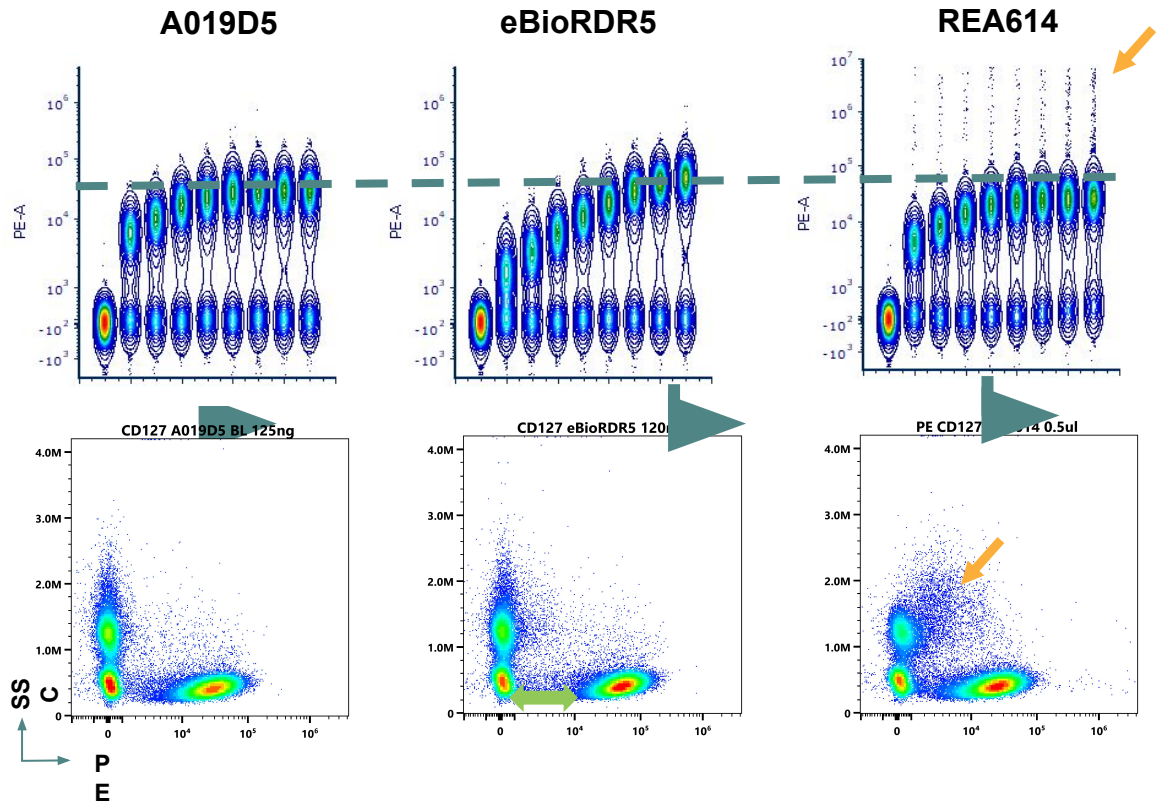
- Level of expression
- Co-expression
- Clone performance



SPECIFICITY	FLUOROCHROME	CLONE	CATALOG #	VENDOR	Titer (ng/test)
Viability	LIVE/DEAD Blue	-	L34962	Thermo Fisher	5 µL of 1:40 dilution of stock
CD45	PerCP	2D1	368506	BioLegend	250
CD3	BV510	SK7	344828	BioLegend	400
CD4	cFluor YG584	SK3	R7-20041-100T	CYTEK	10
CD8	BUV805	SK1	612889	BD Biosciences	125
CD25	PE-Alexa Fluor 700	CD25-3G10	MHCD2524	Thermo Fisher	500
PD-1	BV785	EH12.2H7	329930	BioLegend	500
TCRγδ	PerCP-eFluor 710	B1.1	46-9959-42	Thermo Fisher	500
CD14	Spark Blue 550	63D3	367148	BioLegend	1000
CD16	BUV496	3G8	612944	BD Biosciences	250

Clone Selection Is Key

Anti-human CD127



Gating Strategy Helps Visualizing Co-Expression

Assess Co-expression

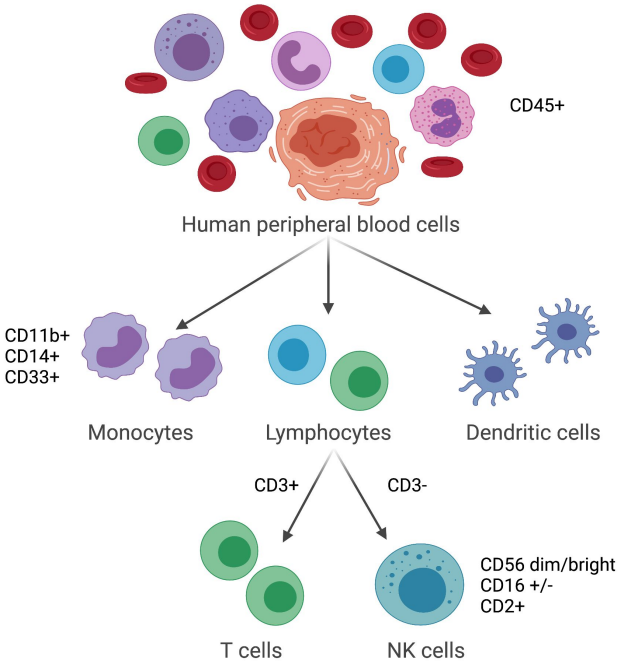
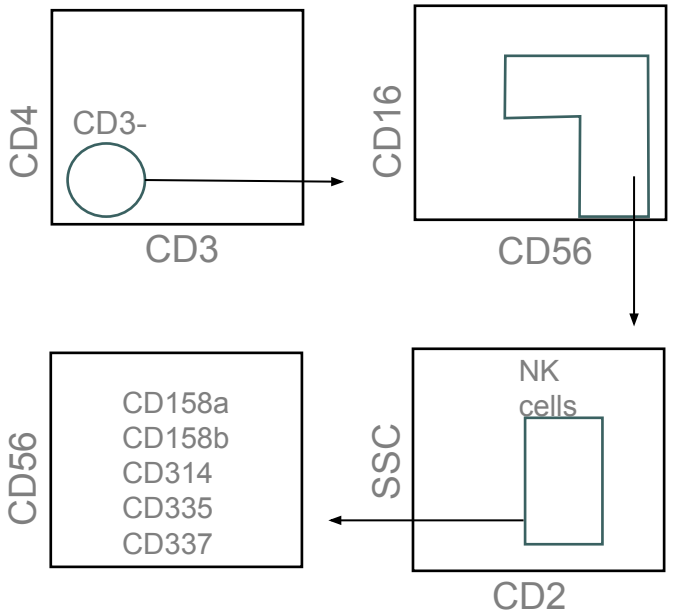


Image created with Biorender.com



Step 2: Fluorochrome Selection

1

Brightness

- Stain index calculation
- Usage of stain index charts

2

Spread

- How to predict spread impact in a panel based on fluorochrome selection

3

Other considerations

- Stability
- Size
- Non-specific binding
- Dye-dye interaction

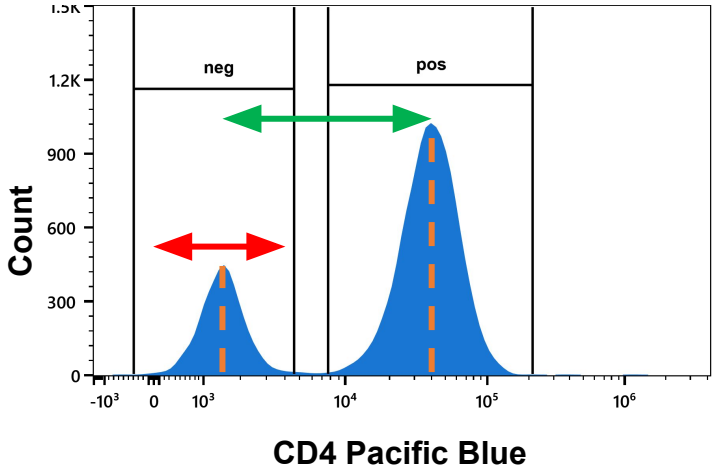
Fluorochrome Brightness

How to quantify fluorochrome relative brightness by flow?

$$\text{Stain Index} = \frac{MFI_{pos} - MFI_{neg}}{2\sigma_{neg}}$$

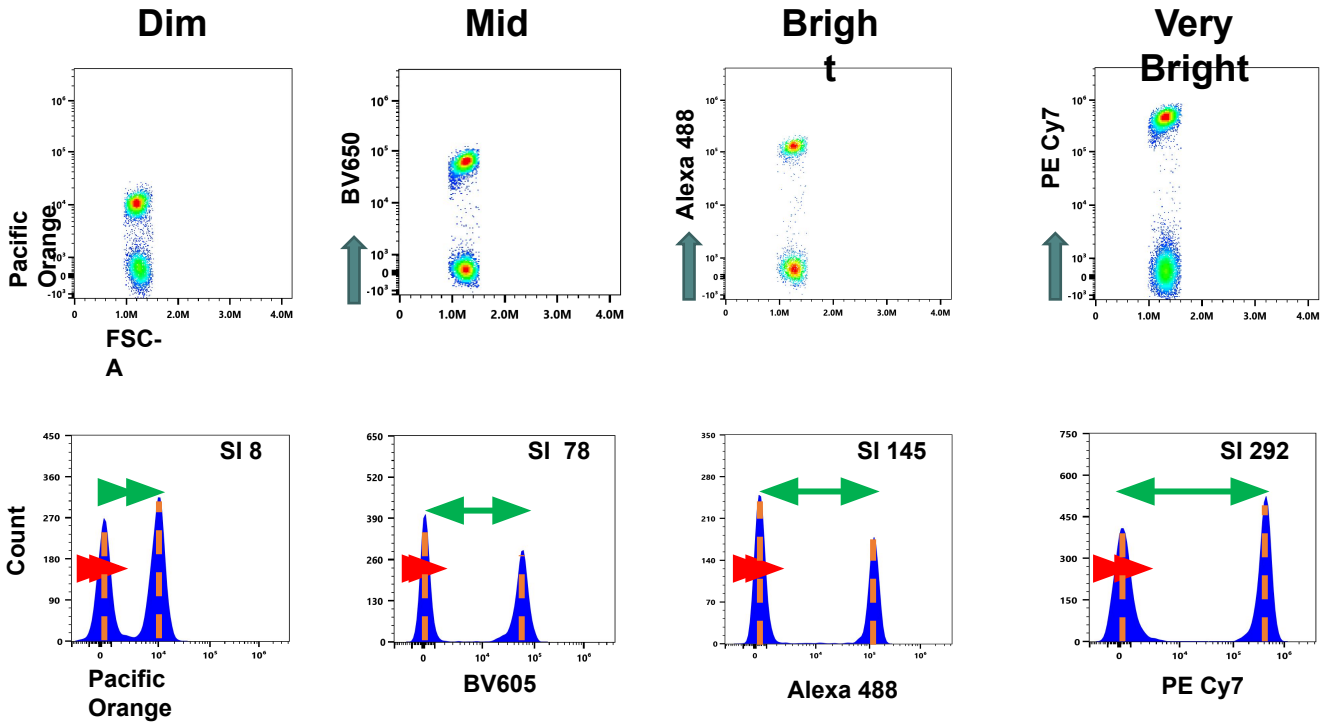
Factors affecting brightness

1. Antigen Expression
2. Fluorochrome Brightness
3. Instrument Setup and Performance

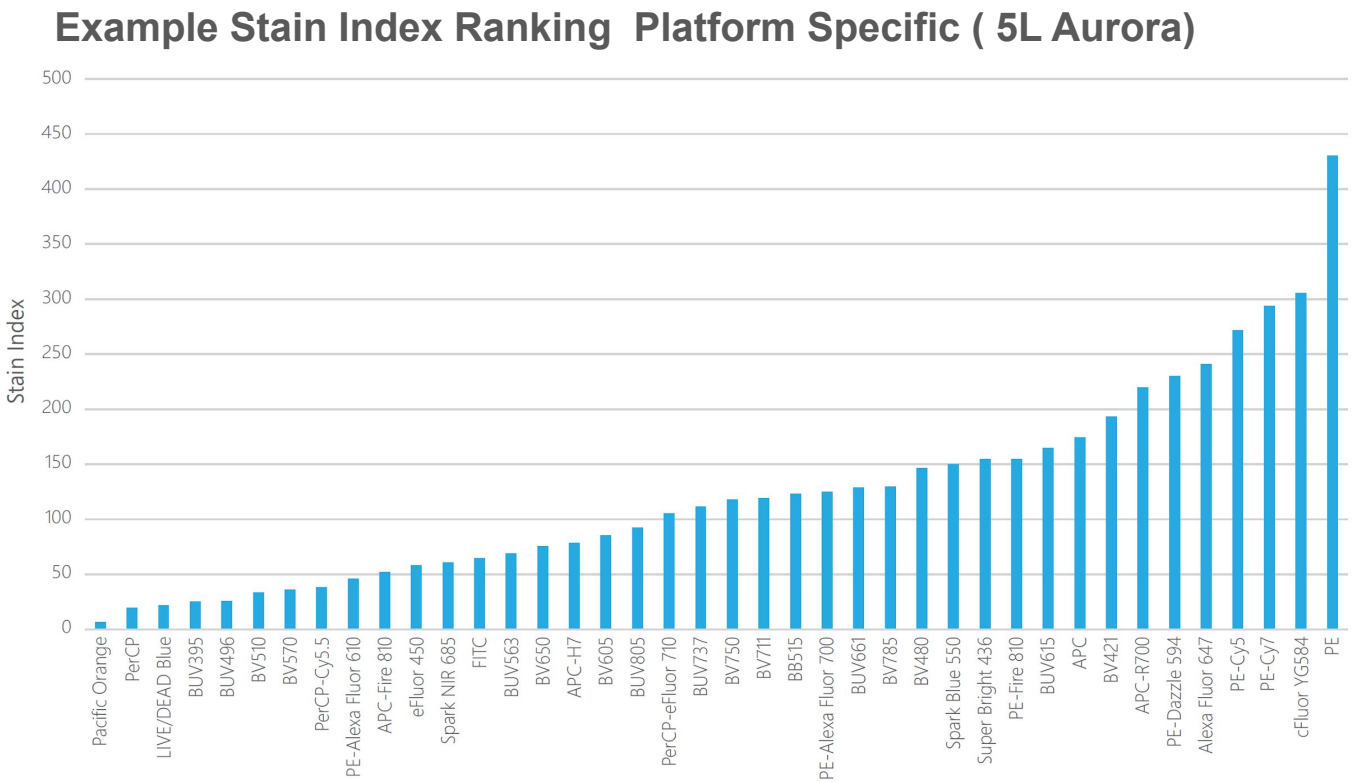


Fluorochrome Brightness (Cont.)

Test running human PBMCs stained with anti CD4 conjugated to different dyes

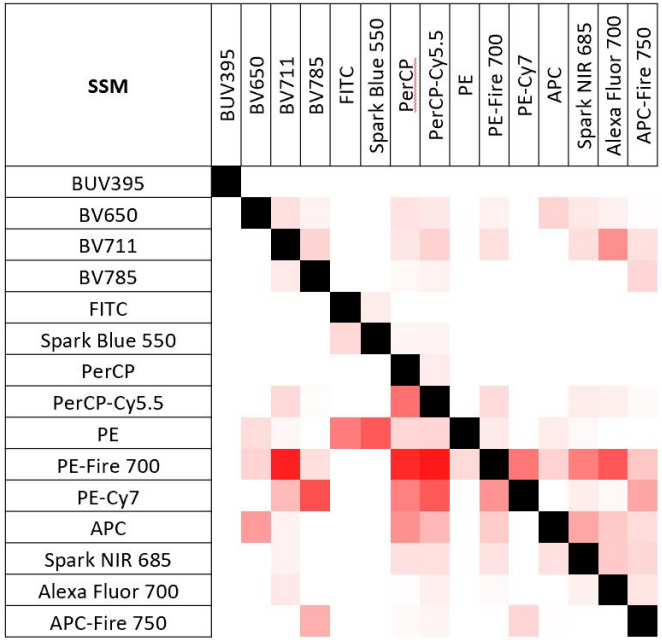


Fluorochrome Brightness: Ranking



Spread ☺

- Characterize spread on your instrument, at your optimal setup, with the fluorochromes most commonly used
- Using anti human CD4s is a practical approach, but remember to interpret data based on antigen level of expression



Step 3: Reagent Availability

- Stick to the research you did on clones and fluorochromes as much as possible
- If using online automated tools, make sure that those allow you to select the clone!

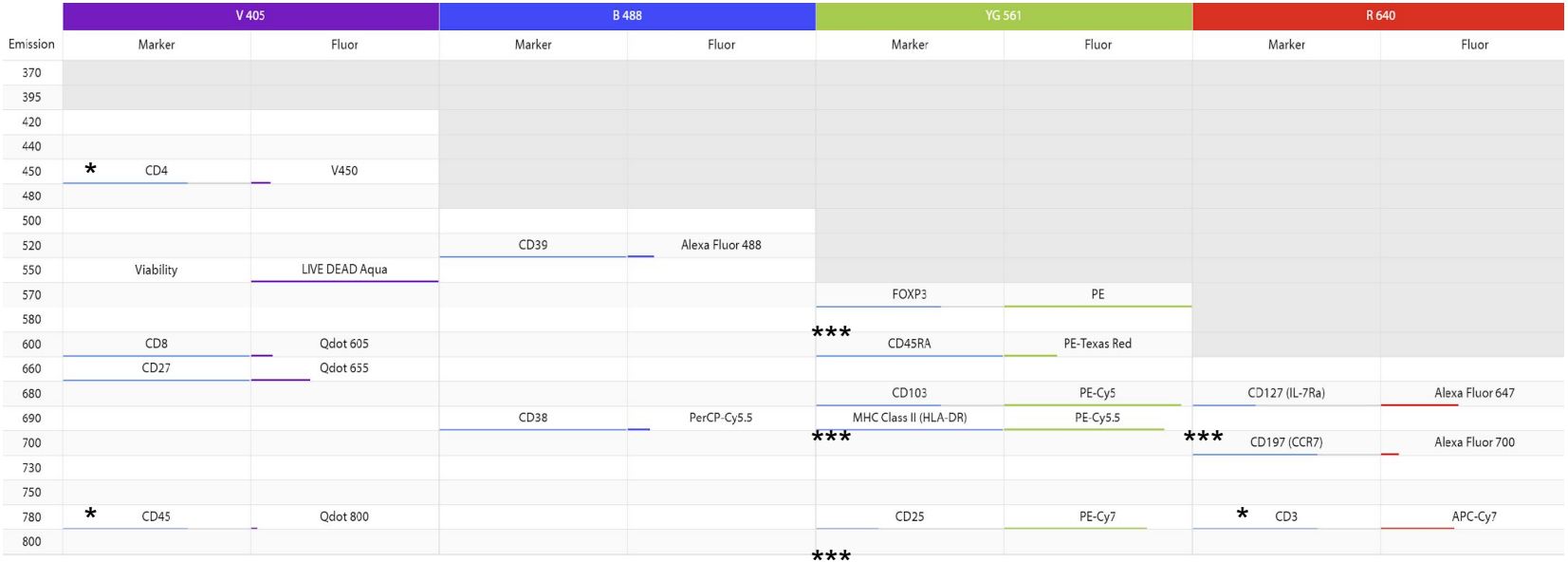
	CD45 (HI30)	CD3 (SK7)	CD4 (SK3)	CD8 (SK1)	CD25 (M-A251)	CD127 (A019D5)
FITC	x	x	x	x	x	x
PE			x		x	
PerCP	x				x	
PerCP Cy55		x	x	x		x
PE Cy7			x	x	x	x
APC		x	x		x	x
APC H7	x	x	x	x		

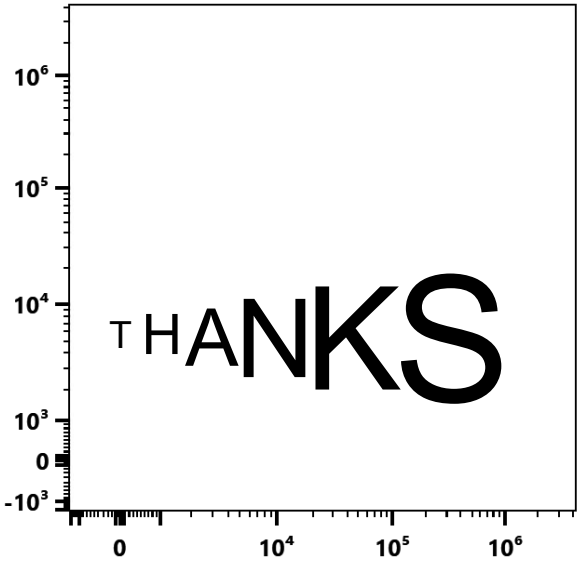
Step 4: Panel Design

- Multiple approaches published
- Practical approach:
  - Start pairing TERTIARY antigens with brightest fluors
  - Assign next the PRIMARY antigens, avoiding spread into the tertiary antigen fluors
  - Secondary antigens paired to remaining fluors, ideally bright or mid and minimizing spread

Panel Design QC

- Are all tertiary antigens assigned to bright fluors?
- For co-expressed markers:
  - Are primary and secondary antigens assigned to fluors that do not introduce significant spread into the fluors assigned to the tertiary markers



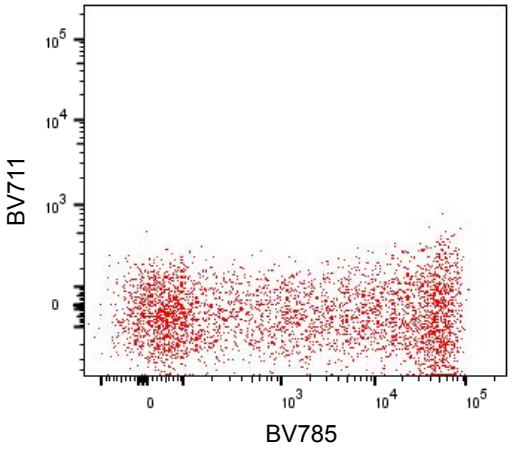

THANKS
10^6
10^5
10^4
10^3
0
-10^3
0
10^4
10^5
10^6

Controls

Maria Jaimes

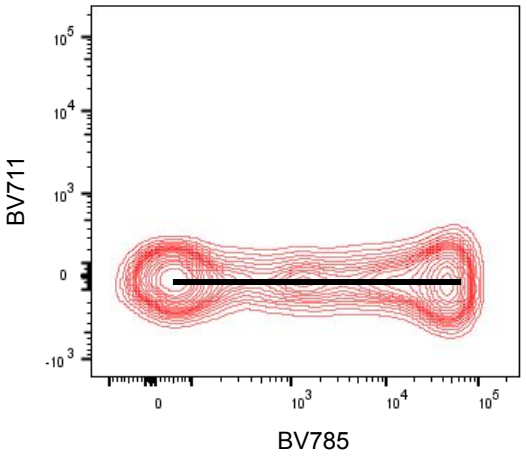
Visualization Of Compensation/Unmixing Accuracy

Dot plot +
Bi exponential scale NOT fully adjusted:



Suboptimal
assessment

Contour plot +
Bi exponential scale fully adjusted:



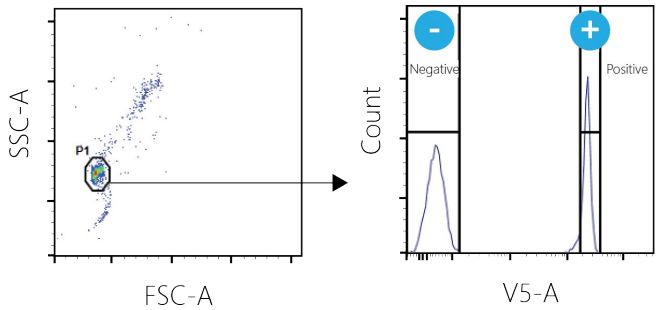
Optimal
assessment

Compensation/ Unmixing Controls Rules

- ✔ Compensation issues are very often caused because of use of inadequate compensation controls

- ✔ A good compensation control:
  - ✔ Uses the SAME fluorochrome than the fully stained sample
    - ✔ Alexa 488 is not FITC!
    - ✔ Tandems need lot specific compensation
  - ✔ Is bright
  - ✔ Negative and Positive populations come from the same particle
  - ✔ Is acquired at the same settings as fully stained sample

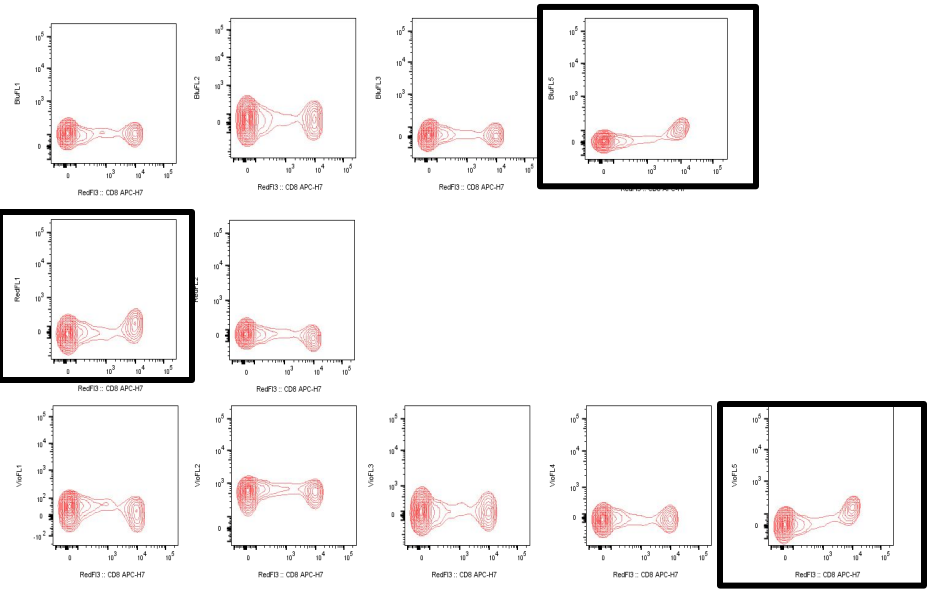
Compensation/Unmixing Controls Rules



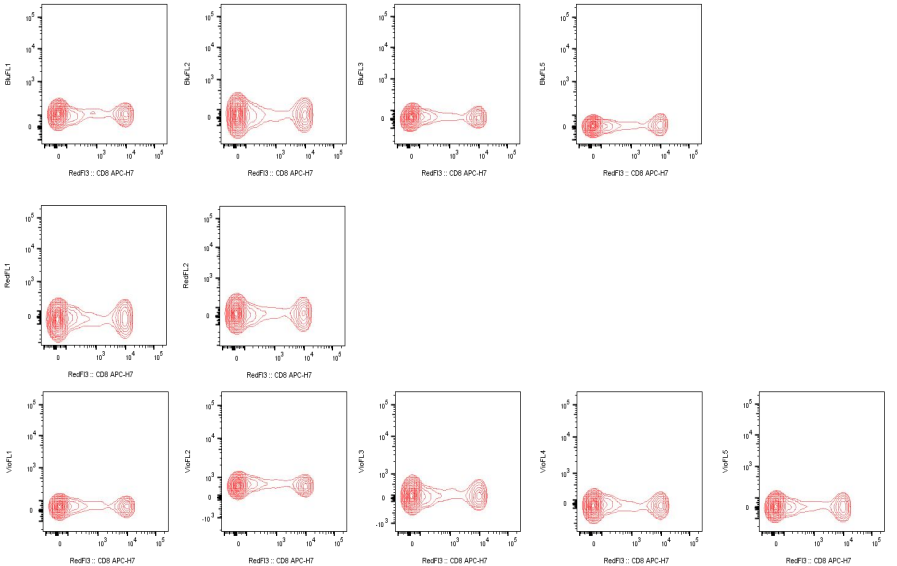
1. Positive and negative particles clearly separated
2. Negative and positive particles with IDENTICAL autofluorescence characteristics
3. Sufficient events for both data points
4. Fluorescence spectrum reference control needs to be IDENTICAL to the one in the multicolor samples

Compensation/Unmixing Controls Are Lot Specific For Tandems

CD8 APCH7 lot A compensation using CD8 APC H7 lot B

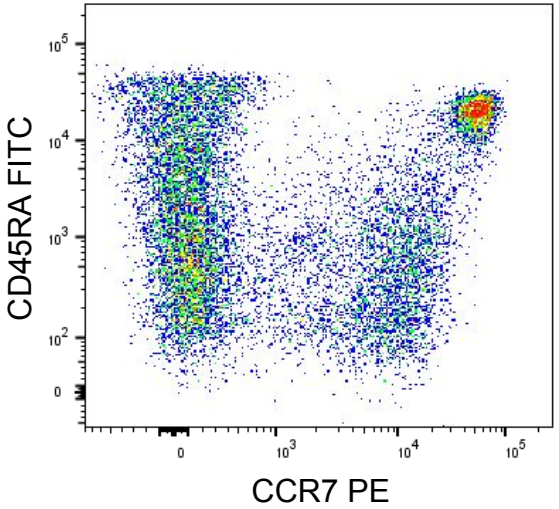


CD8 APCH7 lot A compensation using CD8 APC H7 lot A

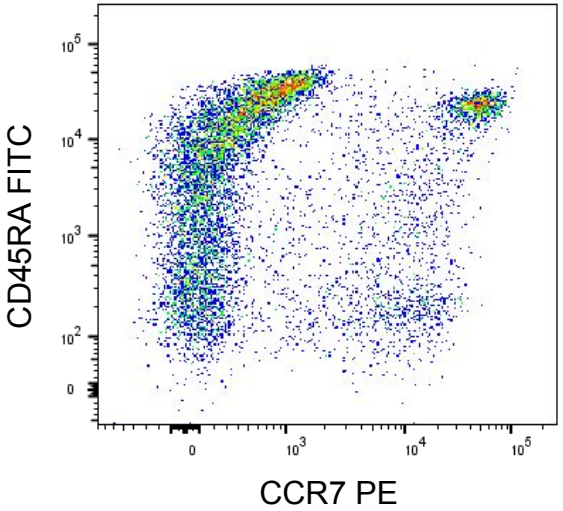


Donor To Donor Variation

Donor 1



Donor 2



% compensation FITC into PE

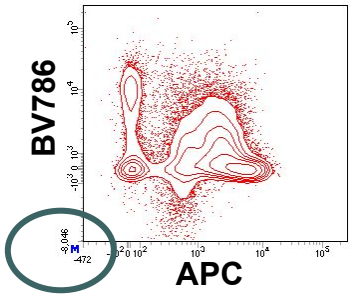
20.8%

23.1%

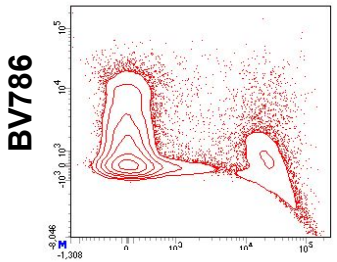
Evaluating Compensation In MC tube: Be Careful!

- ✔ A very large negative biexponential scale is often an indication of compensation issues
- ✔ Problems in pairs that do not have spill between each other is indication of problems elsewhere!

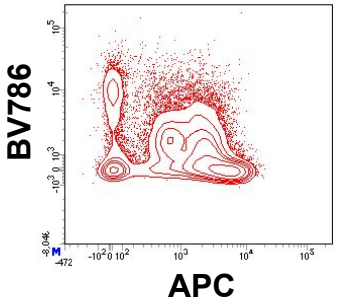
Cannot get events off axis
Biexp is very negative.



Compensation problem is somewhere else



Compensation problem is corrected once other pair is correct



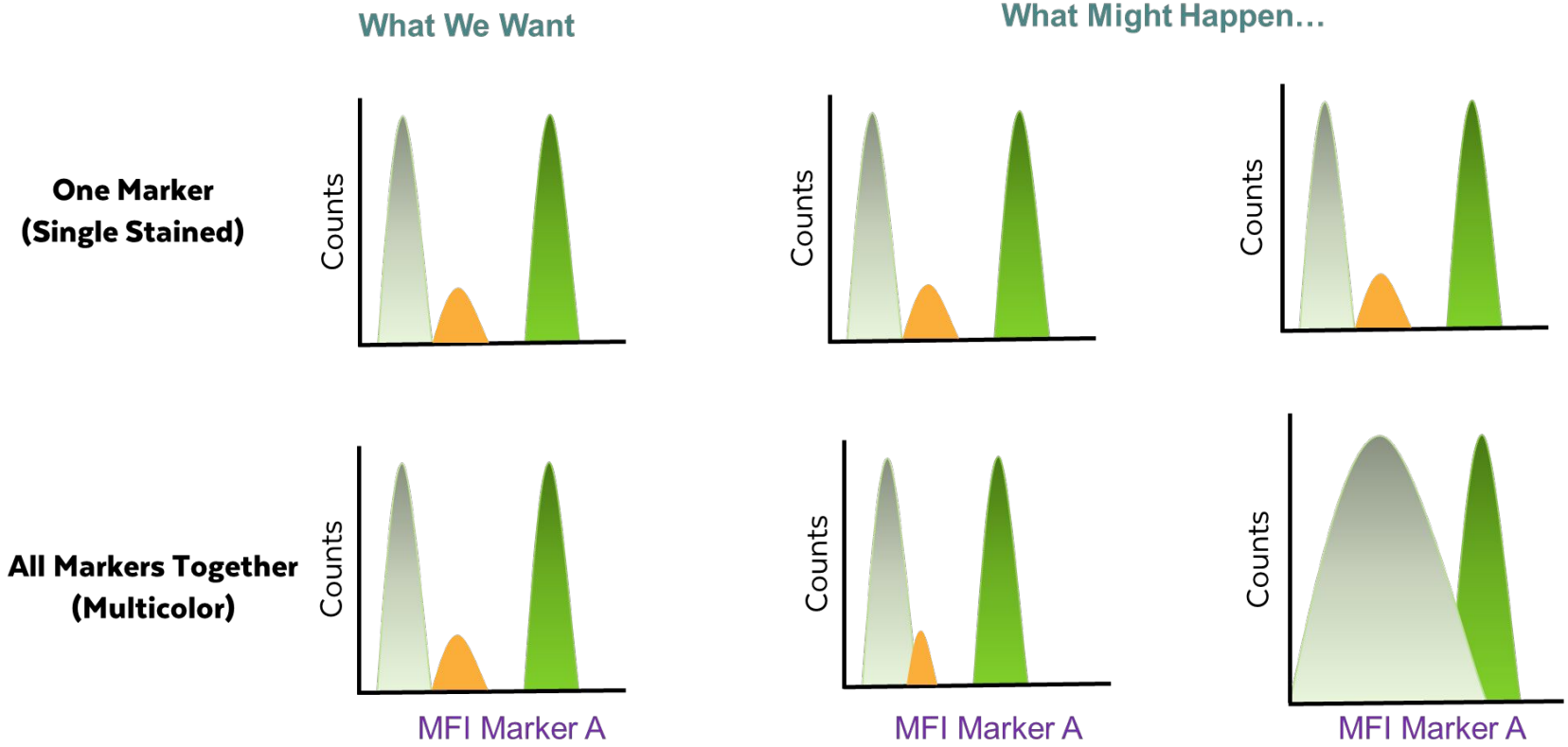
For Which Combinations Should I Expect Spillover?

- ✔ In general, pay attention to adjacent detectors (example: PerCPCy5.5 into PE Cy7)
- ✔ Tandem dyes into their donors (example: APC H7 into APC)
- ✔ Dyes with similar emission (example: PE Cy5 into APC), be aware of cross laser excitation!

	FITC	PE	PerCP-Cy5.5	PE-Cy7	APC	APC-R700	APC-H7	BV421	BV510	BV605
FITC										
PE										
PerCP-Cy5.5										
PE-Cy7										
APC										
APC-R700										
APC-H7										
BV421										
BV510										
BV605										

Combinations with high spillover in a 3 laser, 10-color instrument

Strategy For Assessing Marker Resolution



Suggested Strategy Using Single Stained Controls

Compare resolution of dim/positive populations in Multicolor tube vs single stained control

Very similar

No loss of resolution due to spillover, good panel design

In the multi-color tube the brightness of the positive population is lower than in single stain tube

a. Check mAb titer; might be too low due to dilution effects of multiple reagents
b. Possible competition, steric hindrance

in the multi-color tube spread of the negative population is higher than in single stain tube:

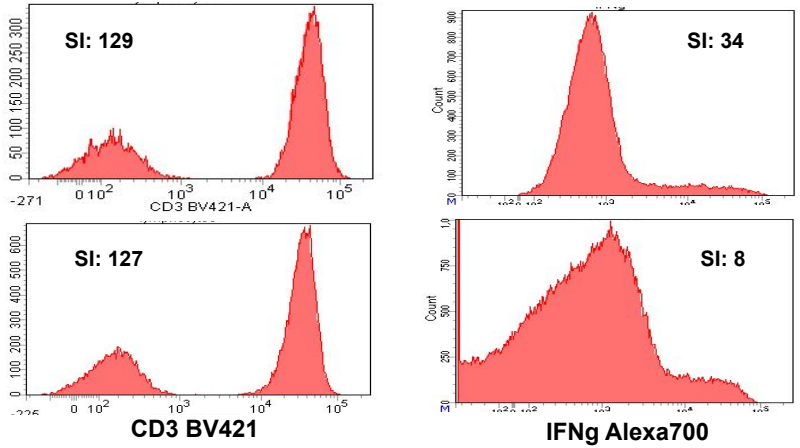
Differences in staining conditions? Spread from spillover of other reagents?

Assessing Marker Resolution: Single Stained vs Multicolor

Single stained cells are an excellent control to assess marker resolution and assay optimization

Single- stained

Multicolor Assay



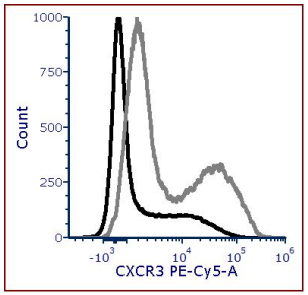
Identical

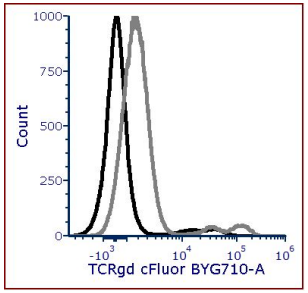
Increased spread negative population

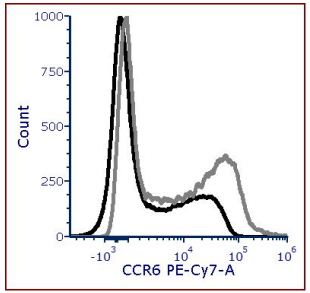
Using A “Reference” To Assess Reagent/Staining Performance

Single stained cells are an excellent control to assess marker resolution and assay optimization

Need to troubleshoot



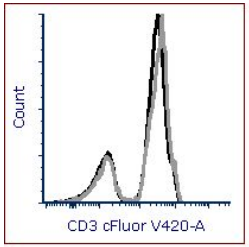


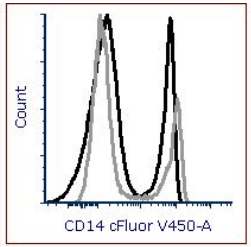


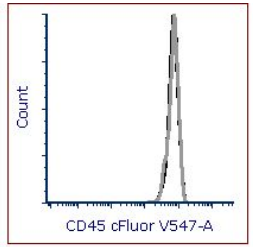
Single Stained

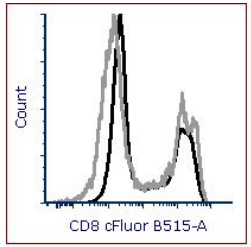
Multicolor

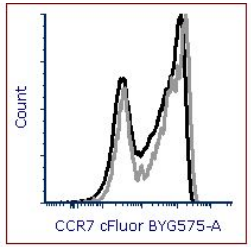
Expected

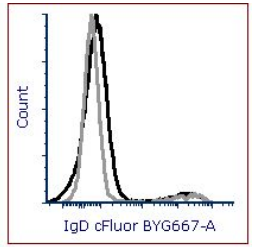








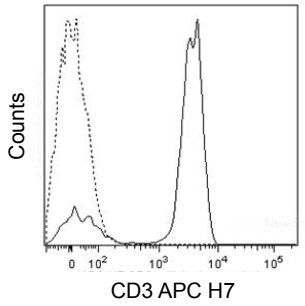


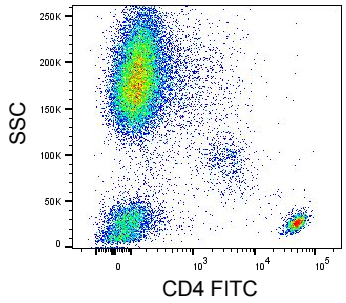


Using A “Reference” To Assess Reagent/Staining Performance

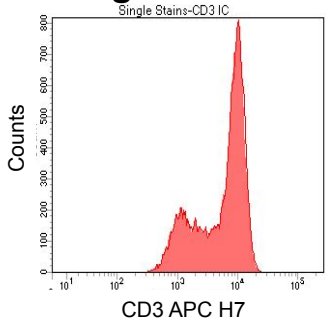
Technical Data Sheets (TDSs) and literature can also be used to assess reagent performance

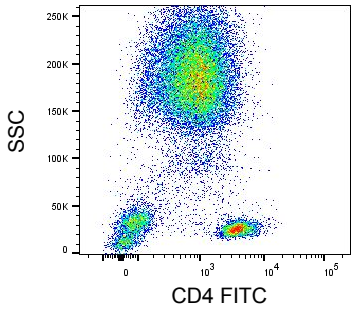
Reference or TDS





Experimental Single Stained



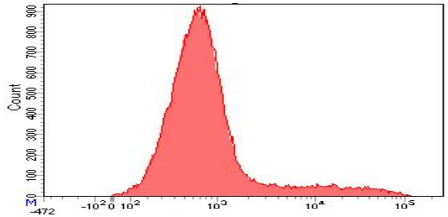


Experimental data shows increased background
Reference data is surface staining. Experimental is intracellular staining.
Conclusion: different staining conditions.
Can IC staining resolution be improved?

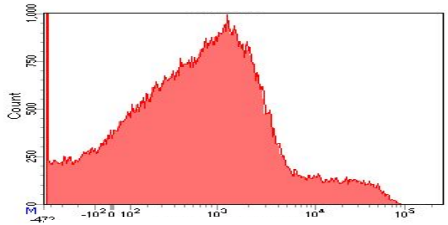
Why is experimental data dimmer compared to reference?
In this example, laboratory chose to use a lower antibody titer, but look, what happen to the monocytes?

Using FMO Control To Assess Reagent/Staining Performance

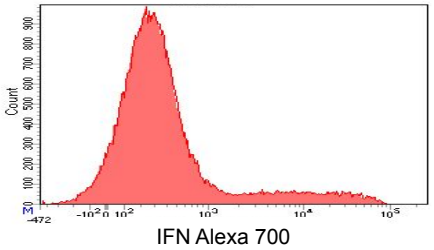
FMO: Fluorescence Minus One. Helps evaluation the impact that one fluorochrome has on the others



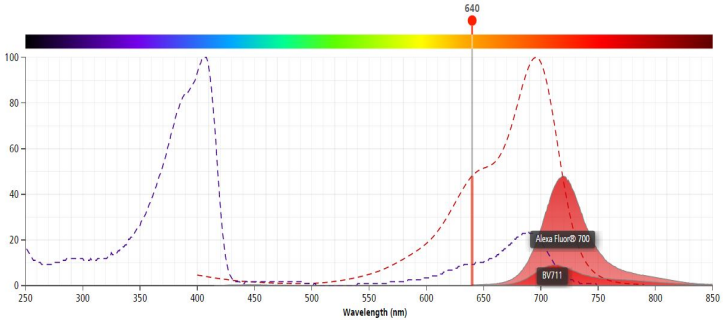
Single Stained Control



Multicolor Tube



FMO BV711
(all Mabs in panel EXCEPT CD8 BV711)



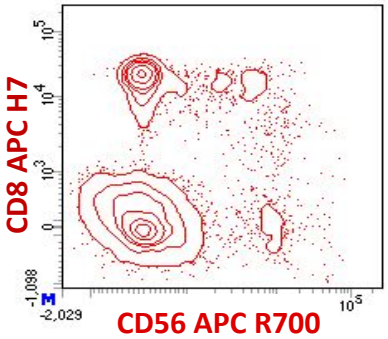
The BV711 FMO control confirms that the loss of resolution in the AlexaFluor®700 detector is due to the BV711 reagent.

The Panel Design did NOT take into consideration this spillover!

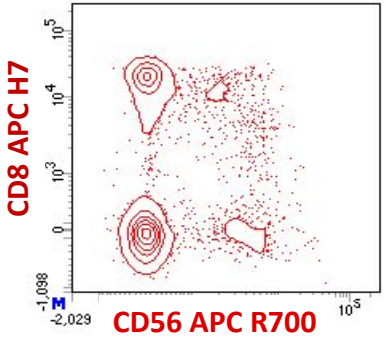
Using FMO Control To Assess Reagent/Staining Performance

Example 1

Full cocktail

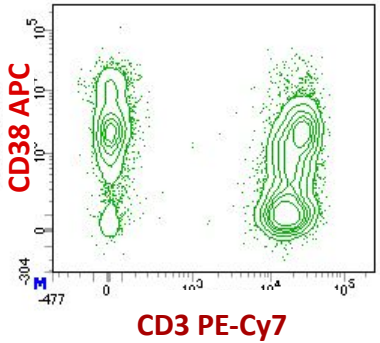


FMO APC (CD4)

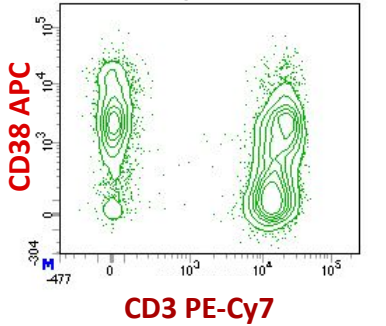


Example 2

Full cocktail



FMO
PerCP-Cy5.5 (CD34)



Summary

- ✔ When evaluating a multicolor assay it is important to be able to distinguish between problems associated with:
  - ✔ the overall execution of the assay
    - ✔ i.e. **instrument setup/performance**, reagent performance, cell preparation and staining protocols)
  - ✔ the design of the multicolor panel itself
    - ✔ i.e. **the choice of the reagents**
- ✔ A careful analysis will ensure that you minimize the number of iterations (save time and money!) needed to optimize a multicolor assay
- ✔ Use of controls (single stained cells and FMOs) facilitates data interpretation and troubleshooting

Questions?

Course Outline

1. **Pre-analytical variables/sample processing (1:00-1:40)**
   a. Sample collection and transport
   b. PBMC isolation or other sample processing
   c. Stimulation/functional assays
2. **Experimental design (1:40-2:45)**
   a. The instrument: lasers, filters, conventional vs. spectral
   b. Panel building and testing: fluorochromes, spillover
   c. Batching
   d. Controls

Break (2:45-3:00)

3. **Instrument setup and acquisition (3:00-3:30)**
   a. Standardizing instrument setup, inter-instrument qualification
   b. Acquisition variables: # of events, speed, collection criteria
4. **Data analysis (3:30-4:30)**
   a. Quality control steps
   b. Manual and automated analysis

Holden Maecker
Maria Jaimes
Caroline Duault

Course Outline

1. **Pre-analytical variables/sample processing (1:00-1:40)**
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4. **Data analysis (3:30-4:30)**
   a. Quality control steps
   b. Manual and automated analysis

Holden Maecker

Maria Jaimes

Caroline Duault

Instrument Setup And Sample Acquisition

Maria Jaimes

Requirements For High Quality Flow Cytometry Data

- Adequate panel design and assay optimization:

- A well-prepared sample
  - good sample integrity/viability
  - optimal staining conditions: antibody titers, incubation length and temperature, choice of buffers, number of washes, etc.

- A flow cytometer well-maintained and performing up to manufacturer specifications:
  - periodic maintenance, performed by users (example long clean) and manufacturer (PMI)
  - optimal warm-up procedure
  - daily Quality Control (QC)
  - optimal instrument setup updated based on daily QC

Workflow For Sample Acquisition

1. Flow cytometer warm-up and cleaning procedure
2. **Flow cytometer QC and assessment of performance parameters (LJs)**
3. Instrument setup
4. Experiment setup (compensation controls, gating strategy, stopping criteria, etc)
5. Sample acquisition

Flow Cytometer Routine (Daily) Quality Control

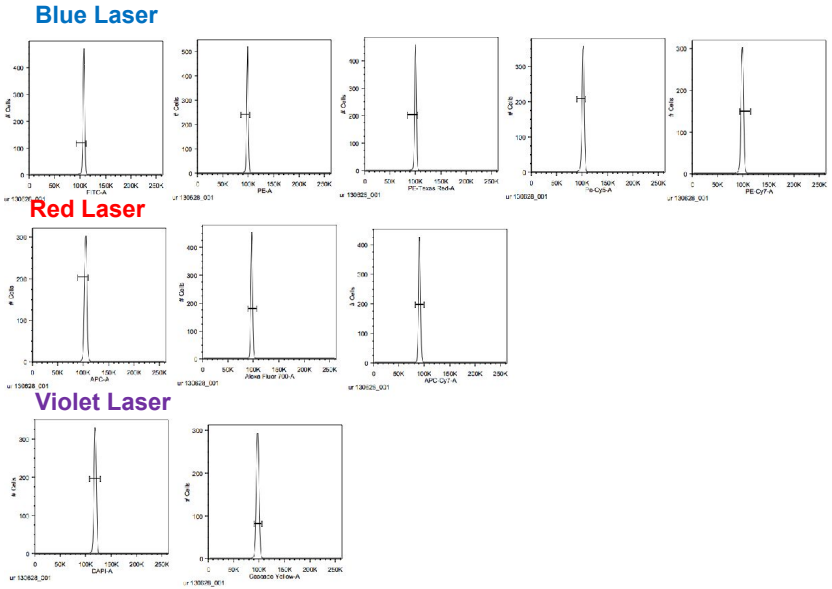
- Two goals:
  - Every time that it is run, it compares measured performance to an established standard: pass/fail criteria
  - Continuous tracking of performance over time: Levey-Jennings (LJs)
- Tools utilized:
  - Hard-dyed, “rainbow”, beads:
    - excited by most common lasers, broad emission
    - very stable, even at room temperature
    - very well characterized
  - Software algorithms that use the beads to calculate different metrics
- Common instrument performance aspects measured:
  - Laser alignment, laser power
  - Noise
  - Laser delays, area scaling factors
  - Conventional cytometers: detector efficiency
  - *Resolution (direct o indirect metrics)*

Laser Alignment and Laser Power

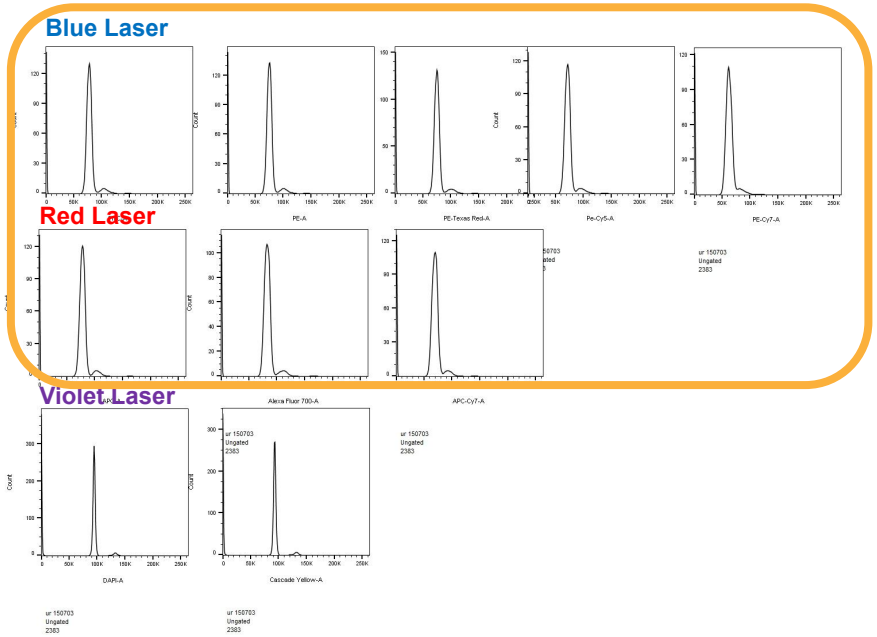
- Laser alignment is assessed using CV of QC beads at a specific wavelength(bright signal):
  - a large CV (usually > 6%) indicates significant loss of light at the flow cell level
- Beads CVs can increase at high flow rates
  - Laser alignment needs to be checked at different flow rates
  - If CV increases significantly at high flow rate, tradeoff between data quality and throughput
- Detector voltages/gains:
  - Many vendors set MFI targets for their QC beads (information available through software “bead lot file”)
  - Voltages/gains are adjusted to place the beads at MFI targets every time QC is run
  - Increases in voltages/gains are an indirect measure of decrease in laser power
- Laser power:
  - More meaningful measurement should be at the level of the flow cell (vs. laser itself)

Laser Alignment and Laser Power (cont.)

Good Laser Alignment



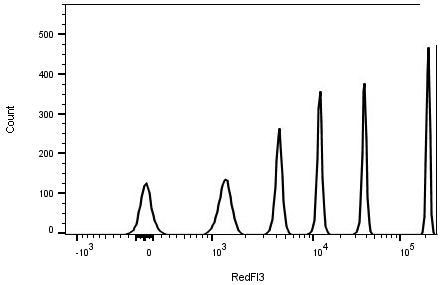
Sub-optimal Laser Alignment



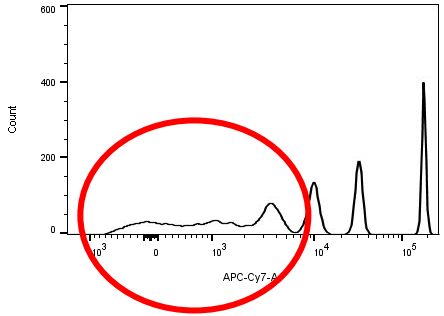
Noise - Resolution

- Two components, optical and electronic noise
- Optical noise: closely related to cleanliness of the flow cell
- Affects resolution at the low-end
- Not always measured directly as part of manufacturer QC, requires multi-level beads

Cytometer A

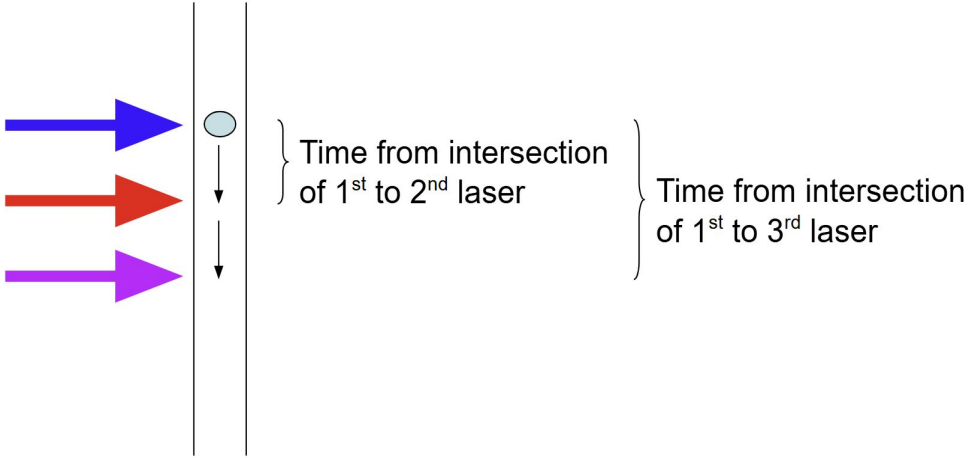


Cytometer B



APC-Cy7 detector (780/60 nm BP filter)

Laser Delay



Laser Delay is the precise microsecond timing interval a cell needs to travel from one spatially separated laser to the next

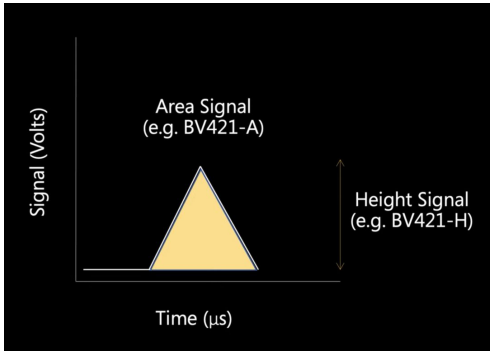
- Laser delays are automatically calculated every time instrument QC is run
- If instrument is performing correctly, and fluidics are stable, values should be very consistent over time
- Incorrect laser delays values will significantly impact multicolor data

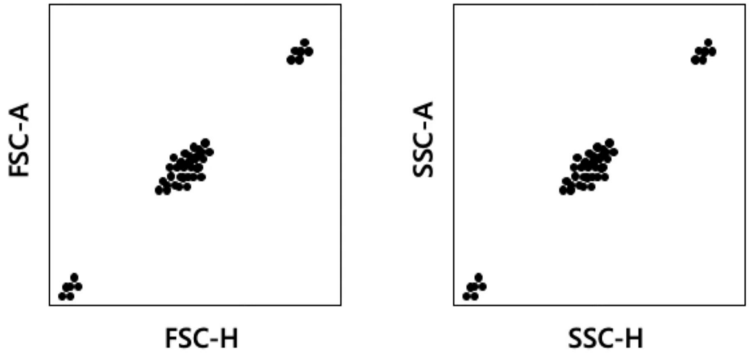
Area Scaling Factor (ASF)

- ASF is a factor that is applied to the pulse area measurement to equalize the height and area scales

- ASFs are calculated for FSC and for each laser in the system. If system is stable, ASFs should be very consistent over time.

- ASFs calculated with QC beads (usually 3 µm size) are not adequate for small particles or large cells

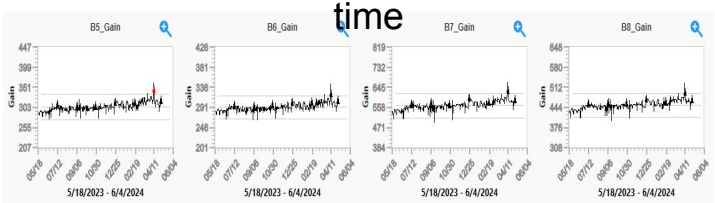




Performance Tracking

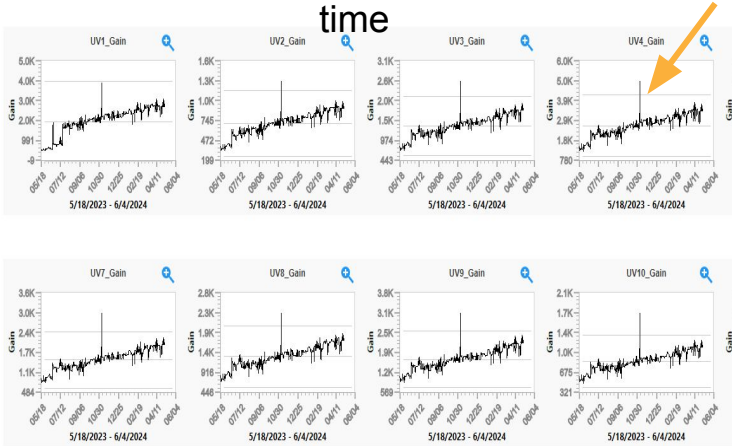
- It is important to monitor CV, voltages/gains, laser delays, ASFs, and other available QC metrics, over time
- Levey-Jennings (LJs) plots (time vs parameter) are available as part of most manufacturer's QC software modules
- If QC fails, it is important to determine if the measurement that failed is an outlier, or if it is a result of changes that have been occurring over time

Stable Gains (voltages) over time



Gain (voltage) increase over time

Outlier measurement



Workflow For Sample Acquisition

1. Flow cytometer warm-up and cleaning procedure
2. Flow cytometer QC and assessment of performance parameters (LJs)
3. **Instrument setup**
4. Experiment setup (compensation controls, gating strategy, stopping criteria, etc)
5. Sample acquisition

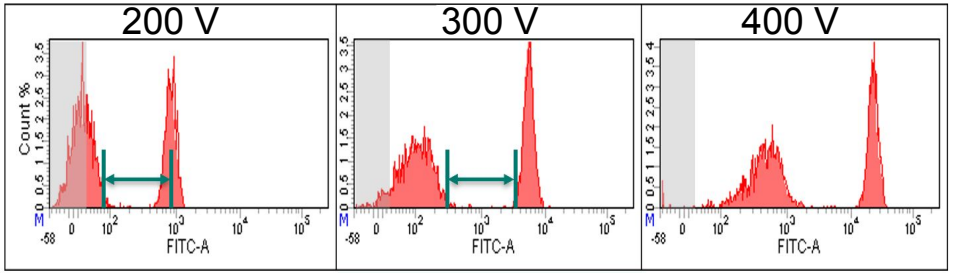
What Constitutes An Optimal Instrument Setup?

- Four important criteria need to be met:
  - Fluorochromes have peak of emission in their primary detector : peak excitation and peak emission wavelength.
  - Optimal resolution of particles labeled with each fluorochrome that is detectable by the system
  - Dim signal are above noise, both optic and electronic
  - Bright signals are on scale and within the linear range of the optical detector
- Careful characterization of instrument performance is required to achieve optimal setup:
  - In some cases, needs to be performed by users.: doable for conventional cytometers (8-20 detectors)
  - In some cases, manufacturer provides recommended settings, expected for spectral cytometers (50+ detectors)

Instrument Setup – “Voltrations”

- The goal is to establish settings for optimal resolution per fluorochrome/detector
- Need to ensure that peak emission is in the primary detector
- Need to evaluate not only resolution but also spread if goal is to use settings for multicolor data acquisition

Example: FITC detector voltration

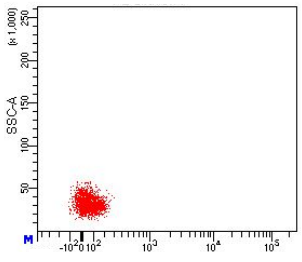


PMT Voltage	370	470	570
Stain Index	15	39	42
MFI Pos Cells	750	5,072	21,183
MFI Neg Cells	15	94	415
rSD Neg Cells	24	64	245

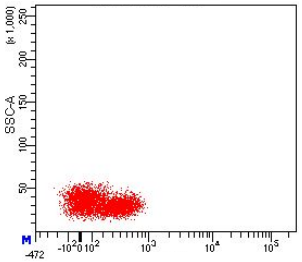
Instrument Setup and Resolution

- Increasing the voltages/gains can improve resolution of dim signals up to a certain point

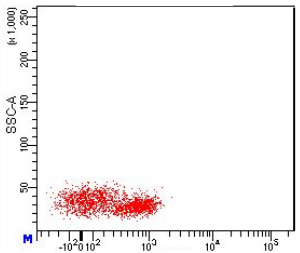
CD4 competition staining using PE labeled and pure antibody, mimicking 313 molecules/cell



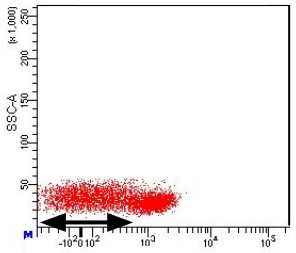
Settings optimized by voltration using CD4 stained cells



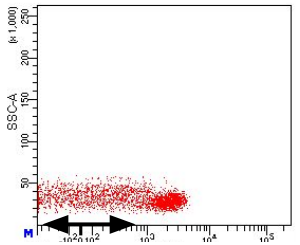
+ 100V



+ 150V



+ 200V



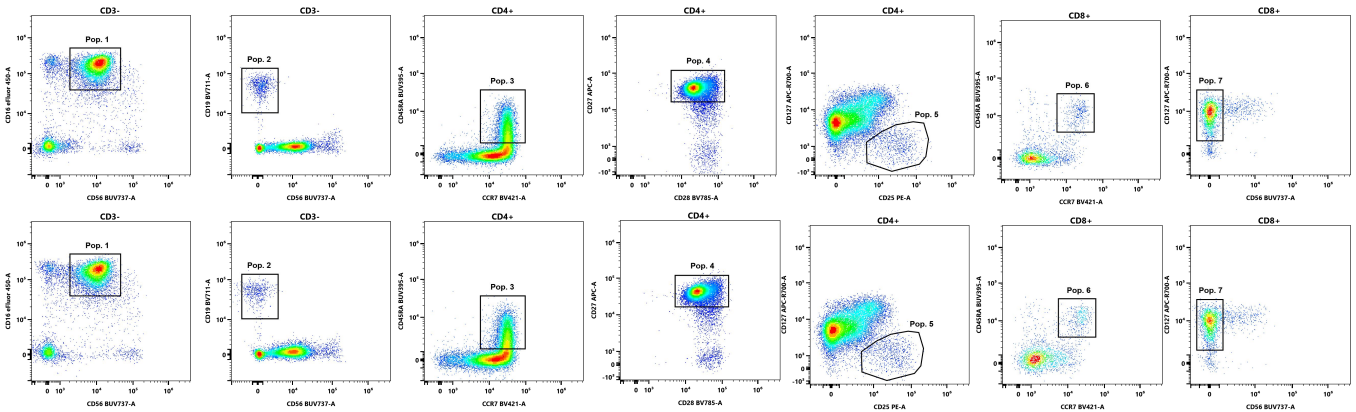
+ 250V

Reproducibility Of Instrument Setup Across Time

- Once optimal settings have been established for the application of interest, they can be saved as MFI targets
- Some acquisition software will allow to save those settings (“user” or “application” settings and for those to be updated upon instrument QC
- Idea is to have consistent setup over time, so that differences in MFIs are merely due to the biology and not to the instrument

Day 1

Day 10



Pre-stained lyophilized cells ran at user optimized settings on the same cytometer

Hard-Dye Beads And Transfer Of Assay MFI Targets

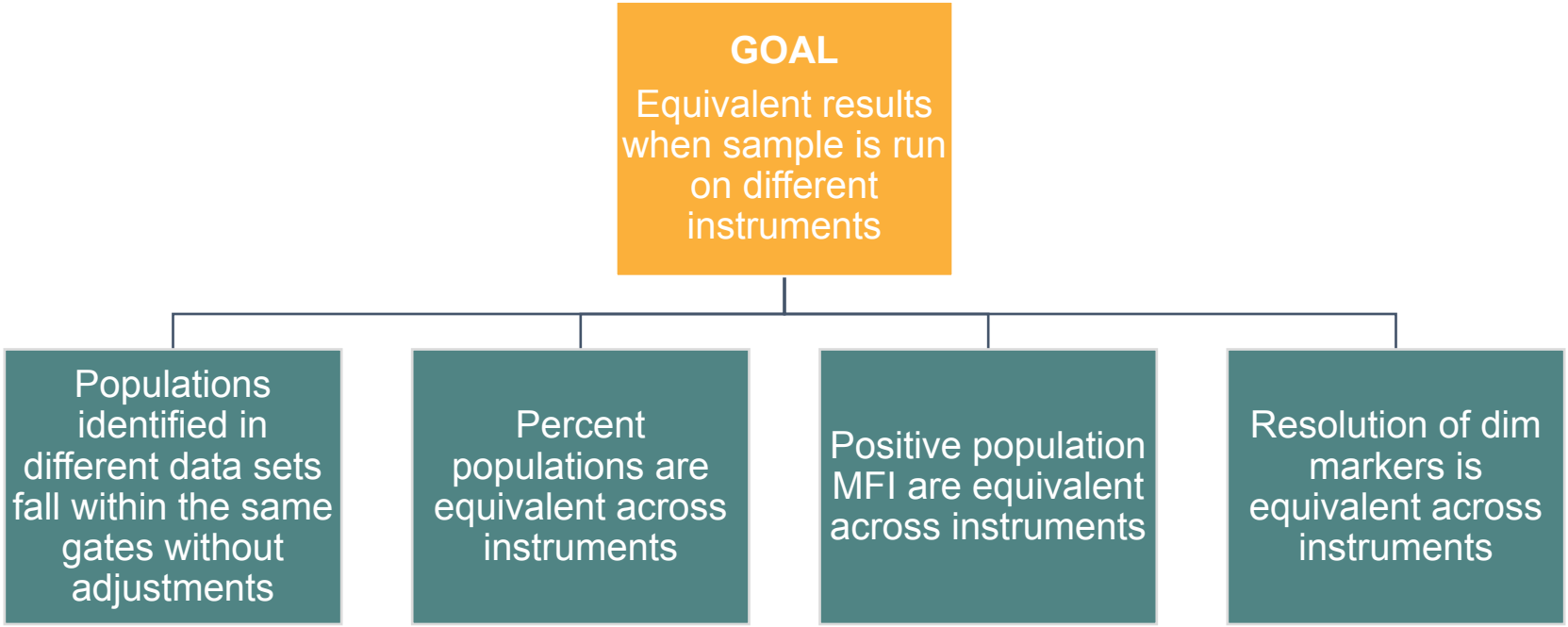
Pros

- Hard-dye beads are highly stable, robust, and low cost
- Hard-dye beads are excited by the most common lasers and have broad emission
- Provide a good tool to achieve setup standardization to a good level of precision for immunophenotyping assays (ex. EuroFlow Consortium)

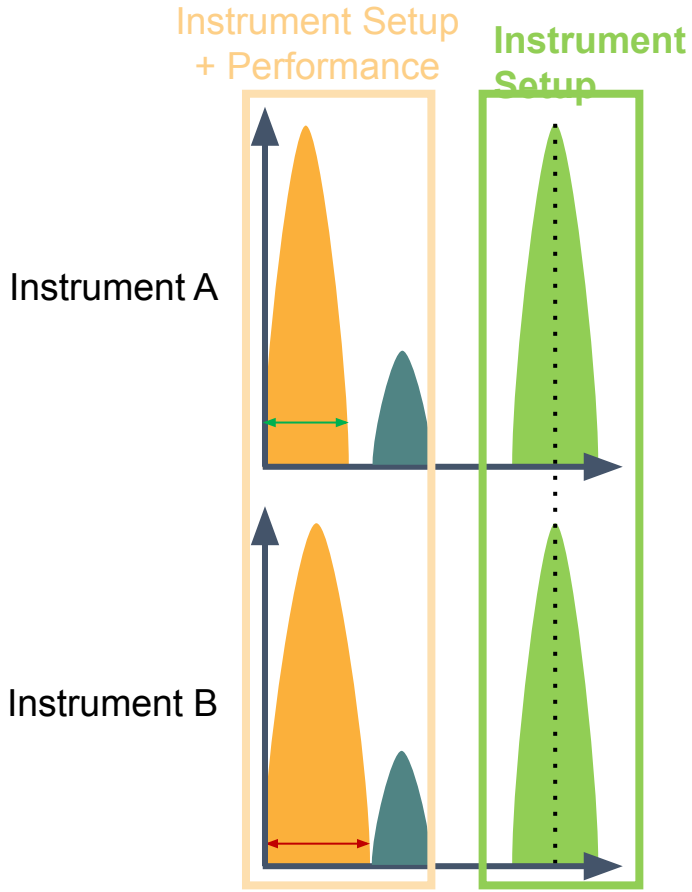
Cons

- Hard-dye beads are not spectrally matched with fluorochrome-labeled particles
- Can expect average of 20-30% variation when using hard-dye beads to set MFI, then moving to actual fluorochromes

Reproducibility Of Setup Across Instruments



Instrument Setup AND Performance Contribute to Reproducibility



Factors Affecting Instrument Sensitivity/Resolution

Positive Signal

Excitation Optics

- Laser power
- Laser wavelength
- Laser alignment

Collection Optics

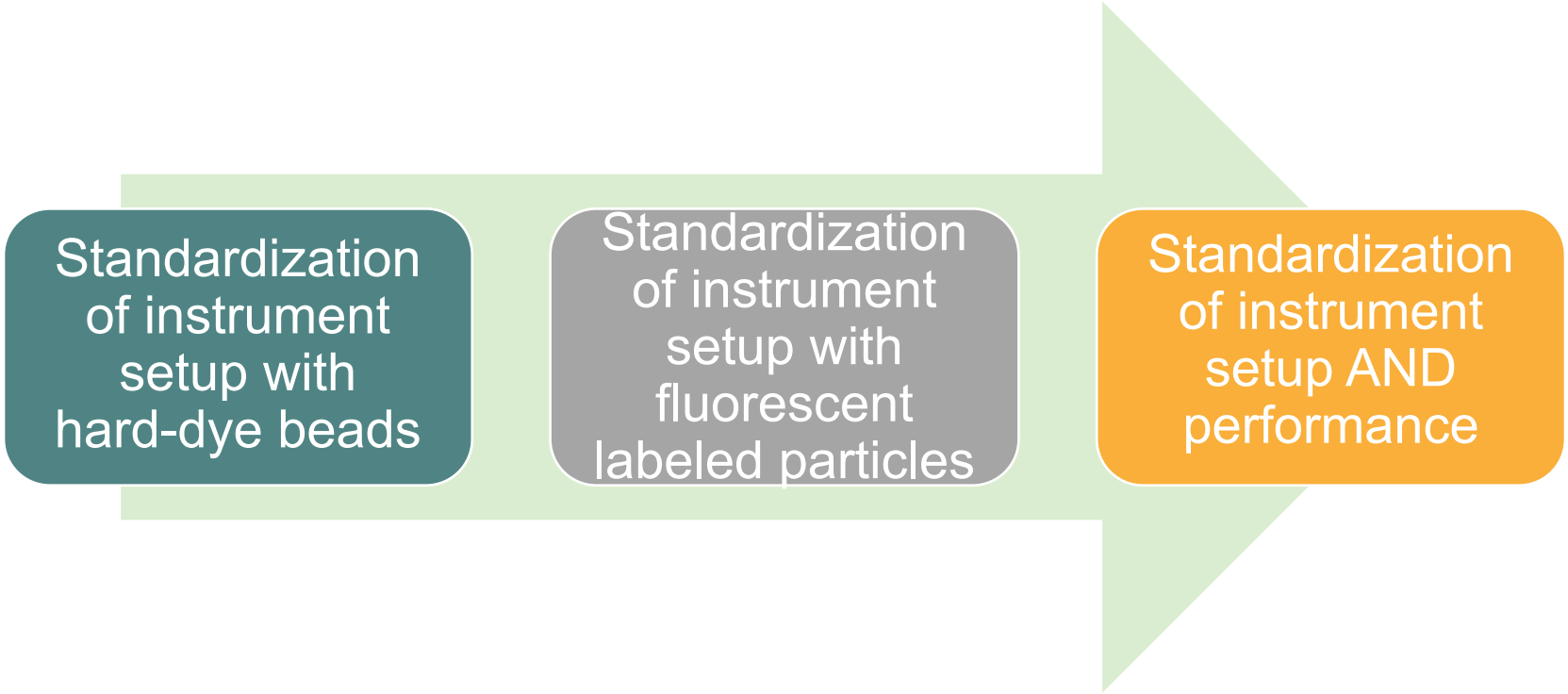
- Optical detector type
- Detector efficiency
- Optical filters
- Laser alignment

Negative rSD

Optical Background

Electronic Noise

Three Different Levels Of Standardization



Example Instrument Setup and Performance Harmonization

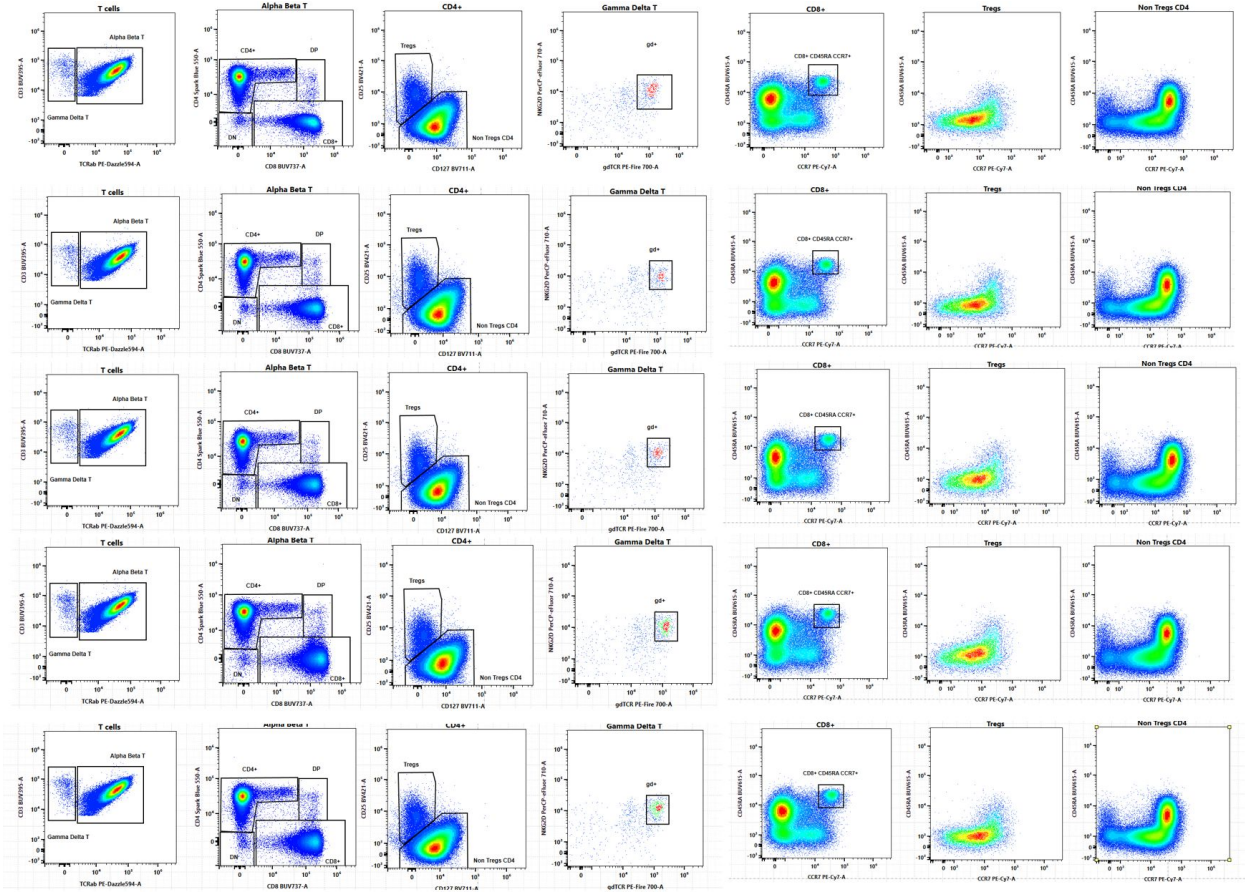
Instrument 1

Instrument 2

Instrument 3

Instrument 4

Instrument 5



Workflow For Sample Acquisition

1. Flow cytometer warm-up and cleaning procedure
2. Flow cytometer QC and assessment of performance parameters (LJs)
3. Instrument setup
4. Experiment setup (compensation controls, gating strategy, stopping criteria, etc)
5. **Sample acquisition**

Considerations For Sample Acquisition: Number Of Recorded Events

- The number of cells needed to perform a flow cytometry experiment need to be carefully assessed:
  - are cells needed for compensation controls?
  - are cells needed for other controls like biological controls or FMOs?
  - how many cells needed to detect with accuracy populations of interest?

TABLE 1 | Total number of cells to collect in detection of rare events.

Frequency of Rare Events (1/x)	% of total	Desired coefficient of variation % (rare events required)			
		30 (11)	10 (100)	5 (400)	3 (1,111)
20	5	222	2,000	8,000	22,222
50	2	556	5,000	20,000	55,556
100	1	1,111	10,000	40,000	111,111
1,000	0.1	11,111	100,000	400,000	1,111,111
10,000	0.01	111,111	1,000,000	4,000,000	11,111,111
100,000	0.001	1,111,111	10,000,000	40,000,000	111,111,111
1,000,000	0.0001	11,111,111	100,000,000	400,000,000	1,111,111,111

For very rare cell populations, number of cells to be analyzed increases substantially.

Lambert et al., Front. Immunol. Volume 11 – 2020
https://doi.org/10.3389/fimmu.2020.02169

Considerations For Sample Acquisition: Event Rate vs Flow Rate

- Maximum event rate (number of events/second) is determined by electronic aborts:
  - Desirable abort threshold: number of aborts/second lower than 10% of total events/second
  - This is a function of the electronics design and performance of the flow cytometer

- Strategies to increase event rate:
  - Increase flow rate: caveat, optical performance can be affected a high flow rates
    - Higher CVs positive populations, affects single color and multicolor samples
    - Higher spread in multicolor samples, affects negative populations and hence impacts resolution of dim signals
  - Concentrate sample and run at low or medium flow rate: desirable

THANK YOU!

QUESTIONS ?

Course Outline

1. **Pre-analytical variables/sample processing (1:00-1:40)**
   a. Sample collection and transport
   b. PBMC isolation or other sample processing
   c. Stimulation/functional assays
2. **Experimental design (1:40-2:45)**
   a. The instrument: lasers, filters, conventional vs. spectral
   b. Panel building and testing: fluorochromes, spillover
   c. Batching
   d. Controls

   Break (2:45-3:00)
3. **Instrument setup and acquisition (3:00-3:30)**
   a. Standardizing instrument setup, inter-instrument qualification
   b. Acquisition variables: # of events, speed, collection criteria
4. **Data analysis (3:30-4:30)**
   a. Quality control steps
   b. Manual and automated analysis

Holden Maecker

Maria Jaimes

Caroline Duault

Flow cytometry course: Data quality control and analysis

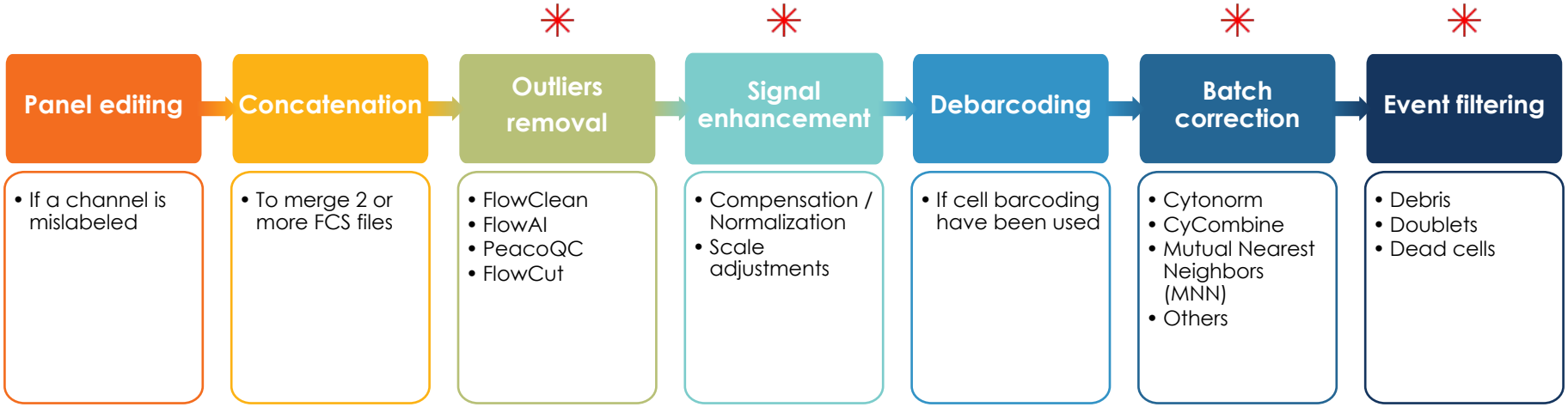
Dr. Caroline Duault, PharmD, PhD
Human Immune Monitoring Center / Maecker lab
Stanford University

The importance of quality controls

- High throughput experiments with measurements of 20-50 parameters = higher complexity of experimental design and data analysis
  - Technical errors vs real biological differences?
- Each step of the experiment should be carefully considered:
  - Experimental design (proper controls, reagents, standardization)
  - Data acquisition (Instrument parameters)
  - **Data QC and transformation (what should be corrected?)**
  - **Data analysis (best practice of gating, best methods of analysis)**

Liechti T. et al., Nat Immunol, 2021

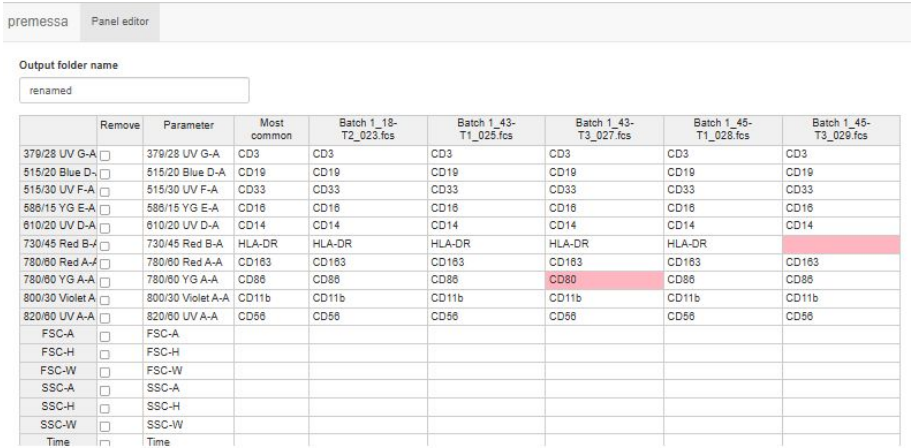
Potential workflow for data QC (flow cytometry)



Tools: R (R studio), FlowJo (including Premessa), GenePattern, Cytobank, etc.

Panel editing

Premessa package in R: https://github.com/ParkerICI/premessa or in FlowJo

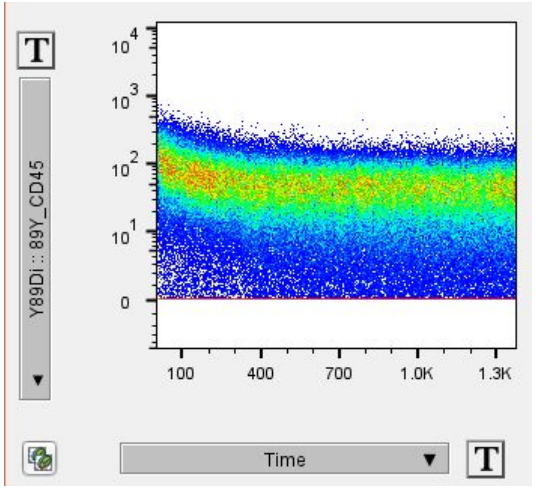


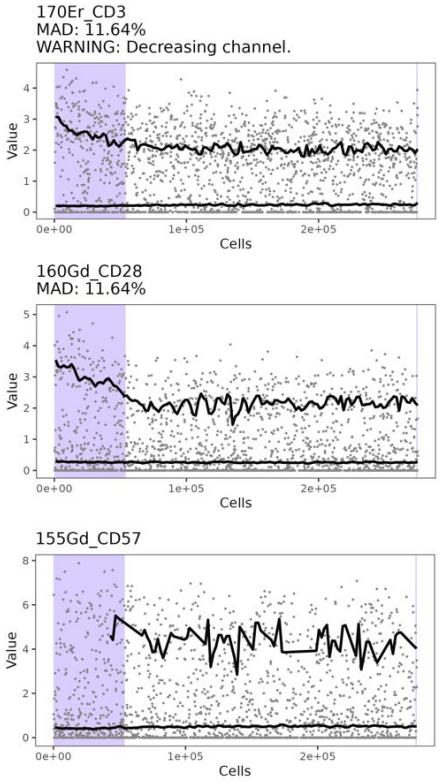
	Remove	Parameter	Most common	Batch 1_18-T2_023.fcs	Batch 1_43-T1_025.fcs	Batch 1_43-T3_027.fcs	Batch 1_45-T1_028.fcs	Batch 1_45-T3_029.fcs
379/28 UV G-A	☐	379/28 UV G-A	CD3	CD3	CD3	CD3	CD3	CD3
515/20 Blue D-A	☐	515/20 Blue D-A	CD19	CD19	CD19	CD19	CD19	CD19
515/30 UV F-A	☐	515/30 UV F-A	CD33	CD33	CD33	CD33	CD33	CD33
586/15 YG E-A	☐	586/15 YG E-A	CD16	CD16	CD16	CD16	CD16	CD16
610/20 UV D-A	☐	610/20 UV D-A	CD14	CD14	CD14	CD14	CD14	CD14
730/45 Red B-A	☐	730/45 Red B-A	HLA-DR	HLA-DR	HLA-DR	HLA-DR	HLA-DR	
780/60 Red A-A	☐	780/60 Red A-A	CD163	CD163	CD163	CD163	CD163	CD163
780/60 YG A-A	☐	780/60 YG A-A	CD86	CD86	CD86	CD80	CD86	CD86
800/30 Violet A	☐	800/30 Violet A-A	CD11b	CD11b	CD11b	CD11b	CD11b	CD11b
820/60 UV A-A	☐	820/60 UV A-A	CD56	CD56	CD56	CD56	CD56	CD56
FSC-A	☐	FSC-A						
FSC-H	☐	FSC-H						
FSC-W	☐	FSC-W						
SSC-A	☐	SSC-A						
SSC-H	☐	SSC-H						
SSC-W	☐	SSC-W						
Time	☐	Time						

FCS files concatenation

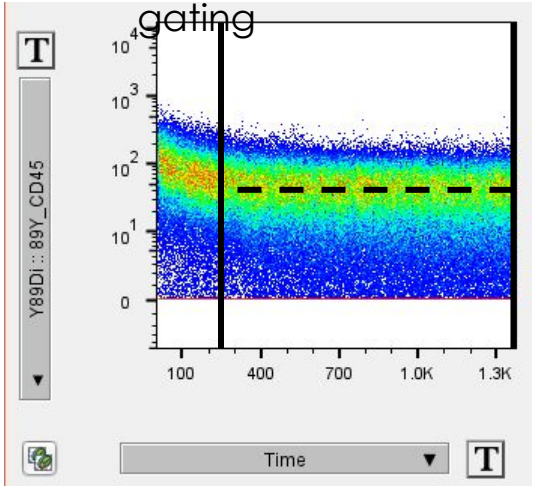
Premessa package in R: https://github.com/ParkerICI/premessa or in FlowJo

Outliers removal

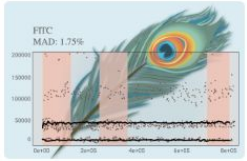




Manual gating

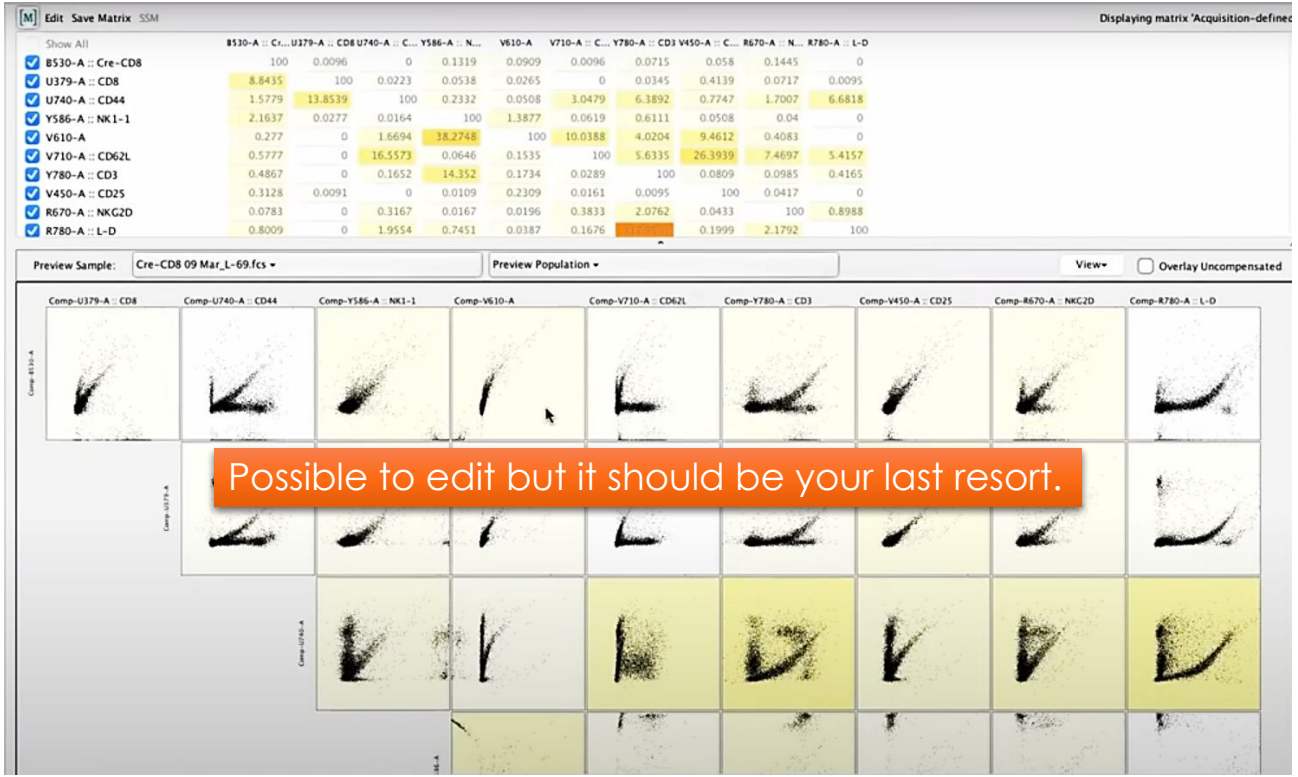


Outliers removal



	flowClean	flowAI	PeacoQC	flowCut
Platform	R, FlowJo, GenePattern	R, FlowJo	R, FlowJo, Cytobank	R, FlowJo
Extracts	Bins with shifts in population distributions	Bins with (1) abrupt changes in the flow rate, (2) instability of signal acquisition, (3) outliers in the lower limit and margin events in the upper limit of the dynamic range.	Bins with events easily separable from rest of data and deviate from median peak density	1. Parameters: MaxOfMeans, MeanOfMeans, MaxContin. 2. Removal bins <10% of the highest density of events 3. Mean, median, 5th, 20th, 80th, and 95th percentiles, second moment (variation), third moment (skewness)
Data type	Fluorescent	Fluorescent Mass without flow rate	Fluorescent and Mass	Fluorescent and Mass
Parameters	Raw	Raw	Compensated	Compensated
References	*Fletez-Brant K, et al. Cytometry A. 2016*	*Monaco G, et al. Bioinformatics. 2016*	*Emmaneel A, et al. Cytometry A. 2022*	*Meskas J, et al. Cytometry A. 2023*

Compensations



@ajarieger_flow

Compensations troubleshooting

1. Double check your controls
   - Are all your controls brighter than your samples?
   - Were your compensation samples made fresh the day of the experiment?
   - Were your compensation controls treated the same than your samples?

2. Are all the controls and samples stained with the same fluorochrome?
   - Live/dead, barcoding dyes

3. Cells vs beads?
   - Sometimes, beads are "too clean"

4. Adjust your positive and negative gates
   - Do you have enough events?

5. Clean up your data
   - Dead cells, doublets, debris, etc. can affect the fluorescence

Compensation improvements

1. **INVEST THE NECESSARY TIME TO PROPERLY PREPARE YOUR CONTROLS**
2. Re-acquire with proper/better controls and apply the compensation matrix to your data
3. Re-check your antibody titers/panel design
4. Try linear regression compensations instead of gate-based: Auto-spill (remove autofluorescence)

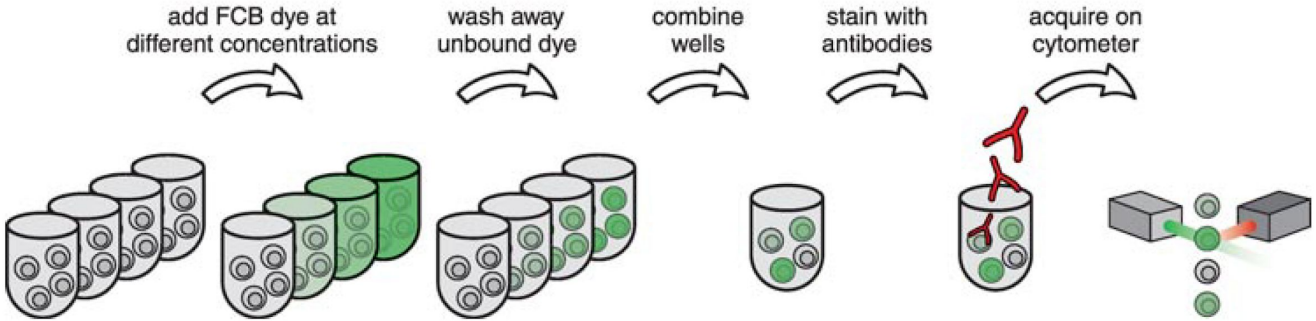
ARTICLE

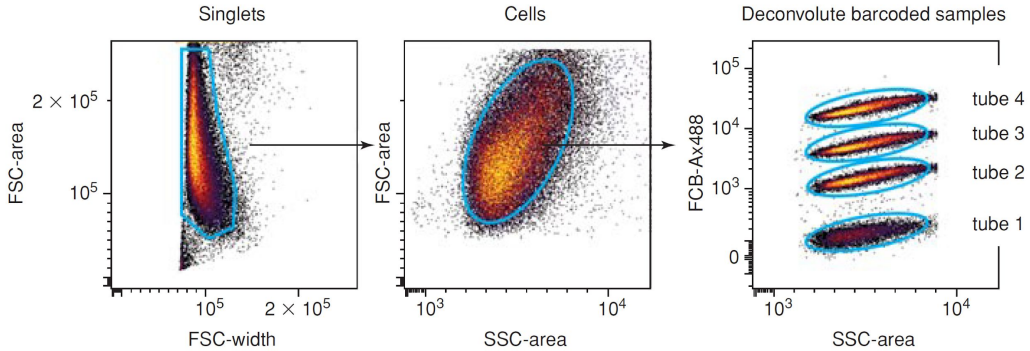
https://doi.org/10.1038/s41467-021-23126-8 OPEN

AutoSpill is a principled framework that simplifies the analysis of multichromatic flow cytometry data

Carlos P. Roca[1,2,3✉], Oliver T. Burton[3], Václav Gergelits[3], Teresa Prezzemolo[1,2], Carly E. Whyte[3], Richard Halpert[4], Łukasz Kreft[5], James Collier[5], Alexander Botzki[5], Josef Spidlen[4], Stéphanie Humblet-Baron[1,2] & Adrian Liston[1,2,3✉]

Cell barcoding (fluorescent or metal-based)





Krutzik, PO et al. Current protocols in cytometry. 2011

Cell Barcoding: Pros and Cons

Pros

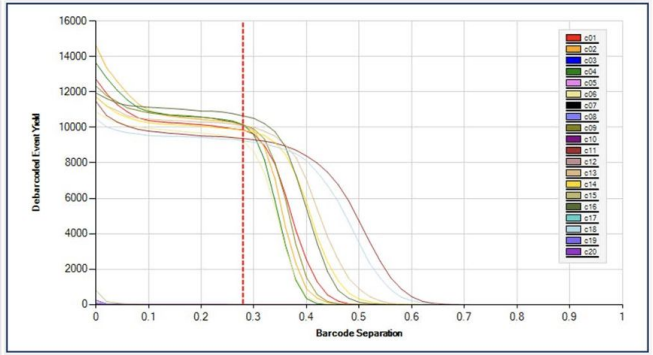
- More samples processed and acquired at once (less batch effect)
- Save on antibodies
- Homogenous staining
- Less technical errors
- Gain on machine usage

Cons

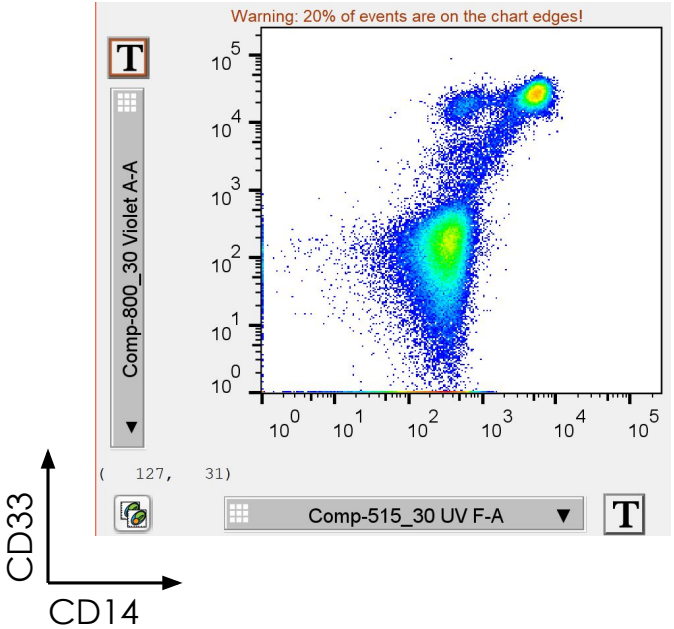
- Tricky development: high risk of spillover at higher dye concentration (easier with CyTOF)
- Need of high quality (viability) samples

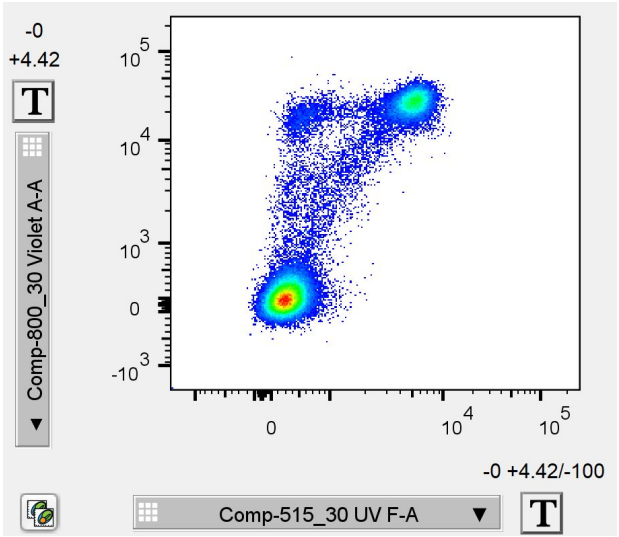
Debarcoding

Example of 6 metal-based barcodes for CyTOF experiment, choose 3 out of 6, 20 barcodes matrix



Scale transformation

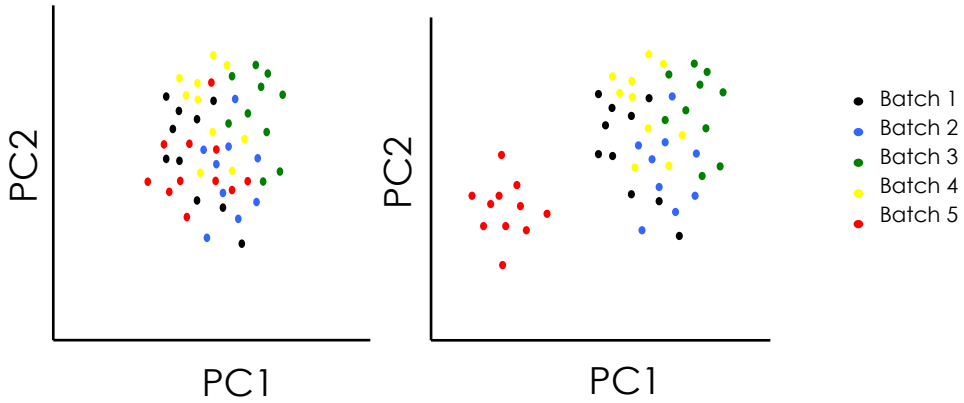




Determinant for the quality of clustering analysis!!!

Normalization / Batch correction

- Longitudinal studies, all samples can not be acquired in the same batch (CyTOF +++)
- Sources of variability:
  - Instruments
  - Operators
  - Reagents



How to minimize and monitor the batch effect?

Instruments

- Proper calibration
- Respect warm up time (laser/plasma)
- Monitor performance over time

Sample processing

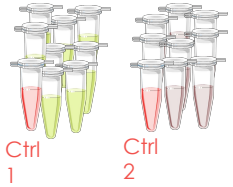
- Standardize staining protocol
- Minimize reagent variability (Ab, buffers)

Batching

- Process all samples from the same patients in the same batch
- Uniform distribution of the various groups

Barcoding

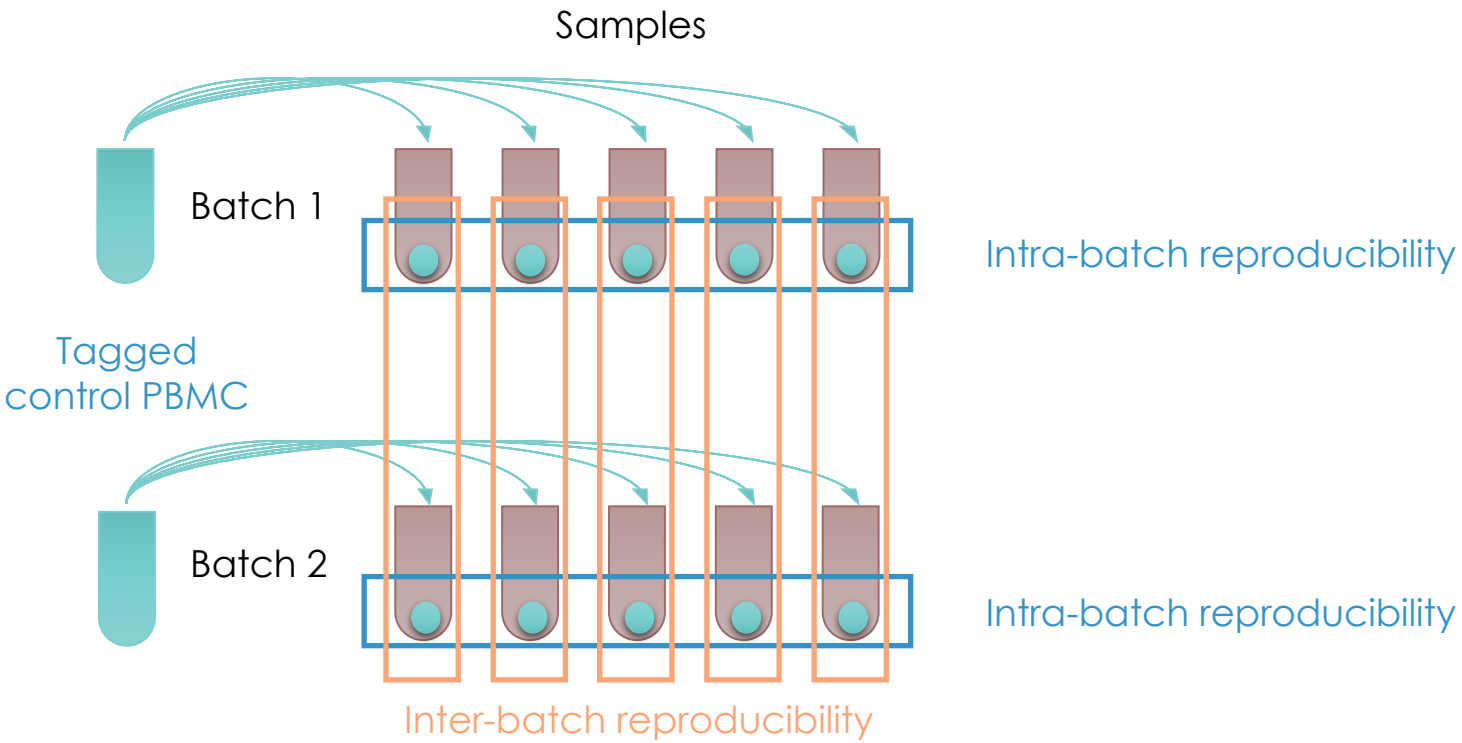
- Minimize greatly the staining and reagent variability



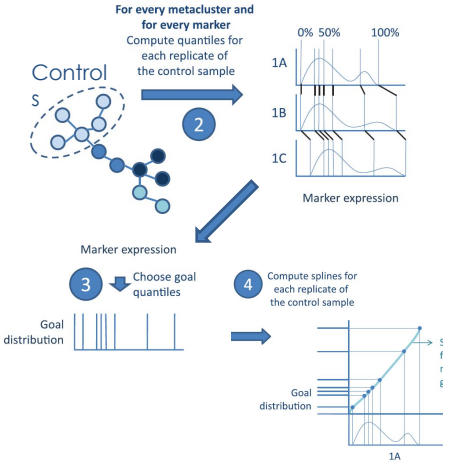
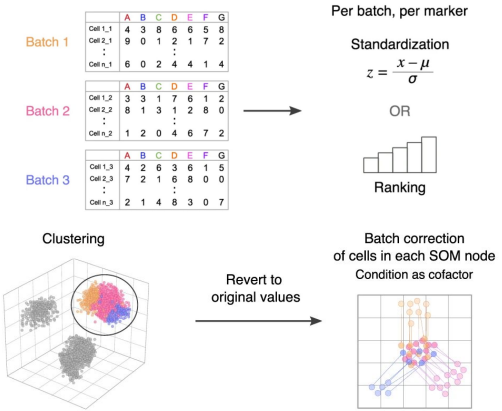
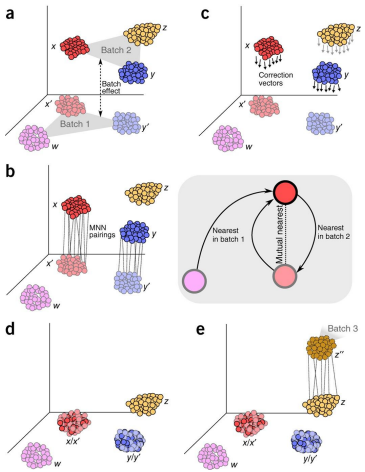
Reference sample

- Stand alone anchor control, processed in the same experimental conditions than your samples
- Spiked-in cells

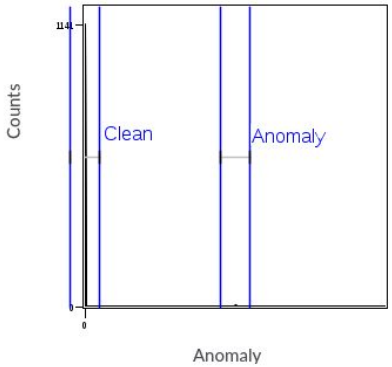
Spiked-in controls



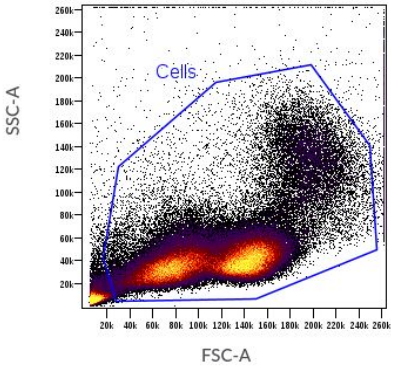
Batch effect removal

	CytoNorm	CyCombine	Mutual Nearest Neighbors (MNN)
Concept			
Anchor sample	Originally yes, but not anymore	No	No
Output	Normalized individual files	Normalized concatenated file	Normalized concatenated file
References	*Van Gassen S. et al. Cytometry A. 2020*	*Pedersen CB et al., Nat Commun. 2022*	*Haghverdi L et al., Nat Biotechnol. 2018*

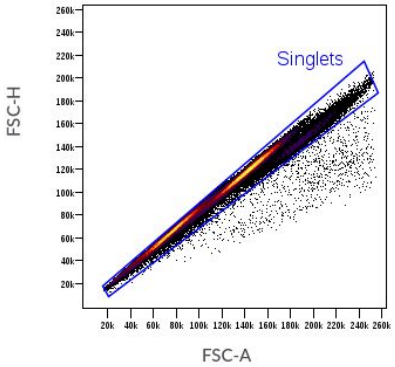
Event filtering



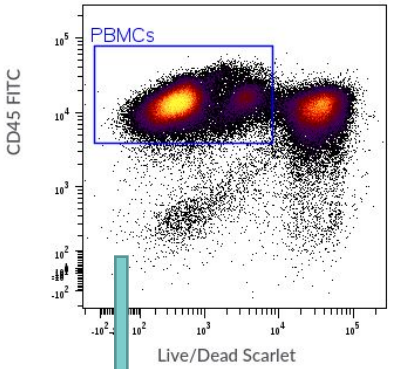
Clean



Cells



Singlets

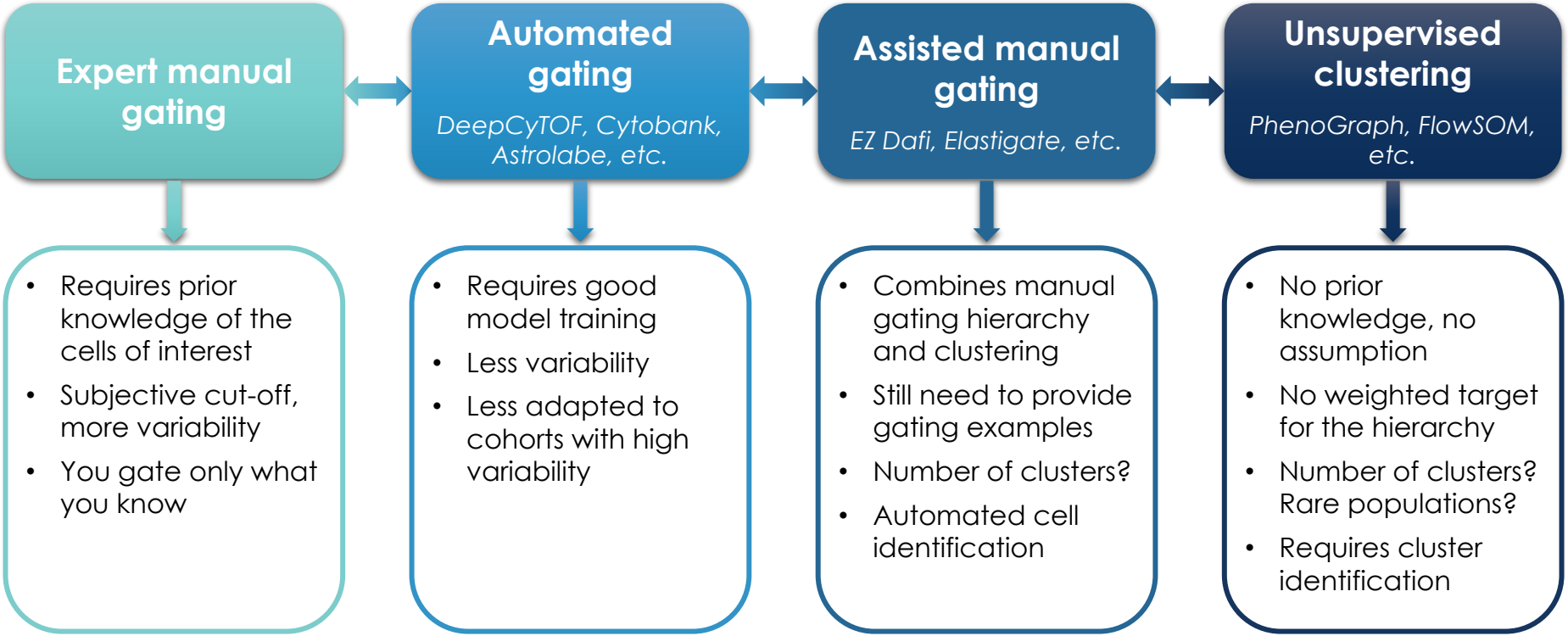


Manual gating or clustering analysis

Conclusions

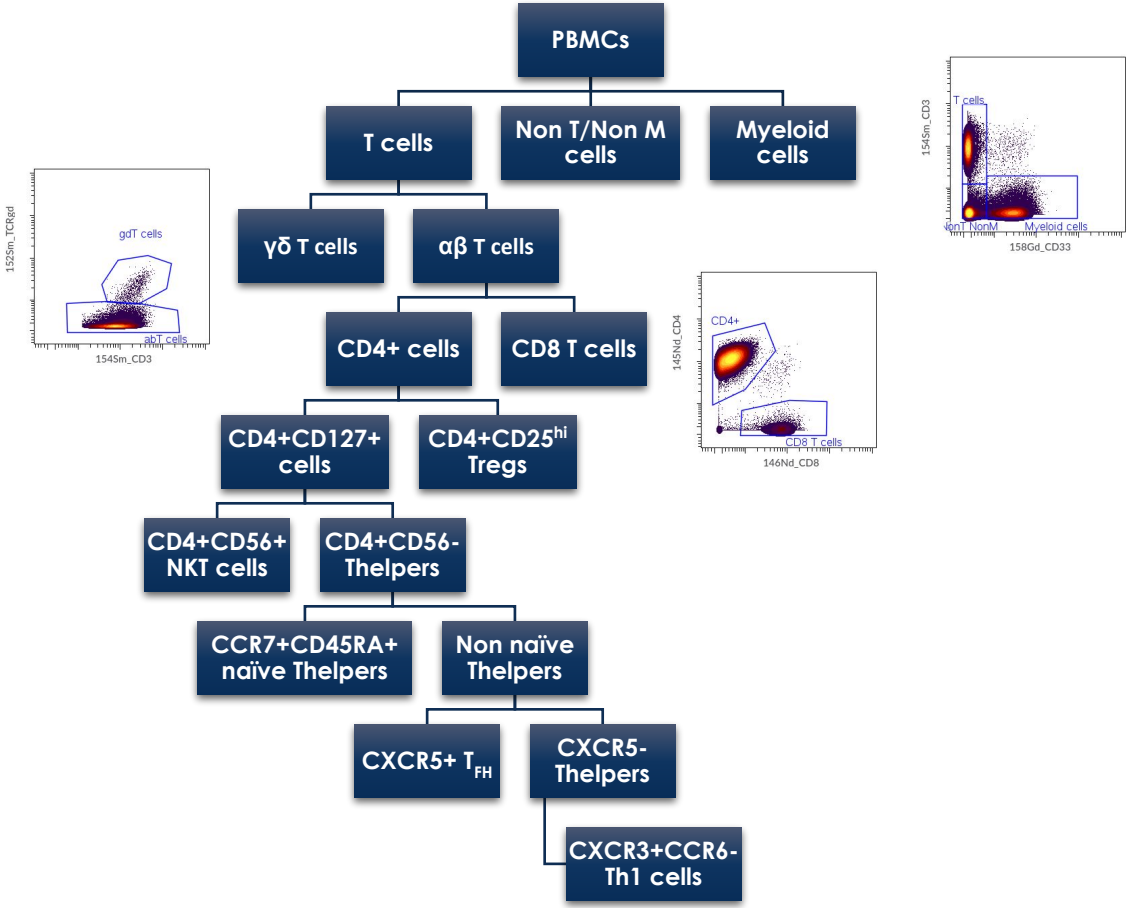
- Data QC is essential to ensure the quality and reliability of subsequent analysis
- The workflow can be adapted depending on the experiment
- No need to overkill the QC! (keep enough events for analysis)

Various options for data analysis

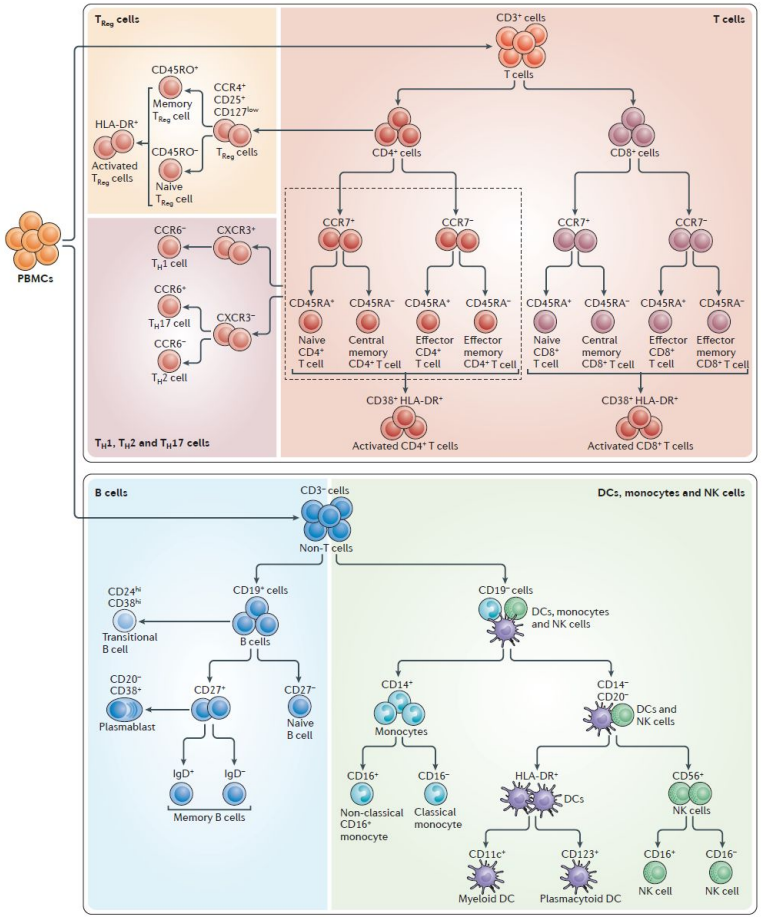


In any case, best practice for event filtering (removal of debris, doublets and dead cells) and data scaling are important

Building an efficient gating strategy



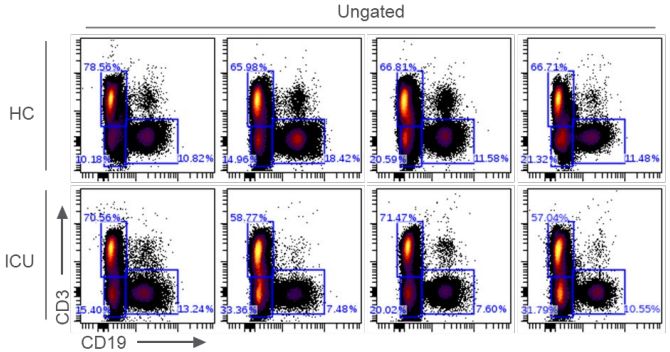
Building an efficient gating strategy

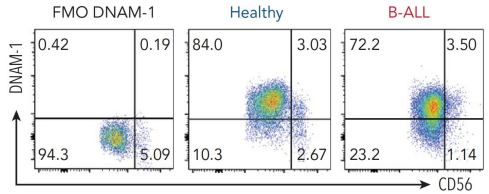


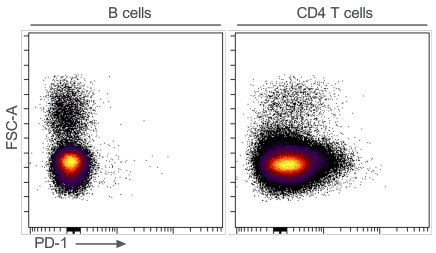
Maecker, H. et al. Nat Rev Immunol, 2012

Gate consistency

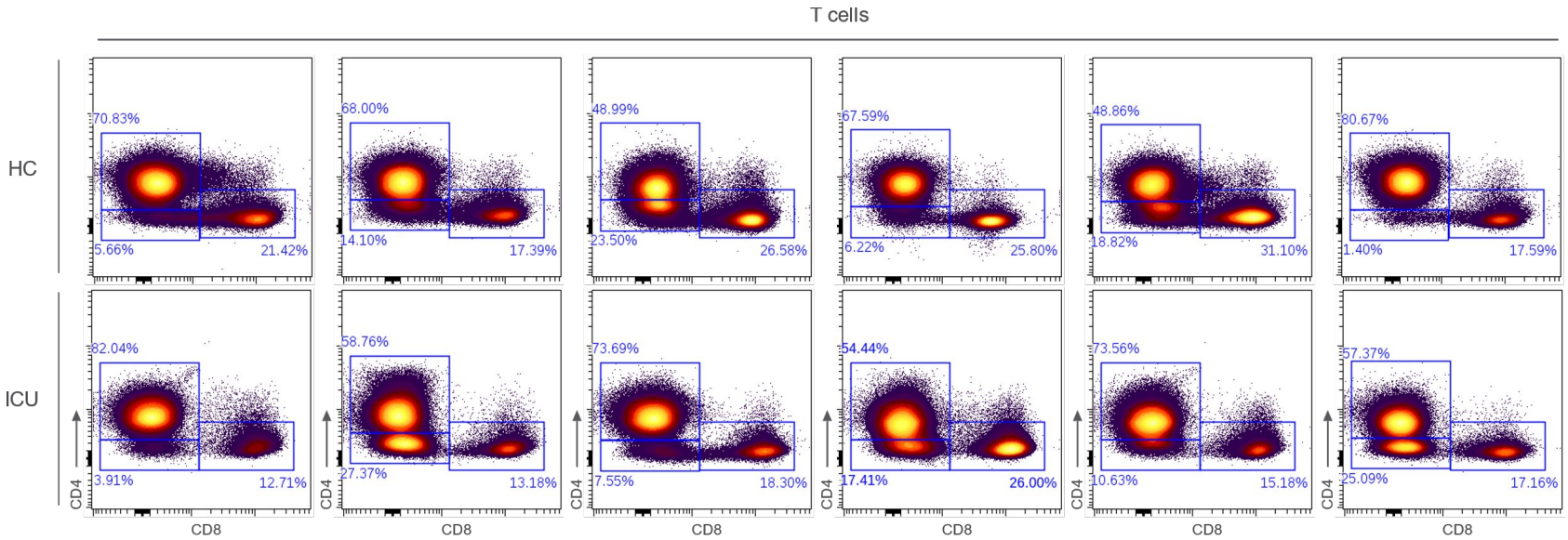
- Check across several samples
- Keep the same shape
- Use your negative controls:
  - **Fluorescence Minus One:** Control sample that contains all the conjugated antibodies but one
  - **Biological negative control:** Using a reference (cell, sample) which does not express the target





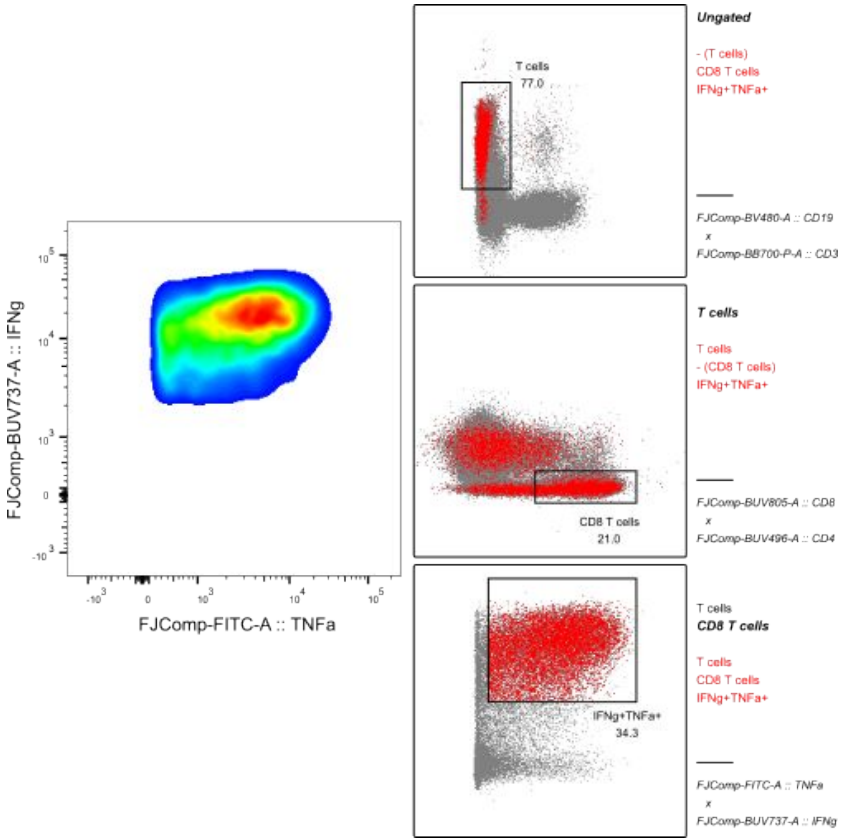


Adjustments for manual gating



Backgating (in FlowJo)

- Allows to track back the final population across the entire gating strategy
- Verifying gate accuracy



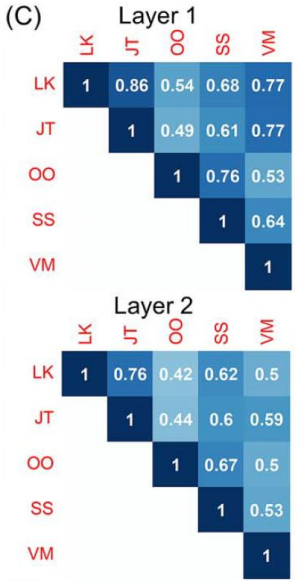
Manual gating variability

OXFORD

Briefings in Bioinformatics, 2025, **26(1)**, bbae633
https://doi.org/10.1093/bib/bbae633
Review

Comprehensive evaluation and practical guideline of gating methods for high-dimensional cytometry data: manual gating, unsupervised clustering, and auto-gating

Peng Liu[1,†], Yuchen Pan[2,†], Hung-Ching Chang[1,†], Wenjia Wang[1], Yusi Fang[1], Xiangning Xue[1], Jian Zou[1], Jessica M. Toothaker[3,4], Oluwabunmi Olaloye[4], Eduardo Gonzalez Santiago[4], Black McCourt[4], Vanessa Mitsialis[5,6], Pietro Presicce[7], Suhas G. Kallapur[7], Scott B. Snapper[5,6], Jia-Jun Liu[8,9], George C. Tseng[1,10,*], Liza Konnikova[4,7,11,12,13,14,15,*], Silvia Liu[8,9,10,16,17,*]

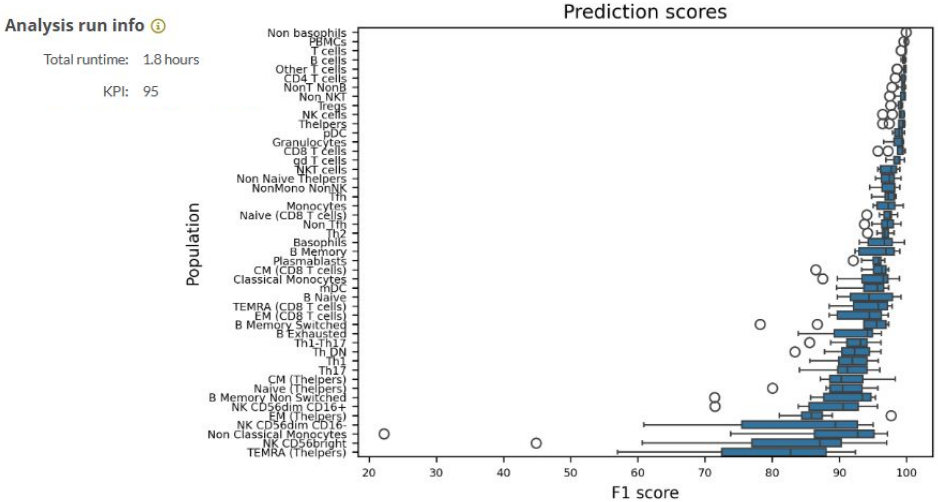


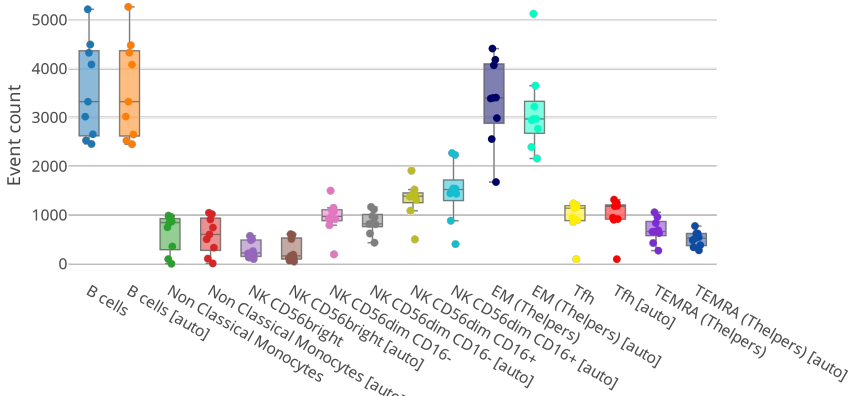
- 4 experts, 1 dataset, no guideline for the gating hierarchy
- 2 layers of gating:
  1. main lineage (7 populations)
  2. more refine with memory subsets (14 populations)

Automated gating workflow

Model training → Model validation → Autogating from trained model

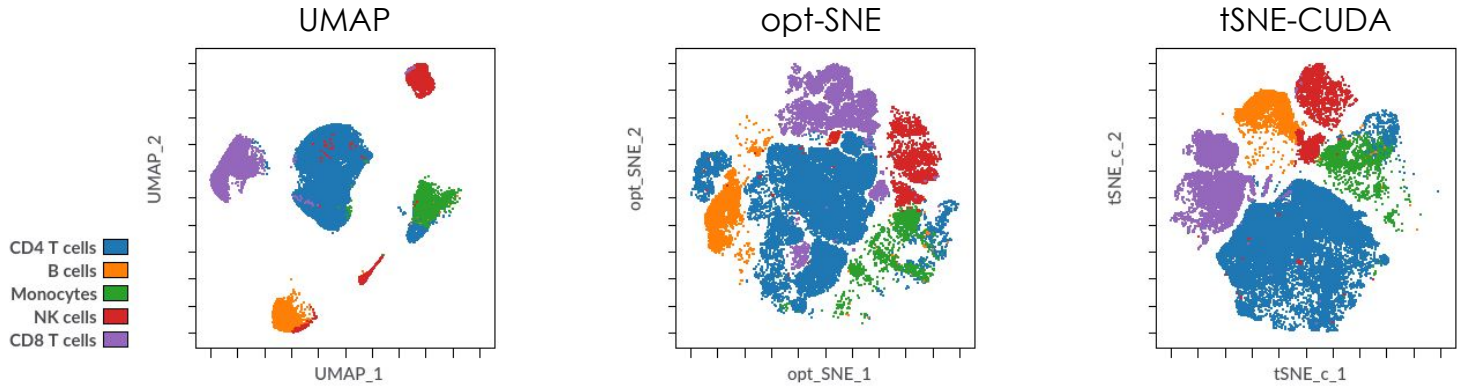
- Model training: selected samples must reflect the overall diversity and variability
- Prefers clinical samples than anchor controls





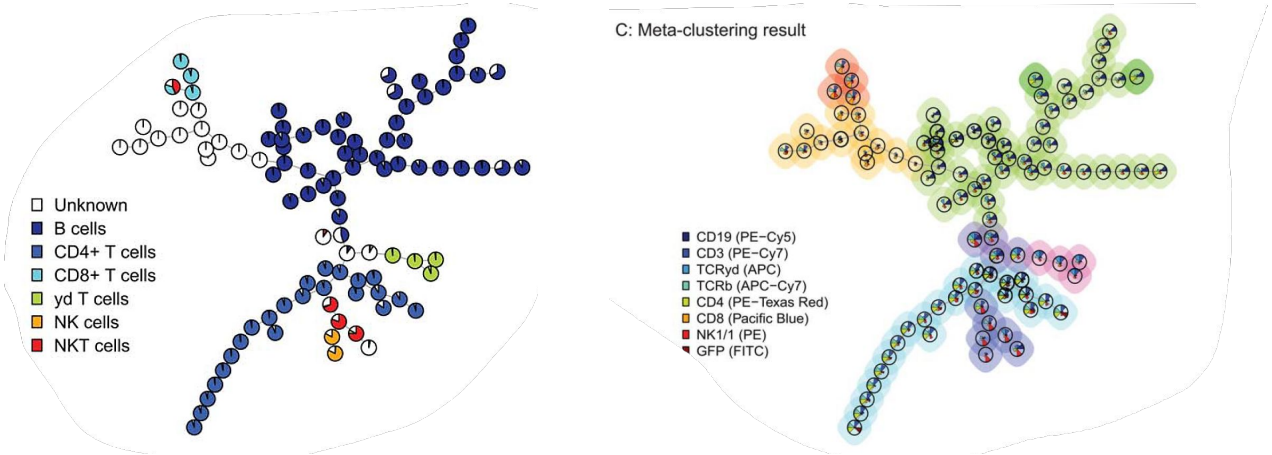
Dimensionality reduction

- Reduces high-dimensional data down to two dimensions
- Clusters are formed by comparing the expression of the phenotyping proteins
- Easy visualization and comparisons, protein co-expression



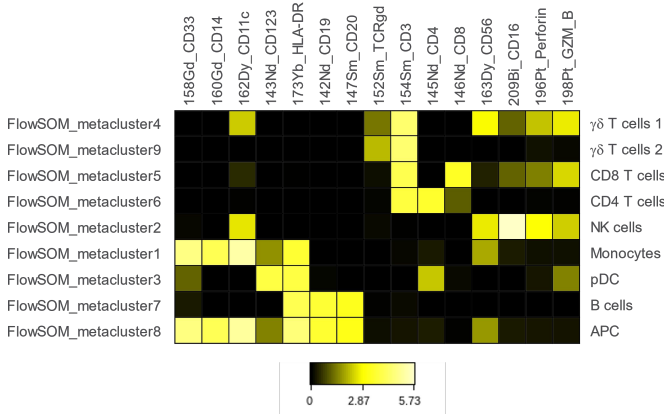
Unsupervised clustering: FlowSOM example

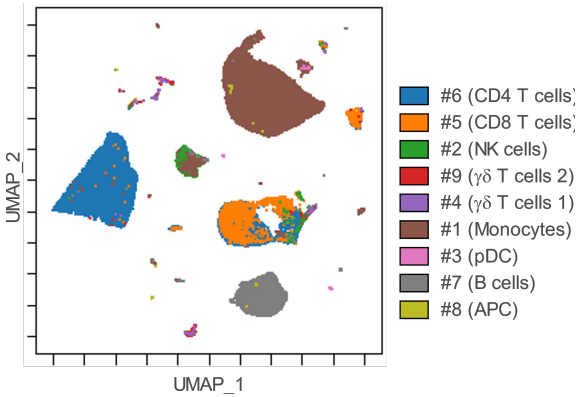
1. Clusters cells based on chosen clustering channels
2. Generates a Self Organizing Maps (SOM) of clusters
3. Produces a Minimum Spanning Tree (MST) of the clusters
4. Assigns each cluster to a metacluster, effectively grouping them into a population

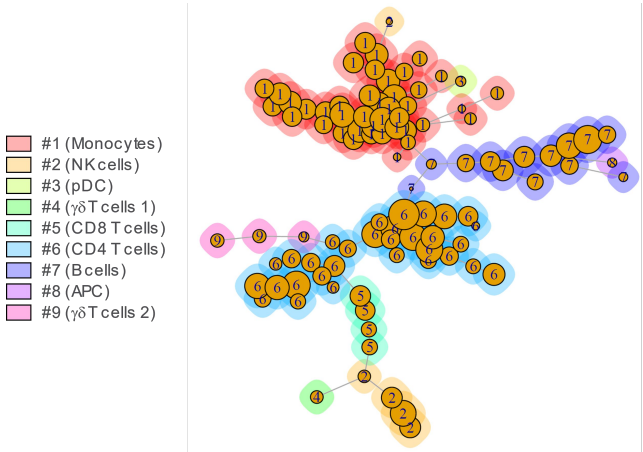


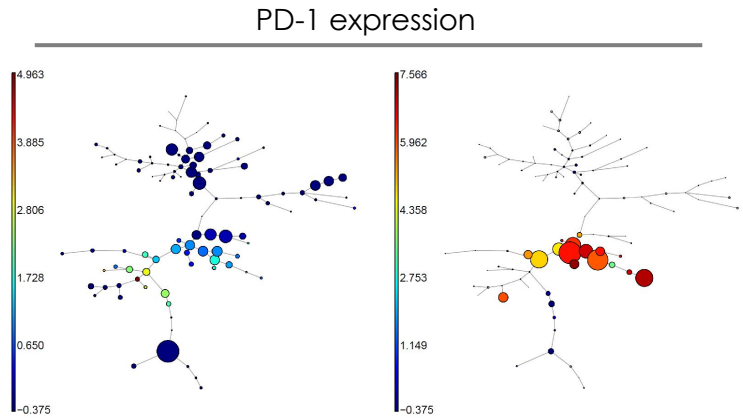
Van Gassen, S. et al., Cytometry Part A., 2015

Unsupervised clustering: FlowSOM example





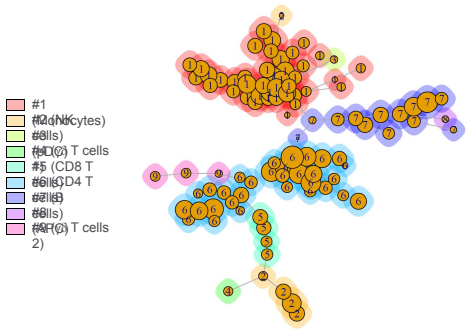




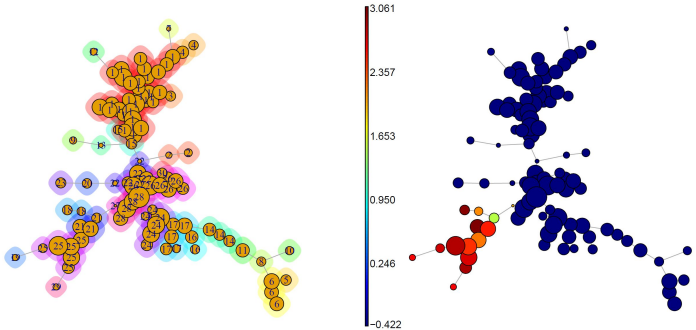
How to determine the optimal number of clusters?

- Underclustered: missing resolution, rare populations are lost
  Overclustered: Redundancy, hard interpretation
- Some clustering tools will automatically adjust the numbers of clusters
- Manual input: how many subsets do you expect? (rule of thumbs)
- INFLECT: tool especially designed to determine the optimal number of clusters (metaclusters) for FlowSOM (*Verhoeff J et al. BMC Bioinformatics. 2022*)

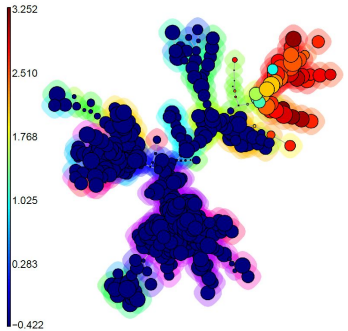
9 metaclusters, 100 clusters



30 metaclusters, 100 clusters



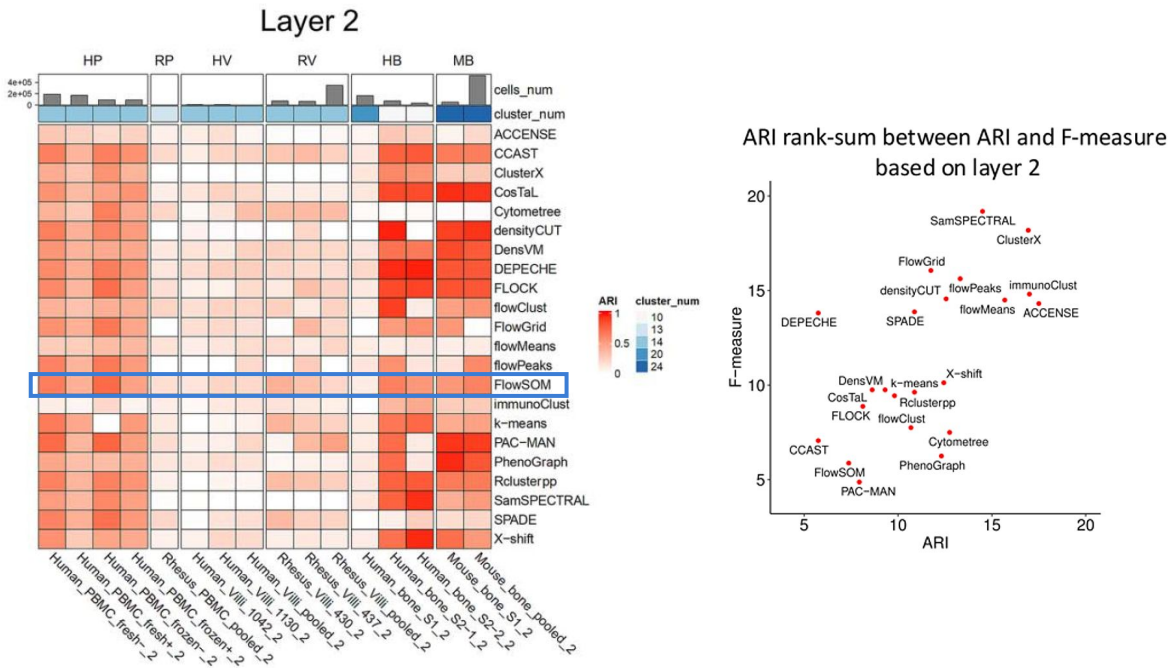
50 metaclusters, 400 clusters



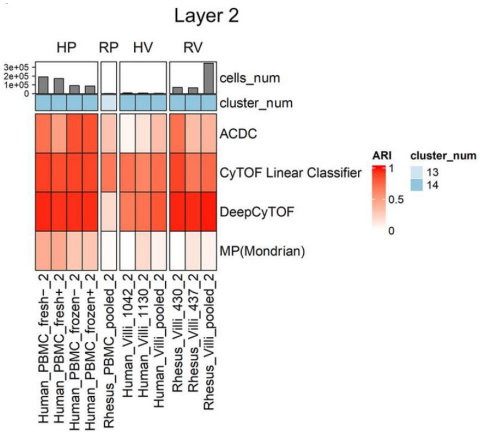
Which methods is the best for clustering or autogating?

Liu P. et al, Brief Bioinform., 2025 (PMID: 39656848)

Unsupervised clustering



Autogating



Astrolabe?
Cytobank?

References

1. **Liechti, T et al. Nat Immunol. 2021 (PMID: 34489590)**
2. Fletez-Brant, K et al. Cytometry A. 2016 (PMID: 26990501)
3. Monaco, G et al. Bioinformatics. 2016 (PMID: 27153628)
4. Emmaneel, A et al. Cytometry A. 2022 (PMID: 34549881)
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6. Roca, CP et al. Nat Commun. 2021 (PMID: 34001872)
7. Krutzik, PO et al. Curr Protoc Cytom. 2011 (PMID: 21207359)
8. Van Gassen, S et al. Cytometry A. 2020 (PMID: 31633883)
9. Pedersen, CB et al., Nat Commun. 2022 (PMID: 35361793)
10. Haghverdi, L et al., Nat Biotechnol. 2018 (PMID: 29608177)
11. Maecker, H et al. Nat Rev Immunol, 2012 (PMID: 22343568)
12. **Liu, P et al, Brief Bioinform., 2025 (PMID: 39656848)**
13. Van Gassen, S. et al. Cytometry A. 2015 (PMID: 25573116)
14. Verhoeff J et al. BMC Bioinformatics. 2022 (PMID: 36384426)

Resources

- Introduction to flow cytometry:
  - https://www.youtube.com/watch?v=5Tz5ya6RdXY (BD)
  - https://www.youtube.com/watch?v=PaGR1kgzO_M (ISAC)
- Purdue cytometry board:
  - http://www.cyto.purdue.edu/content/cytometry-list
- Flow cytometry: an introduction (A. Givan):
  - Methods Mol Biol. 2011;699:1-29. doi: 10.1007/978-1-61737-950-5_1.PMID: 21116976
- Panel design:
  - Current protocols in cytometry. 2020: 92(1), e70